

Original Article

## IN VITRO CYTOTOXICITY EFFECT ON MCF-7 CELL LINE OF CO-ENCAPSULATED ARTESUNATE AND CURCUMIN LIPOSOME

SARVESH SHARMA<sup>1</sup>, VIMAL KUMAR<sup>2</sup>

<sup>1</sup>Department of Pharm, Biotechnology, LNCP, Bhopal, <sup>2</sup>Institute of Pharmacy Nirma University, Ahmedabad  
Email: sarbiotech@yahoo.co.in

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### ABSTRACT

**Objective:** Cancer drug delivery has focused using novel carrier system due to their high drug loading and release controlled property and minimum side effect. Artesunate (ART) and curcumin (CUR) are proved natural herbal lead chemotherapeutics used as Chinese and Indian food. But due to its low absorption and the poor bioavailability limits its clinical efficacy. The object of the present work was to investigate the cytotoxic vs apoptotic effect of these herbal lead molecules in the co-encapsulated liposome formulation.

**Methods:** ART and CUR liposome were prepared by thin film hydration method. Because of their opposite solubility nature, ART loaded in the aqueous phase at pH 7.4 while CUR is present in the central core through lipophilic interaction. *In vitro* cytotoxicity action of the free and liposomal formulation was performed by MTT assay.

**Results:** Physicochemical parameters were evaluated and compared to the co-encapsulated formulation of containing both molecules. The mean diameter around 200 nm, low polydispersity index and co-encapsulation efficiency were found to be 90%. Co-encapsulation in nanometer size is beneficial for uptake and photo-stability of a drug. The *in vitro* cytotoxic effect of the co-encapsulated formulation was shown a better result than individual drug and also gives the clue for apoptosis. IC<sub>50</sub> value for ART, CUR and Liposome was found to be 297.61 µg/ml and 60.60 µg/ml respectively. The result explained the co-encapsulation of curcumin with artesunate is show an ameliorative effect for repositioning therapeutic efficacy of the drug.

**Conclusion:** We observed that human breast cancer MCF-7 cells were relatively resistant to ART and sensitive to CUR. Treatment with ART plus CUR had a synergistic cytotoxic, and apoptotic effect was mediated by up regulation of DR4 and DR5 mRNA expression.

**Keywords:** *In vitro* cytotoxicity, Lipophilic interaction, MCF-7

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### INTRODUCTION

Liposomes are versatile drug delivery carriers which can be used to solve problems of drug solubility, instability and rapid loss. Hydrophilic and hydrophobic drugs can be associated with liposomes. Liposomes can function as sustained release system for drugs and the rate of release can be manipulated. Substantial changes in pharmacokinetics which often accompanies the association of the drug with liposomes. New formulations of liposomes sterically stabilized with substance like surface-grafted polyethylene glycol have circulating half-lives in humans of up to 2 d. In the direction of enhancing the therapeutic effect and reduce toxicity and frequency of drug administration, different novel drug delivery carriers have been developed. Breast cancer in women is a form of malignancy due to changing lifestyle; internal hormonal or biochemical changes are a probable cause of high incidence and mortality for this disease. The characteristic feature of breast cancer can be presence or absence of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor-2 (HER-2).

Tumor necrosis factor related apoptosis inducing ligand (TRAIL), a tumor necrosis factor superfamily member has been shown to induce apoptosis [11]. TRAIL can bind to 5 different receptors *i.e.* TRAIL-R1 (death receptor 4 DR4), TRAIL-R2 (DR5), TRAIL-R3 (decoy receptor, DcR1), TRAIL-R4 (DcR2), and osteoprotegerin (OPG). DR4 and DR5 are the death receptors that signal apoptosis, whereas DcR1, DcR2, and OPG are considered antagonistic because they are unable to induce such signalling due to lack of intracellular death domain or because they are secreted molecule. TRAIL triggers apoptosis through binding to its receptors DR4 and or DR5 in various cancer cell types but not in most normal cells [9, 10].

