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Comparative study of cytotoxic properties of nanoliposomal form of Curcumin or vitamin E towards laryngeal carcinoma

Naglaa M. Ismail[1]*, Medhat W. Shafaa [2], Maha R. Elsyed[1]

[1]Physics Department, Biophysics Division, Faculty of Women for Arts, Science and Education. Ain Shams University. Cairo. Egypt.

[2]Physics Department, Medical Biophysics Division, Faculty of Sciences, Helwan University, Cairo, Egypt.

Abstract

This study aimed to investigate in vitro the effect of Curcumin and Vitamin E by using liposome as a drug-delivery system and evaluate the cytotoxic potential of these liposome using laryngeal carcinoma Hep-2 cell line. Curcumin and Vitamin E liposomes were characterized in terms of size distribution, ζ-potential, Fourier transform infrared (FT-IR) spectroscopy and Differential scanning calorimetry (DSC). In the cell count assay, the viability of cells decreased as the concentration of the compounds increased. The cell viability declined to about 4.5% at a concentration of 400 µM for Curcumin and Curcumin-loaded liposomes, while declined to about 52.1% and 53.6 % at a concentration of 400 µM for Vitamin E and Vitamin E loaded liposomes respectively. Finally, this result indicates that Curcumin and its liposomal form have strong inhibition of cell growth and continually increasing inhibition with increased concentrations against Hep-2 cell line. vitamin E showed limited effect on Hep-2 cancer cells, while Curcumin combined with Vitamin E or its liposomal forms indicated a significant reduction in the cell viability when tested at the same concentration (400 µg/ml).

Keywords: Curcumin, vitamin E, hep-2, liposomes, ft-ir, cytotoxicity

1. Introduction

Cancer is a main cause of death worldwide. It is a group of a large number of diseases characterized by genetic mutations and epigenetic changes that can be caused partly by environmental factors such as oxidative stress **[1].** Head and neck carcinoma is a major type of cancer that causes mortality in humans, ranking sixth in the incidence rate of all types of cancer **[2]**. One of the most common malignancy types of head and neck carcinoma is Laryngeal cancer, which ranking second in the incidence rate of all respiratory tract neoplasms behind head and neck squamous cell carcinoma, which accounts for 95% of all types of head and neck carcinoma **[3]**. Chemotherapy is considered the most effective treatment for Laryngeal squamous cell carcinoma.

Curcumin (CUR; diferuloylmethane), is a natural yellow pigment compound extract from the turmeric plant (fig.1). It possesses a variety of biological activities and pharmacological actions, including anti-inflammatory, anti-carcinogenic, and anti-virus properties, as well as promising clinical applications due to its low toxicity, it is a lipophilic molecule that can permeate the cell membrane easily **[4,5,6]**.

Curcumin has anticancer activity against various cancers, such as leukemia and lymphoma, gastrointestinal cancers (colon, gastric, pancreatic), genitourinary cancers (prostate), breast cancer, ovarian cancer, head, and neck squamous cell carcinoma, lung cancer, and sarcoma **[7,8]**. However, curcumin has limited effectiveness due to poor aqueous solubility, low bioavailability, rapid metabolism, and systemic elimination **[9]**. Taking the advantages (namely, anticancer activity) and seeking to overcome the disadvantages (namely, solubility or toxicity) **[10]**. Recently, Curcumin has been proven to suppress cell proliferation in a diversity of human cancer cell lines *in vitro* **[11,12,13]**. Curcumin is effective against head and neck cancers in vitro **[14,15]**.

*Corresponding author e-mail: E-mail: naglaa.mostafa@women.asu.edu.eg .

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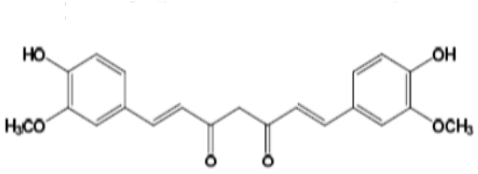

Fig.1. Chemical structure of Curcumin

Vitamin E exists as eight natural isomers, all of which have potent anticarcinogenic properties, including antioxidant and apoptotic characteristics. α-Tocopherol, the essential component of Vitamin E, is a lipid-soluble hydrocarbon compound that divides into cell membranes and lipid storage organelles. It is an efficient scavenger of lipid peroxyl radicals and, hence, it can break peroxyl chain propagation reactions in cellular membranes preventing lipid peroxidation. These properties of vitamin E may make it an ideal supplement to standard cancer treatments such as chemotherapy as well as immunotherapies that modify the tumor microenvironment.

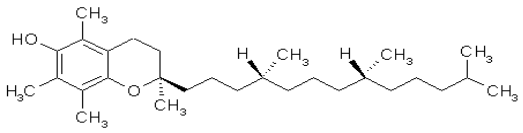

Fig.2. Chemical structure of vitamin E

Liposomal membranes are like cell membranes that compose an aqueous core that is surrounded by lamellae or lipid bilayers. Phospholipids can spontaneously form vesicles upon hydration with aqueous media due to the amphiphilic molecular structure possessing a hydrophilic phosphatidyl head group and hydrophobic fatty acid tails **[16].**

The lipid composition, surface charge, size, and preparation method all influence liposomal membrane properties. Thus, the choice of the lipid components determines the rigidity, fluidity, and charge of the lipid bilayer and consequently the properties of the liposome **[17]**.

The goal of this study is to investigate in-vitro the effect of Curcumin and Vitamin E by using liposomes as a drug-delivery system and estimate the cytotoxic potential of these liposomes using a Hep-2 cell line. The aim of the present study also is to investigate how Curcumin or Vitamin E modulates the physical structural properties of model lipid membranes and to estimate the subtle perturbation of the lipid bilayer structure using transmission electron microscopy (TEM), Zeta potential, Dynamic light scattering (DLS), as well as differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy.

2. Materials and methods

2.1. Chemicals

Curcumin and Vitamin E with a molecular weight of 368.38 and 430.7, respectively were purchased from EIPICO (Egyptian International Pharmaceutical Industries Co, Egypt). The molecular structure of Curcumin and Vitamin E are shown in **Fig. 1 and 2**. Ethyl alcohol was bought from DaeJung Chemicals (Seohaean-ro, Gyeonggi-do, Korea), with an absolute 99.9%. L-α- Phosphatidylethanolamine from sheep brain with a molecular weight of 691.515 of purity ≥98% in powder form bought from Sigma (ST. Louis, Mo, USA) is presented in (**Fig. 3**), Tris base in powder form, a molecular weight of 121.1 was purchased from CDH, New Delhi, India. Dimethylsulphoxide (DMSO), RPMI-1640 medium, Sodium bicarbonate, Trypan blue: An isotonic solution of 0.05 % trypan blue in normal saline, Penicillin / Streptomycin, Trypsin, Acetic acid, Fetal bovine serum (FBS), Sulphorhodamine-B (SRB), 0.4 % SRB dissolved in 1 % acetic acid, Trichloroacetic acid (TCA), 100 % isopropanol was bought from (Sigma Chemical Co., St. Louis, Mo, USA). In distilled ultra-pure water, solutions were prepared. HPLC grade was used with all the other preparations used in this study.

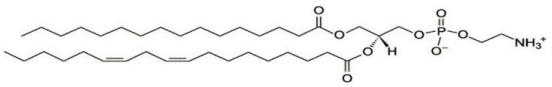

Fig.3. Schematic chemical structure of L-α-Phosphatidylethanolamine.

2.2. Preparation of liposomes (PE)

PE: Curcumin or Vitamin E or a combination of the two at a molar ratio of 7:2 was used to prepare neutral MLVs using Bangham thin-film hydration method **[18]**. 30 mg of PE and 4.566 mg of Curcumin or 5.85 mg of Vitamin E or a combination of the two at a molar ratio of 2:7 to PE were transferred to 100 ml round bottom flask. Then 20 ml of ethanol (EtOH) were added, and the flask was shaken until all components were dissolved in the EtOH. Using a vacuum rotary evaporator in a warm water bath (50 °C), the organic solvent was progressively extracted to create a uniform thin film of lipid on the inner wall of the flask. The lipid film was hydrated with a Tris buffer (0.2 M, pH 7.2 at 37 ° C) to form multilamellar vesicles in a water bath at 50 °C for 15 minutes (MLV). Then the flask was put for mechanical shaking at 50 °C for 1 hr and then nitrogen stream was used to flush the flask and closed immediately. Empty liposome serves as control was prepared using the same approach as mentioned above, using only PE mass aliquots previously used in the preparation.

2.3. Liposome morphology by transmission electron microscopy

The size and morphology of blank liposomes and liposomes doped with either Curcumin or Vitamin E or Curcumin coupled with Vitamin E were analyzed using a negative stain transmission electron microscope (JEOL, JEM-2100, Japan) operating at 200 kV. An aqueous solution of phosphortungstic acid stain (1% w/v) was used as a negative staining agent. The liposome samples were first diluted (1:10) in Tris buffer pH 7.4 at 37 ° C and 20 µl of aliquot was added to a transmission electron microscopy (TEM) grid (carbon-coated copper grid). The solution was then left for 1 minute before being filtered to remove any excess from the grid. Images of the TEM were collected and examined.

2.4. Dynamic light scattering and Zeta potential

Zeta potential, mean particle size and size distribution of liposomes loaded with either Curcumin or Vitamin E or Curcumin combined with Vitamin E and blank liposomes were calculated by using "Nanotrac Wave II, Microtrac, USA" (particle sizing method) for DLS (dynamic light scattering) in 7.4 PH of tris buffer at 25 °C. The experiment was repeated three times, and the results are shown as mean ± standard deviation.

2.5. FT-IR spectra

Lyophilized samples were prepared as KBr disks:1mg of lyophilized compounds were mixed with 100 mg of dried and ground KBr, the vibrational spectra of the compounds were taken using IR spectrometer of type Jasco-6300 FT-IR plus, Japan. The samples were run over wavenumber range 400-4000 cm^{-1} and the background spectrum was that of KBr.

2.6. Differential scanning calorimetry (DSC)

DSC scans were obtained with DSC-50 Shimadzu scanning calorimeter made in Japan. The samples and reference cells of DSC were measured at room temperature, and the degassed lyophilized compounds of weight 0.5 mg. The scan was started (2°C/min scan rate), the compounds should settle to the bottom of the calorimeter cell during the equilibration period. The scan was run to 100-102°C.

2.7. Cell culture

HEP-2 cell line was obtained from Nawah Scientific Inc., (Cairo, Egypt). Cells are maintained in DMEM media containing 100 mg/mL of streptomycin, 100 units/mL of penicillin, and 10% of heat-inactivated fetal bovine serum in humidified, 5% (v/v) CO_2 atmosphere at 37 °C.