Artesunate is an effective antitumor drug used to the management of breast cancer. Besides its clinical utility is limited by its optimum

dose calculation, toxic effects like myelo-suppression, nausea, vomiting and renal and cardiotoxicity [1]. Similarly, Curcumin is well-known chemo preventive and chemotherapeutic choice of the drug candidate to control different types of cancer, but their low solubility and bioavailability rapid intestinal and hepatic metabolism are the constraints, so incorporation of these molecules in novel delivery system approaches is demanded to improve its potential. Previous research suggests the positive effects of the combination of curcumin and some polyphenols in oxidative stress markers and chemoprevention [2, 3].

The mechanism of action of artesunate against cancer is not clear but *in vitro* testing for various cell lines proved its cytotoxic behavior [4]. But Curcumin has the variety of biological activity and pharmacological action, such as anti-inflammatory, anti-carcinogenic and anti-virus properties. Recent research suggests curcumin has been inhibiting cell proliferation in a various human cancer cell line and has been used to prevent as well as treat cancer [5].

Several limitations related to systemic administration of single-drug chemotherapy like high clearance, low bioavailability, and multidrug resistance. Single drug therapy may not potent to suppress all cancers cell due to unequal distribution within tumors. Co-encapsulation of these herbal lead molecules in liposome gives the prospect of newer approaches to treating cancer. The vesicular systems are capable of stabilizing photo labile substance, controlling drug release, improved effectiveness and increase the biodistribution of the drug. In the present research, we investigate the comparison of free drug combination and drug combination in liposome through *in vitro* study. So first we evaluated the physicochemical characteristic of formulations and then investigated the cytotoxic effect of the drug. We formulated liposome containing artesunate and curcumin in equimolar concentration. The *in vitro* cytotoxicity was investigated using a spectrophotometric method [8].

## MATERIALS AND METHODS

### Material

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Aldrich (Germany). Roswell Park Memorial Institute (RPMI 1640) foetal bovine serum (FBS) and trypsin were purchased from GIBCO (Germany). All solvents used were HPLC grade from Renkem, India. Curcumin and Artesunate were obtained as gift sample from IPCA, Ratlam Laboratories. Cholesterol, soya phosphatidylcholine (SPC) was purchased from sigma-aldrich Bangalore. Potassium dihydrogen phosphate, Disodium hydrogen phosphate, sodium chloride chloroform and methanol were purchased from Qualigance Chemicals Mumbai, India.

### Preparation of liposome suspension

Conventional liposomes were prepared according to the thin film hydration methods according to the optimised molar ratio of lipid and cholesterol [7]. Accurately weighed quantities of curcumin (10 mg), soya lecithin (09 mg) and cholesterol (01 mg) were dissolved in chloroform and methanol (2:1) (10 ml). A thin film of the lipid-containing drug was allowed to form on the walls of the round bottom flask by evaporating solvent under reduced pressure using a rotary vacuum evaporator (BUCHI type, Lab India, Mumbai) at a temperature 60 °C and 30 rpm. Residual solvent was evaporated overnight by storing the thin film in a vacuum desiccators. To form MLVs, this layer was hydrated with 10 ml of saline containing 10 mg artesunate, while mixing the flask, which was maintained at a temperature 70 °C and 30 rpm for 1 h. The liposomal suspension was then centrifuged at 5000 rpm for 30 min to separate the free drug and then the pellet of MLVs was resuspended in normal saline. The resulted suspension was sonicated by probe sonicator (Lark Ultrasonicator) for 10 min and filtered with 0.2 microns to get small unilamellar vesicles.

### Size, polydispersity, zeta potential profile and microscopy

All the liposomal formulation were examined for their morphological attributes using a trinocular compound microscope (Optics India) at different magnification and selected formulation to go for further evaluation.