2.8. Cytotoxicity assay

SRB assay was used to assess cell viability. Aliquots of 100 µL cell media for 24 h. Cell suspension ($5x10^3$ cells) were plated in 96-well plates and incubated in complete were treated with another aliquot of 100 µL media containing drugs at different concentrations ranging from (0.01,0.1,1,10,100 ,200, 400 ug/ml). After 72 h of drug exposure, cells were fixed by replacing media with 150 µL of 10% TCA and incubated at 4 °C for 1 h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70 µL SRB solution (0.4% w/v) were added and incubated in the dark at room temperature for 10 min. Plates were washed 3 times with 1% acetic acid and allowed to air-dried overnight. Then, protein-bound SRB stain was dissolved by adding 150 µL of TRIS (10 mM); the absorbance was measured at 540 nm using a BMG LABTECH®-FLUO microplate reader (Ortenberg, Germany).

3. Results and Discussion

The entrapment efficiency percentage was found to be higher than 90% for all prepared liposomal suspensions when the drug was mixed with the lipid powder before dissolving it in ethanol.

TEM images showed that, as shown in **Figure 4**, the morphology of all liposomes prepared in this work was almost spherical in shape, well dispersed and less aggregated for empty and encapsulated vesicles. The size of empty liposomes was concentrated around 142±20.02 nm while curcumin-doped liposomes were within the range of 140±45.46 nm (**Figures 4A and B**). Vitamin E doped with liposomes was in the range of 186±59.96 nm **(Figure 4C)**. In the TEM image of liposomes encapsulated curcumin coupled with Vitamin E, the size was in the range of 241±46.23 nm **(Figure 4D)**.

TEM findings showed that Curcumin can be physically correlated with disrupting the membrane packing property of liposomes on the surface. The size of liposomes that trap Curcumin decreased significantly from 142±20.02 nm to 140±45.46 nm in the TEM image of liposomal Curcumin. The presence of curcumin in liposomes reduced the spacing between the neighboring bilayers, resulting in lower-sized liposomes relative to the controls. The reduction in particle size may be due to stronger drug interactions with the lipid bilayer of liposomes through hydrogen bonding. It is supported that Curcumin could be inserted in the hydrophobic region of the bilayer and these findings are in good agreement with the data observed by the DSC and FTIR results.

Colloidal suspension particle homogeneity is effectively accounted for by the polydispersity index (PDI). Values above 0.7 mean that the sample has a very wide size range and that the dynamic light scattering technique is therefore not stable **[19]**.

Fig(5A) showed the size distribution of a pure PE sample that concentrated around **170.4±111.266** nm and mean size diameter with **0.405** PDI. **Fig(5B)** displays a decrease in mean size diameter of pure PE to **139.63±88.98** nm with **0.299** PDI upon the encapsulation of Curcumin into PE and

this probably could be attributed to the electrostatic attractive force between the positive charge of PE $N(CH_3)_3^+$ group and the negative charge of OH^-group of Curcumin where Curcumin drug is mainly considered as a lipophilic drug and could be entrapped in the hydrophobic core of the bilayer.

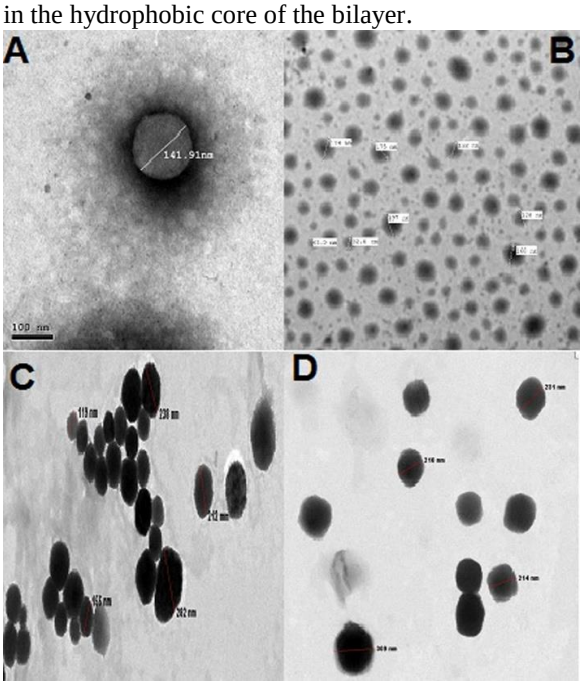

Fig. 4. TEM images for blank liposomes (A), Curcumin-loaded liposomes (B), Vitamin E-loaded liposomes (C) and Curcumin combined with Vitamin E into liposomes (D).

The inclusion of Curcumin into liposomes decreased the spacing between the adjacent bilayers resulting in the formation of liposomes smaller in size compared with the control ones. The incorporation of Vitamin E into PE resulted in an increase in the calculated mean size diameter of blank liposomes to **187.8±117.06** nm with **0.265** PDI. The inclusion of Vitamin E into liposomes was increased the spacing between the adjacent bilayers resulting in the formation of liposomes larger in size compared with the control ones. The increase of particle size may be due to stronger drug interactions with the polar head group of phospholipids via hydrogen bonding near PO_2^- group. **Fig(5C)**, **Fig(5D)** showed that after incorporation of Curcumin associated with Vitamin E together into PE liposomes, the calculated mean size of blank PE was increased to **187.73±90.33** nm with **0.379** PDI. These results indicate that the liposomes may be physically associated with Curcumin or Vitamin E at the core and the molecule of them tends to have interacted to large extent with the lipid bilayer and perturbed them. The results of the particle size depicted by TEM in **Figure (4)** agree with the results obtained by DLS measurements (**Figure 5**).

Table 1 : Summary of data obtained by dynamic light scattering (DLS) by intensity with Polydispersity index (PDI)and zeta potential for all formulations.

Sample	DLS		Zeta Potential
	Mean size ± SD (nm)	PDI	Mean Zeta Potential ± SD (mV)
Empty liposomes	**170.4±111.266**	**0.405**	**-25.43±8.293**
Liposomal Vit E +Curcumin	**187.73±90.33**	**0.379333**	**-37.1333±8.59**
Liposomal Curcumin	**139.6333±88.98**	**0.299**	**-40.76±9.3**
Liposomal Vit E	**187.8±117.06**	**0.265**	**-51.66±7.36**

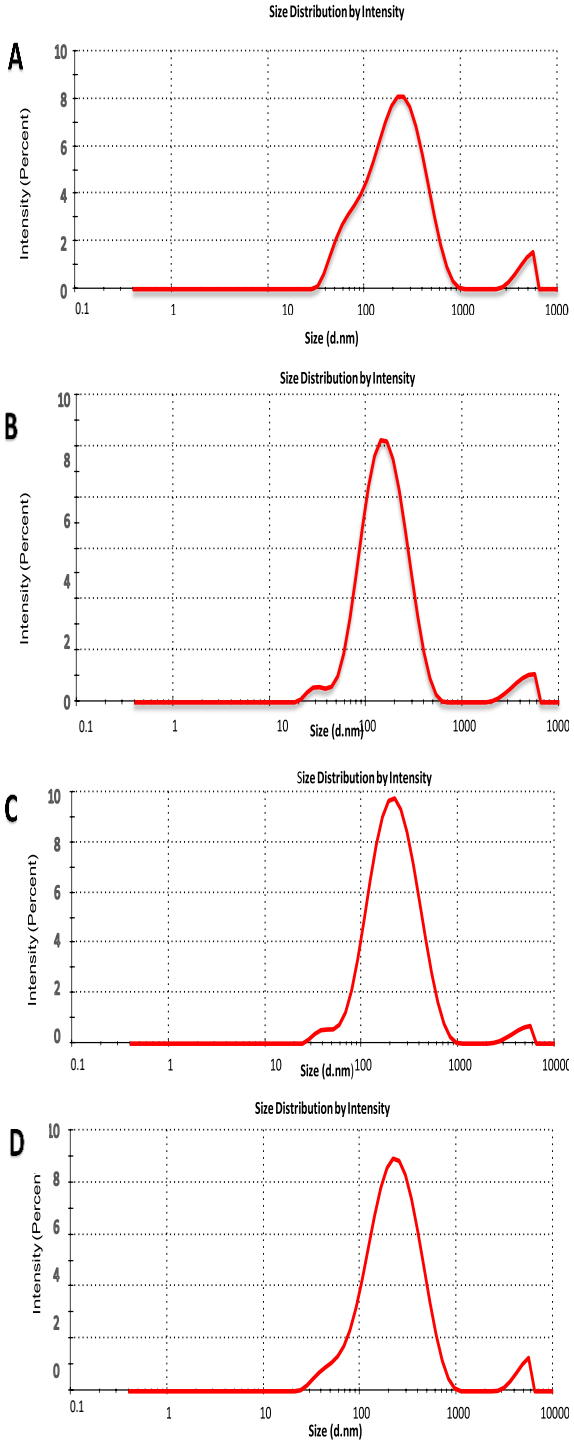

Fig.5. Size distribution of liposomes determined with dynamic light scattering (DLS) for (A) empty PE liposomal sample, (B) Curcumin-encapsulated liposomes, (C) Vitamin E-encapsulated liposomes and (D) Curcumin combined with Vitamin E into liposomes.