Size, polydispersity and zeta potential were measured by photon correlation spectroscopy. For this determination, 20 µl of liposomal dispersion were diluted 100 folds with saline (0.9 % Sodium chloride). Measurement was carried out at the set temperature of 25±2 °C using a Zetasizer (Malvern 6.01, Instruments. UK)

### Transmission electron microscopy (TEM)

It was employed to observe the internal structure of liposome sample with negative staining methods. The sample was diluted 30 folds with distilled water. Equal volume of diluted sample and 2% ammonium molybdate solution were combined and left of 3 min at ambient condition. A drop of this solution was placed on a formvar-carbon coated copper grid (200 mesh, 3 mm diameter HF 36) for 5 min. The excess liquid was drawn off by filter paper. After drying the grid at room temperature for 5 min, micrograph was made using Philips CM 20 (TEM) camera.

### Cell culture

The human breast cancer cell line (NCCS, Pune) was cultured in RPMI-1640 medium supplanted with 10% v/v fetal calf serum and 100 U/ml ampicillin and 100 mg/ml streptomycin and 2 mmol L-Glutamine incubated in 5 % CO<sub>2</sub> at 37 °C.

### MTT cytotoxicity assay

Cytotoxicity was analyzed by MTT assay. A blank control group (RPMI-1640), a negative control group (untreated cells), alone Artesunate and Curcumin treated group was designed for this experiment. In short 100 µl exponentially growing cancer cell suspension (1X10<sup>6</sup>) were seeded into each well of 96-well plate. Artesunate and Curcumin were then added to each well. The final concentration of Artesunate and Curcumin was 10 to 100 µg/ml. During incubation at 37 °C for 24 h, 20 µl MTT (5 mg/ml final concentration) was added to each well. The plates were then

incubated at 37 °C for an additional 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized at 150 µl DMSO at 37 °C for 10 min. The absorbances of reaction were measured at 570 nm using a microplate reader (Bio-Red model 680). Cell Viability was calculated by the formula: Cell Viability (%) = (absorbance of the treated wells-absorbance of the blank control wells)/(absorbance of negative control well-absorbance of the blank control wells) x 100. All MTT experiments were performed in triplicates.

The 50% inhibitory concentration (IC<sub>50</sub>) of a 24 h exposure, defined as the drug concentration resulting in 50% reduction of cell viability, were then determined from curves of reagent concentration *versus* cell viability at 24 h of incubation for the cell line analyzed. Cell sensitivity to the drug was evaluated by the IC<sub>50</sub> value. An IC<sub>50</sub>-80 µg/ml indicated that the cell was sensitivity to the drug, while an IC<sub>50</sub> ≥80 µg/ml indicated that cells were relatively resistant to the drug [20].

### Toxicity of the combination of artesunate and curcumin

The concentration of Artesunate and Curcumin combination were set as follows:

Artesunate and Curcumin (10-80 µg/ml) on the basis of the effect on cell viability. Cell viability after 24 h exposure was then assayed by the MTT assay. A blank control group, a negative control group, Artesunate alone group, Curcumin alone group, Artesunate with Curcumin group (Cell treated with Artesunate and Curcumin) and Liposomal formulation were employed for this experiment.

### Evaluation of synergistic effect

The synergistic effect of Artesunate and Curcumin was analyzed by the Webb coefficient [6]. Predicted cell viability (C) was calculated by the equation  $C = a \times b / 100$ , where a and b indicate cell viability after the use of each agent. Synergism of drug interaction was indicated by a cell viability of ≤ 70% of predicted value. Based on the synergistic effect observed, the optimum concentration of 0.1 µg/ml Artesunate and 0.5µg/ml Curcumin were chosen for all later studies.

### Cell morphology

Experiments were divided into four groups as follows: Artesunate alone group (cells treated with 0.1 µg/ml ART), Curcumin alone group (cells treated with 0.5 µg/ml CUR), combination group (cells treated with 0.1 µg/ml ART and 0.5 µg/ml CUR), and negative control group (untreated cells). Exponentially growing tumor cells were seeded onto 96-well plate a density of 1 x 10<sup>6</sup>/ml cells/well. Artesunate and or Curcumin were added to each well. After incubation at 37 °C for 24 hr, morphological cell changes were observed under a phase contrast trinocular microscope at 1000X (A55-Olympus).