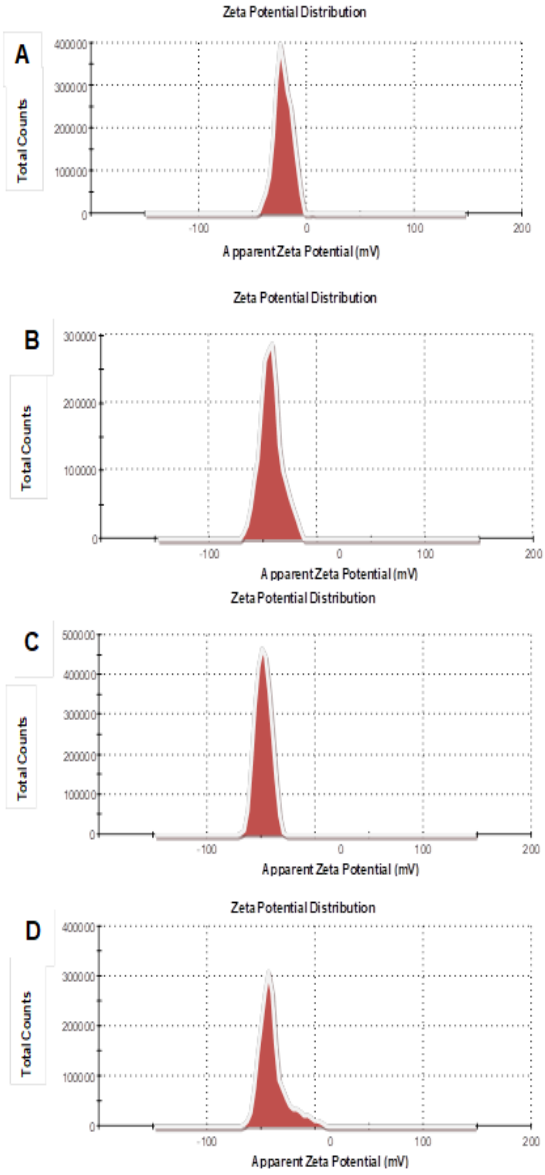

Fig.6. Zeta potential for (A) empty PE liposomal sample, (B) Curcumin-encapsulated liposomes, (C) Vitamin E-encapsulated liposomes and (D) Curcumin combined with Vitamin E into liposomes.

The zeta potential analysis was used to determine the stability of the synthesized NPs. The zeta potential analyses the drug charges on the surface of the material. This charge contributes significantly to the increased stability of NPs.

The magnitude of the zeta potential provides an indication of the colloidal system's potential stability. The repulsion between particles would be greater as the zeta potential increases, resulting in a more stable colloidal dispersion. If all suspended particles have a strong negative or positive zeta potential, they will appear to repel each other, and the particles will not tend to come together [20].

According to the observation of others, empty liposomes displayed negative zeta potential (**-25.43±8.293** mV) [21,22,23,24]. Curcumin or Vitamin E-loaded liposomes had higher negative zeta potential (**-40.76±9.3** mV and **-51.66±7.36** mV, respectively) than blank liposomes due to the integration of both into the PE liposomal membranes. Particles with zeta potentials of +30 mV or more positive ethanol than -30 mV are generally considered stable in general. The incorporation of Curcumin or Vitamin E appears to increase the density of negative charge and hence made the zeta potential negative. The liposomal formulation in the presence of Curcumin combined with Vitamin E recorded the lowest zeta potential value among drug formulations (**-37.1333±8.59** mV) as the repulsion phenomenon between particles are smaller, thus leading to a less stable colloidal dispersion (**Fig.6**).

Due to altered interactions between the encapsulated drugs and liposomes, the Differential Scanning Calorimetry is used to characterize the melting and crystallization activity of crystalline materials [25].

The PE vesicles were used as a model membrane because this phospholipid can mimic many characteristics of biological membranes. Pure PE vesicles after dehydration displayed a significant major endothermic peak (T_m) at 144.638 °C (**Fig.7)** when submitted for DSC examination, in accordance with [26,27,28]. For pure PE liposomes, the pre-transition temperature (T_p) was about 70 °C.

The presence of compound in the membranes of the PE may affect the vesicle transition's thermotropic parameters. The introduction of Curcumin into PE liposomes showed a small shift to a higher temperature at 145.2 °C compared to the main endothermic peak (T_m) of empty PE that presents at 144.638 °C, which iAAndicates that the phase-transition temperature of lipid membrane directly influences its liquidity, which in turn affects the release of curcumin from liposomes. Meantime, the drug molecules might disrupt hydrogen bonds spanning adjacent head-groups, thereby destroying the specific structural arrangement of a particular polar head group region, further reducing the melting point of the liposomes [29] Curcumin had a substantial effect on the PE bilayer of acyl chains causing a conformational disorder within the phospholipids and decrease the transition cooperatively of lipid acyl chains [30,31]. The pre-transition temperature (T_p) peak for Curcumin liposomes is shifted to lower degree (68.99 °C), which revealed that Curcumin interacted with the polar head group of phospholipids.

Interestingly, upon the incorporation of Curcumin combined with Vitamin E into PE liposomes did not cause any noticeable shift to the major characteristic endothermic peak of pure PE that exists at 144.638°C. The pre-transition temperature was found to be shifted from 70 °C to 65.99 °C for PE liposomes doped Curcumin combined with Vitamin E which revealed a membrane rigidizing effect. The insertion of the drug between the polar heads of the PE will facilitate the creation of a less orderly liquid crystalline phase than the gel phase and as observed by DSC, slightly reduce the transition temperature of the gel-to-liquid crystal phase [32]. The main endothermic peak of pure PE, which occurs at 144.638°C, remained unchanged when Vitamin E was incorporated into PE liposomes. It has been found that the pre-transition temperature for Vitamin E liposomes was also not changed. Using DSC, it has been found that mixtures of PE and Curcumin or Vitamin E display a single peak, indicating that they are miscible [33].

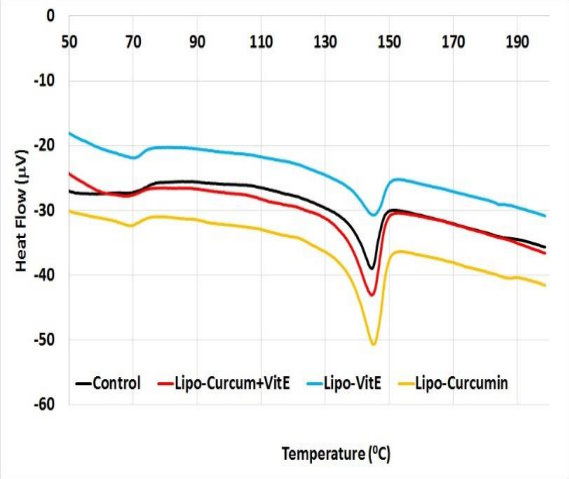

Fig.7. DSC curves for pure PE, liposomes doped with either Curcumin or Vitamin E or both of them.