### DNA fragmentation assay

MCF-7 cells (2 X 10<sup>6</sup>) were suspended in a minimal volume of DMEM medium, 2µl trypsin and different drug and formulation concentration were added and then make the final volume to 2 ml. It was incubated at 37 °C for various h (from 1-24 h). After incubation centrifuge, the cells at 10,000 rpm for 10 min, discard the medium and wash the cells for 2 times in NTE buffer. Now suspend the cells in 2 ml NTE buffer and 2% trypsin (100µg/ml) and add 20% SDS (25µl/ml) and proteinase K (100µg/ml). Again incubate the cells at 37 °C for overnight. Add 1 ml NTE buffer-saturated phenol and 1 ml chloroform and shake the vial 12 times (turning up and down slowly) centrifuge at 10,000 rpm for 10 min (2 phases are seen) and transfer the upper portion to another vial and add 1 ml chloroform, repeat this for 3 times. To this RNase was added and incubated at 35 °C for 2 h. Centrifuge the vial at 10,000rpm for 10 min and decant the supernatant and take the pellet (DNA) and dissolve in TAE buffer. Then the dissolved DNA is subjected to horizontal electrophoresis (Genei, Bangalore) on 0.8 % agarose at 2 V/cm<sup>2</sup> for 16 hr and visualized under UV trans-illuminator (Sunshine Instruments) after staining with ethidium bromide. [19]

### Western blot analysis

Cells incubated with ART (0.1µg/ml) or CUR (0.5µg/ml) alone or in combination for 24 hr were lysed in lysis buffer. Protein concentration was determined by bi-cinchonic acid methods. 40 µg

of cell lysate protein was subjected to 4-15% gradient SDS-PAGE using a Tris-glycine system and then the gel was electroblotted onto polyvinylidene difluoride membranes for 45 min. The membranes were then incubated with 5% non-fat dry milk in PBS for 1 hr in order to block nonspecific binding sites, and then incubated with the appropriate primary antibody concentration (1:2000 for caspase-9, 1:400 for Bax and 1:2000 for  $\beta$ -actin) for 2 h at 37 °C in 5% non-fat dry milk. The membrane was subsequently rinsed in PBS and then incubated for 2 h at 37 °C with secondary antibody (HRP conjugated anti-rabbit or anti-mouse IgG) at 1:2000 dilution. After incubation, membranes were rinsed and blots were visualised by incubation with enhanced chemiluminescence detection reagents. Signal density was obtained by scanning exposed X-ray films on a Bio-Rad imaging system. Normalized density was obtained by dividing the rough density values of a sample band by those of the loading control band ( $\beta$ -actin). Immunoblotting data were quantitated from at least three experiments.

#### Statistical analysis

All of the tests were done in triplicate. The result was expressed as a mean  $\pm$  standard deviation. A probability level less than 5% ( $p < 0.05$ )

was considered significant. The student t-test, one-way ANOVA performed by Microsoft Excel.

#### RESULTS

In order to overcome the undesirable side effects of drugs, synergistic combination of two or more drugs is a flexible strategy (16). In comparison with single drug delivery system, co-delivery of drugs through nanocarriers and convey them to the same cancer cell simultaneously (17, 18). All formulation were analyzed in terms of size (nm), Polydispersity (Pd) and zeta potential were measured by photon correlation spectroscopy, as summarized in the fig. 1. The correlation coefficient is larger to 0.9 it indicates the signal to noise ratio is good.

The mean diameter of Artesunate based liposome was 100 nm and it is suitable for the intra-peritoneal administration. Pd is a dimensionless measure of the broadness of size distribution and value were calculated for each peak as peak width/mean diameter. Zeta potential is around -30mV; this is an evidence for a bigger dispersion electrostatic stabilization in physiological solution.

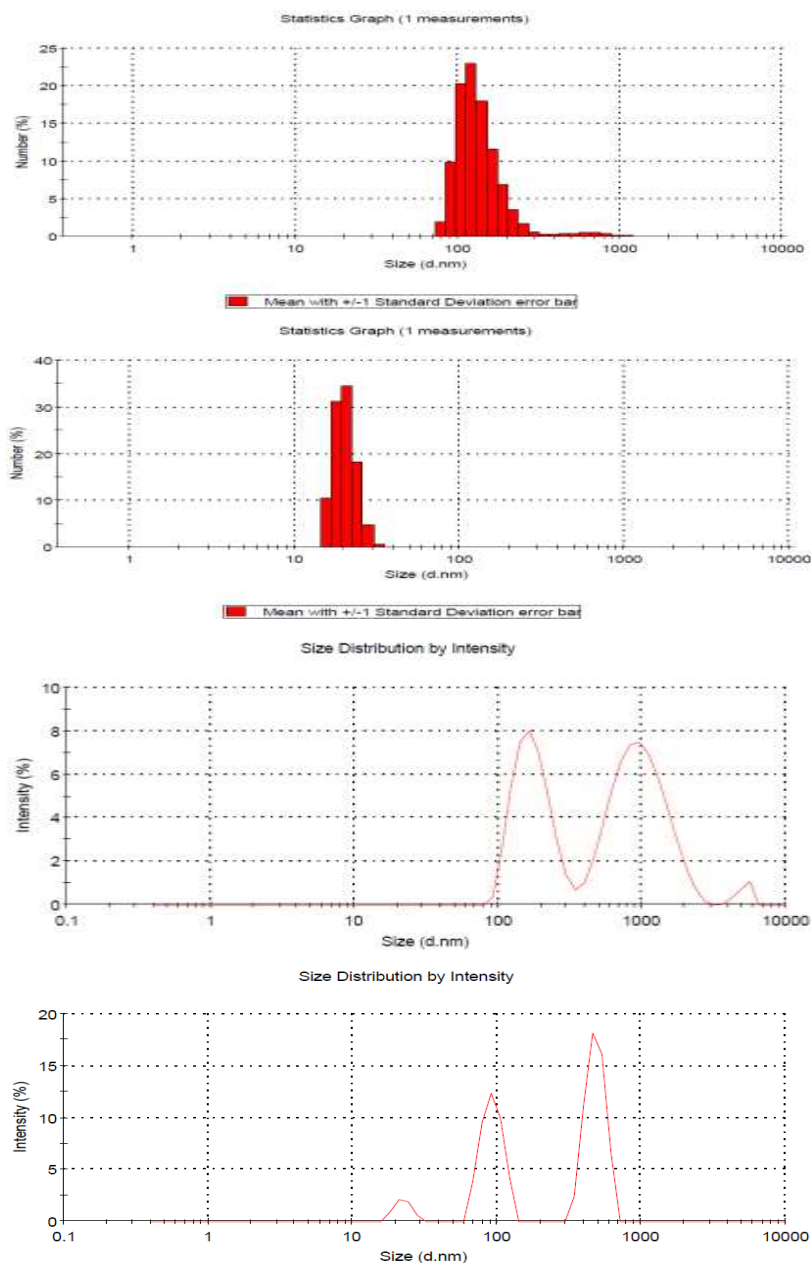
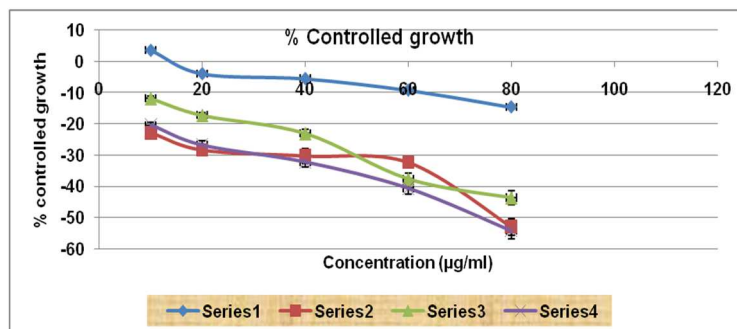
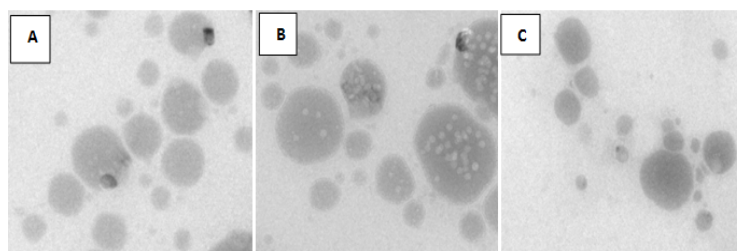