FT-IR spectroscopy was used to analyze possible changes in the structure of PE by recording the wavenumber of different functional groups considering the acyl chains and the lipid molecule head group region in the presence or absence of foreign molecules.

In three different spectral areas, namely 3600-2800 cm^{-1}(**Fig.8**), 1800-1400 cm^{-1} (**Fig.9**), and 1400-800 cm^{-1} (**Fig.10**), FT-IR spectra of empty PE liposome was compared with Curcumin or Vitamin E or both of them/ PE liposomal samples

The highest absorption FTIR characteristic peak was shown by the PE liposome spectrum [34]. Encapsulation of Vitamin E or Vitamin E combined with Curcumin into the PE liposomes caused a change in the wavenumber of the antisymmetric CH_2 stretching bands in the acyl chain **(Fig.8)**, indicating that Vitamin E or Vitamin E combined with Curcumin produce a conformational disorder within the phospholipid's acyl chains. In other words, they affected the order of the membrane. The peak at 2917

cm^{-1} for the pure PE is shifted towards higher wave number 2919.155 cm^{-1} for both Vitamin E or Vitamin E combined with Curcumin liposomes. This may indicate that the number of gauche conformers is increasing, implying a rise in a bilayer disorder [35]. Interestingly, the signal intensity of Curcumin or Vitamin E or a combination of them-loaded liposomes has become more intensive. After the incorporation of Curcumin into PE, the peak at 2917 cm^{-1} for the pure PE is remained stable, suggesting a stabilization of the system in the gel and liquid phase.

The peaks of CH_2 symmetric and asymmetric stretching vibrations have been used as a sensitive alkyl chain ordering indicator. There are substantial changes in the CH_2 stretching band wavenumber, showing that both Vitamin E and Vitamin E combined with Curcumin liposomes increased the number of gauche conformers, suggesting an increase in bilayer conformational disorder [36,37].

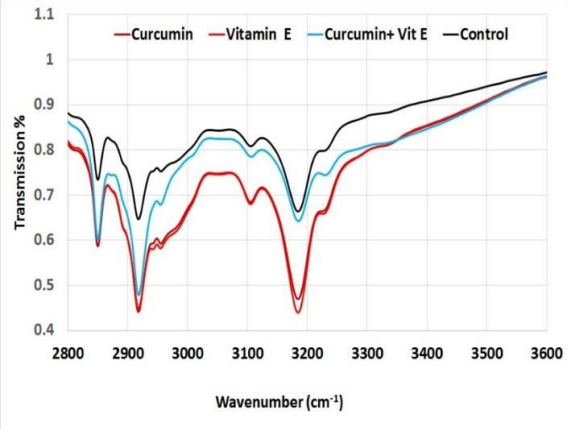

Fig. 8. The magnified part (3600–2800 cm^{-1}) of FTIR spectra of empty PE and PE / Curcumin or vitamin E or both liposomal samples.

For the interaction between Curcumin or Vitamin E or both of them and the glycerol backbone near the head group of phospholipids in the interfacial zone, the C =O stretching band is analyzed [38]. As seen from (**Fig.9**), the wavenumber value of C=O group at 1736.66 cm^{-1} is decreased for the liposomal samples containing Curcumin (1728.42 cm^{-1}), with an evidence of hydrogen bonding formation. In the glycerol backbone region of the PE molecule, shifts in the contours of ester C =O stretching regulated the degree of hydrogen bond formation.

As seen from (**Fig.9**), the wavenumber value of C=O group at 1736.66 cm^{-1} was shifted to higher frequencies at 1738.72 cm^{-1} for the liposomal sample containing Vitamin E or a mixture of Vitamin E with Curcumin, without any evidence of hydrogen bonding formation. According to empirical rules, decreasing frequency values mean that existing hydrogen bonds are being strengthened or that new hydrogen bonds are being formed between the components [35].

The absorption bands of the C =O ester are vulnerable to changes in the polarity of their immediate surroundings and are affected by hydrogen bonding and other interactions. Therefore, any change in the spectrum of this area may be due to the interaction between Curcumin or Vitamin E or mixture of them and a polar/ polar interfacial region of the membrane [35].

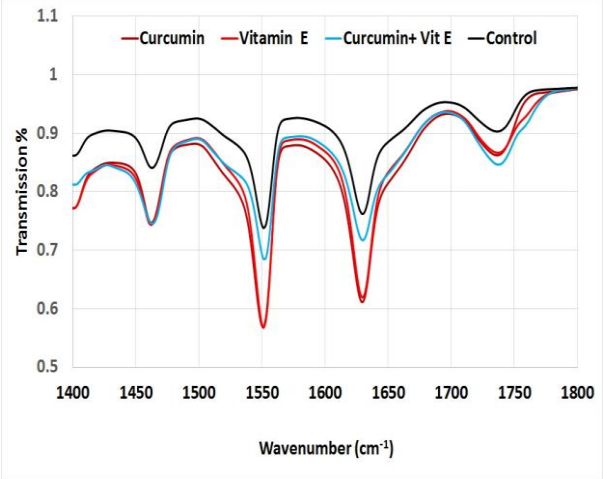

Fig. 9. The magnified part (1800–1400 cm^{-1}) of FTIR spectra of empty PE and PE / Curcumin or vitamin E or both liposomal samples.