Fig. 1: Size and zeta potential profile of Liposome containing combination of Artesunate and curcumin

**Table 1: Human breast cancer cell line (MCF-7) % controlled growth**

S. No.	Drug and formulation	Drug concentration ( $\mu\text{g/ml}$ )				
		10	20	40	60	80
1	Artesunate	3.6 $\pm$ 0.18	-3.9 $\pm$ 0.19	-5.5 $\pm$ 0.27	-9.2 $\pm$ 0.46	-14.6 $\pm$ 0.73
2	Curcumin	-22.9 $\pm$ 1.14	-28.4 $\pm$ 1.42	-30.3 $\pm$ 2.46	-32.4 $\pm$ 1.62	-53.1 $\pm$ 2.65
3	Artesunate+Curcumin	-11.8 $\pm$ 0.59	-17.2 $\pm$ 0.86	-23.0 $\pm$ 1.15	-37.6 $\pm$ 1.88	-43.6 $\pm$ 2.18
4	Liposome Formulation	-20.4 $\pm$ 1.02	-26.8 $\pm$ 1.34	-32.2 $\pm$ 1.61	-40.6 $\pm$ 2.03	-54.2 $\pm$ 2.71
5	Adriamycin (Doxorubicin)	-35.1 $\pm$ 1.7	-40.7 $\pm$ 1.9	-44.4 $\pm$ 2.2	-46.2 $\pm$ 2.3	-49.3 $\pm$ 2.5

**Fig. 2: Effect of single drug and combination on cell growth of human breast cancer cell line MCF-7 [series 1-ART, series 2-CUR, series 3-ART+CUR and series 4-Liposomal formulation of ART+CUR]****Fig. 3: TEM image of A. ART liposome B. CUR liposome C. ART+CUR liposome****Effect of artesunate or curcumin alone on MCF-7 cell growth**

Twenty h exposure of MCF-7 cells to ART at a range of concentration from 0.01 to 10  $\mu\text{g/ml}$  induce limited cell death, and there was no correlation between cell growth and ART concentration ( $r=0.814$ ,  $P>0.05$ ). IC 50 was 297.61  $\mu\text{g/ml}$  ( $>10 \mu\text{g/ml}$ ), advising that MCF-7 cells were relatively resistant to ART. Similarly 24-h exposure of MCF-7 cells to CUR at a range of concentration from 0.05 to 50  $\mu\text{g/ml}$  strongly induced cell death in a dose-dependent manner ( $r=-0.16$ ,  $P<0.05$ ). IC 50 was 60.60  $\mu\text{g/ml}$  suggesting that MCF-7 cells were sensitive to CUR. fig. 2.

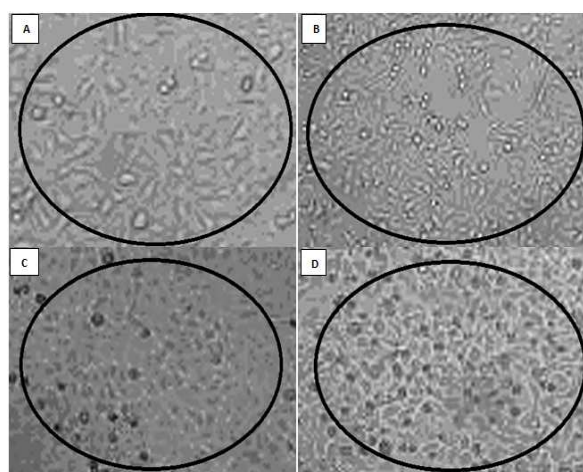
**Effect of combination artesunate and curcumin on MCF-7 cell growth and evaluate the synergistic effect**

Compared to ART alone a group, cell growth showed no significant difference in the combination groups of 0.005  $\mu\text{g/ml}$  Curcumin and 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$  ART, respectively ( $P>0.05$ ). When 0.05 or 0.5  $\mu\text{g/ml}$  CUR was combined with 0.01, 0.1, 1 and 10  $\mu\text{g/ml}$  ART, respectively, showed synergistic cytotoxic effect ( $P<0.05$ ) which was stronger in the group treated with the combination of 0.1  $\mu\text{g/ml}$  ART and 0.5  $\mu\text{g/ml}$  Cur ( $P<0.05$ ) fig. 2.