The interaction between Curcumin or Vitamin E or a mixture of them and the head group of PE liposomes was studied through the PO_2^- antisymmetric stretching band, which is located at 1219.57 cm^{-1}. **Fig.10** shows the PO_2^- antisymmetric stretching band for PE liposomes formulations in the absence and presence of Curcumin or Vitamin E or a mixture of them.

As seen from (**Fig.10**), the wavenumber was shifted to higher values after the addition of Curcumin (1223.69 cm^{-1}) into PE liposomes. This implied the absence of hydrogen bonding between the liposome head group and curcumin. The decrease in the value of the wave number implies that existing hydrogen bonds are strengthen or new hydrogen bonds between the components are formed[35]. The wavenumber was shifted to lower values after the addition of Vitamin E (1215.45 cm^{-1}) or Vitamin E mixed with Curcumin (1209.27 cm^{-1}) into PE liposomes. This implied the presence of hydrogen bonding between the liposome head group and Vitamin E or mixture of Vitamin E with Curcumin.

Table 2: The chemical shifts observed for Curcumin or Vitamin E or a mixture of them after the incorporation into PE liposomes.

Peak assignment	Wavenumber (cm^{-1})	Wavenumber (cm^{-1})			
		Control	Curcumin	Vitamin E	Curcumin mixed with vitamin E
Symmetric stretching vibration of CH_2 in acyl chain	(2800–2855)	2849.1	2849.1	2849.1	2849.1
Antisymmetric stretching vibration of CH_2 in acyl chain	(2916–2921)	2917	2917	2919.155	2919.155
Carbonyl stretching vibration C=O	(1730–1740)	1736.66	1728.42	1738.72	1738.72
CH_2 bending vibration	(1456–1470)	1462.66	1462.66	1462.66	1462.66
Aliphatic phosphates (P-O-C stretch	(920-1088)	1036.22	1036.22	1036.22	1036.22
Antisymmetric PO_2^- stretching vibrations	(1215–1260)	1219.57	1223.69	1215.45	1209.27
$(CH_3)_3N^+$ symmetric deformation	1405	1396.74	1398.80	1398.80	1402.92
$(CH_3)_3N^+$ antisymmetric stretching	972	972.36	972.36	972.36	972.36

In addition, for all molecules studied in PE liposomal samples, the value of N $(CH_3)_3^+$ symmetric deformation band at 1396.74 cm^{-1} is modified (**Fig.10)**. This may be attributed to the presence of new intermolecular hydrogen bond formation between Curcumin or Vitamin E and N $(CH_3)_3^+$.

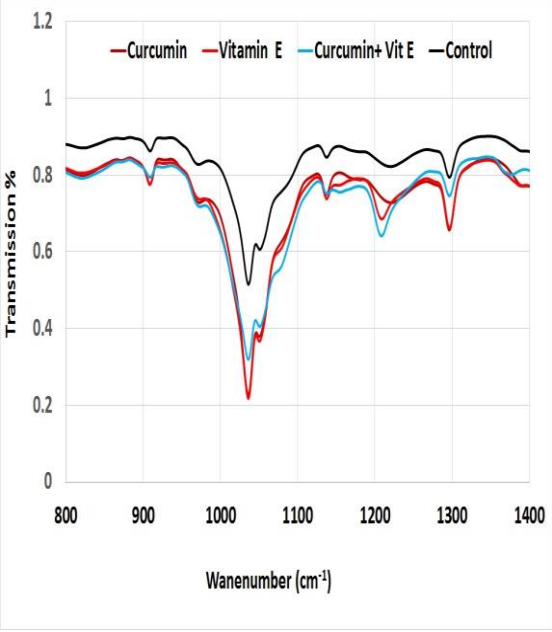

Fig.10 . The magnified part (1400–800 cm^{-1}) of FTIR spectra of empty PE and PE / Curcumin or vitamin E or both liposomal samples.

The efficacy of the drug delivery system was investigated using cell viability assay (*In-Vitro* cytotoxicity SRB) at different drug concentrations of Curcumin, Vitamin E, Curcumin with Vitamin E and separately their liposomal formulations against laryngeal carcinoma (Hep-2) cell lines [39]. Untreated cells acted as monitors at the zero concentrations of each drug. Separately, the Hep-2 cell lines were incubated at different drug concentrations with the same sequence 0.01, 0.1, 1, 10, 100, 200, and 400µg/ml (**Fig.11**). The assay was terminated after 72 hours and measurements of cell viability were performed.

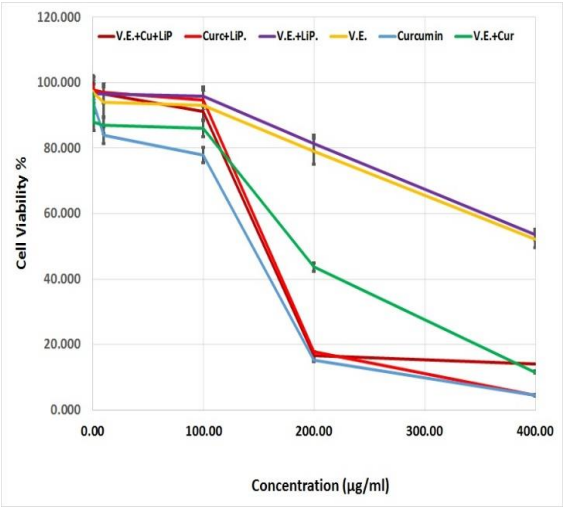

Fig.11.Cytotoxicity of free Curcumin, free Vitamin E, Curcumin combined with Vitamin E and separately their liposomal formulations against laryngeal carcinoma (Hep-2) cell line).

Curcumin and its liposomal form had shown little cytotoxicity when the concentrations of curcumin were less than 10 µg/ml [40]. After the drug concentrations exceeded 100 µg/ml, all of them had shown strong inhibition of cell growth. Curcumin liposomes maintained a stable cell viability of about

80% up to 100 µg/ml, but Curcumin had shown continually increasing inhibition with increased concentrations against Hep-2 cell lines. This result agrees with [41] which shows that curcumin inhibits cell proliferation, and increased caspase-3 activity and promotes apoptosis of human laryngeal cancer cells. Free Curcumin, Curcumin liposomes shown similar anti- Hep-2 cell effects at a high concentration (*e.g.*, 200 µg/ml) with viabilities of 15.7±8.32%, and 17.2±6.50% at 200 µg/ml, respectively. Therefore, Curcumin *in vitro* shows high anti-Hep-2 cancer cell effect. While free Vitamin E treated cells, the cell viability was roughly 52% and for Vitamin E loaded liposome, the viability was roughly the same 53.6 % at the same concentration (400 µg/ml) , this result evidence for the possible effect of Vitamin E on head and neck cancers is limited [42]. For Vitamin E loaded liposomes treated cells, the increase in cell viability compared to the free vitamin E could be attributed to the sustained release of Vitamin E from liposomes.

Interestingly, Curcumin combined with Vitamin E or its liposomal forms indicated a significant reduction in the cell viability when tested at the same concentration (400 µg/ml) against Hep-2 cell lines. The cell viability was roughly 11.6% and 14% for Curcumin + Vitamin E and the lipo-state of Curcumin + Vitamin E as a mixture, respectively. It seems that the combination of Curcumin with Vitamin E has the capacity to act in synergism.

Cytotoxic activity among various drug formulations at higher concentrations displayed the order of free Curcumin or its liposomal form> Curcumin + Vitamin E >lipo-state of Curcumin + Vitamin E > free Vitamin E> liposomal Vitamin according to (**Fig.11)**.

At the lower concentration approximately at 100 µg /ml, Hep-2 treated cells with free Curcumin displayed cell viability 77% relative to their liposomal form of about 95%, while 86% and 91% of the cell remained viable for Curcumin + Vitamin E and its liposomal form, respectively (**Fig.11)**.

The IC50 value for free Curcumin in cytotoxic assay with Hep-2 treated cells has counted a minimum of 146 µg/ml, while liposomal Curcumin treated cells for Hep-2 treated cells were counted as 157µg/ml. IC50 was 190.5 µg/ml for Hep-2 treated cells with free mixture of Curcumin + Vitamin E and 153µg/ml for lipo-state of Curcumin + Vitamin E. This improved efficacy can be attributed to the lipo-solubilized state of the drug due to its entrapment within multiple lipoidal domains of vesicles. Based on the above results and depending on the cancer cells type, free Curcumin showed the highest therapeutic efficacy against Hep-2 cell line (**Fig.12**). It can be noticed that the IC50 of Vitamin E and its liposomal form were not applicable in cytotoxic assay with Hep-2

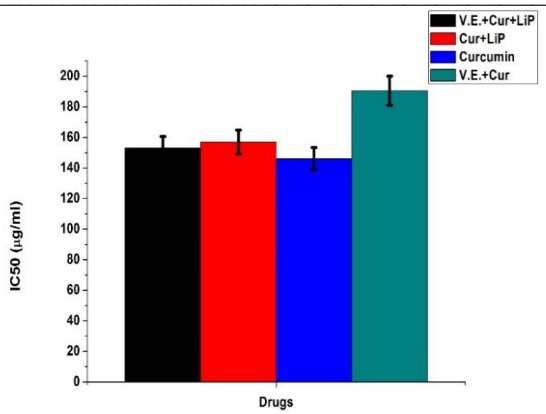

Fig.12. IC_{50} chart for free Curcumin, free Vitamin E or their integration into PE liposomes against laryngeal carcinoma (Hep-2) cell line by using SRB assay, 72 h post-treatment

Conclusion

The worldwide investment and interest in nanomedicine accelerated in the early 2000s and has continued. Because of these efforts, innovative nanodrugs are already available on the market. These sophisticated, effective agents were developed because of an increase in financial investment and partnership among industry, academia, and governments to integrate multiple technologies. One successful approach has been to use drug carriers like liposomes to alter the pharmacokinetics and biodistribution of anticancer drugs. In general, liposome encapsulation of drugs results in (sometimes dramatic) reductions in their volume of distribution and significant increases in tumor accumulation. Liposomes, as carriers for anticancer drugs, have been shown to decrease significantly nonspecific toxicities and to deliver an increased amount of drug effectively to the tumor. Curcumin and its liposomal form inhibited cell growth strongly and the inhibition continually increased with increased concentrations against Hep-2 cell lines. Vitamin E showed a limited effect on Hep-2 cancer cells, while Curcumin combined with Vitamin E or its liposomal forms indicated a significant reduction in the cell viability when tested at the same concentration (400 µg/ml).

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Conflict of interest

The authors have no conflicts of interest to declare.

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