**Cellular changes of MCF-7 after drug treatment**

24 treatments with ART, some cells were floating and became rounder and smaller and refraction also decreased; some cell debris was observed in the medium. The cells left on the wall became rounder and smaller (fig. 4 A) In CUR alone group, most cells were adhesive. The cells left on the wall underwent significant changes in morphology; the original shape was gone, cells became rounder and larger, and the cytoplasm became rougher (fig. 4 B). In the combination group, the majority of cells were floating and had

irregular cells walls, and there was some cell debris in medium (fig. 4 C), whereas MCF-7 cells in the negative control group did not show obvious morphological changes (fig. 4 D).

**Fig. 4: Morphological changes of human breast cancer cell line MCF-7 after treatment, MCF-7 cells were treated with ART (0.1  $\mu\text{g/ml}$ ) and/or CUR (0.5  $\mu\text{g/ml}$ ) for 24 h and the morphological changes of cells were observed under an inverted microscope (1000X)**

As shown in fig. 7 Lane 3 (Liposome of ART+CUR) had higher fragmentation than Lane 4 free ART+CUR



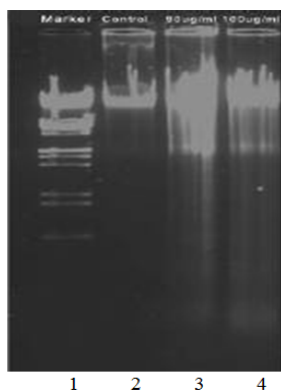


Fig. 5: DNA fragmentation of ART+CUR in MCF-7 cells

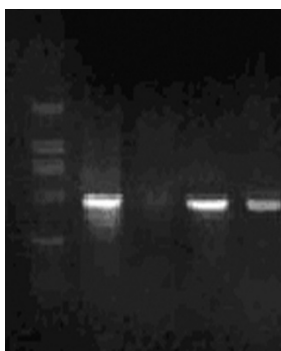


Fig. 6: Expression of ART+CUR mRNA receptor in MCF-7 cells  
Lane M, DL2000; lane 1, DcR2 (453 bp); lane 2, DcR1; lane 3,  
DR5 (502 bp); lane 4 DR4 (506 bp)

## DISCUSSION

Liposome containing artesunate and curcumin were produced and showed consistent characters. Co-encapsulation did not change the properties of the formulation containing an individual drug.

It is essential to interpret the structural characteristics of liposomes such as their size and shape with the incorporation of a combination of the drug. Hence, in order to study the morphological characteristics and incorporation of Artesunate and Curcumin into the bilayer, Zeta-potential and TEM analysis was performed. The morphological characteristics of liposomal formulations at an equimolar concentration of individual drug are shown in fig. 3 (A, B, C).

The release behaviour of the drug *in vitro* can be controlled by regulating the dissolubility, pH and zeta-potential of the formulation. Phosphatidyl-cholein could interact with the negatively charged (acidic) drug when incorporated into films, and this might affect drug release characteristics in the microenvironment. Cationic charge on liposome has also been shown to reduce their biodistribution between the tumor microvasculature and interstitium without impacting overall tumor uptake. The hydrophobic interaction between the drug residues provides for additional stabilization. Faster release of curcumin was also observed for curcumin loaded liposome, which may have more peripheral drug localization and hence faster release.

In this study, we observed that effect of artesunate or curcumin alone and the combination of artesunate and curcumin on MCF-7 cell growth and evaluate the synergistic effect by webb coefficient equation. The % controlled growth and concentration relation was shown in the graphical representation in fig. 2.

After one-day treatment with ART alone group, some cells were floating and became rounder and smaller and refraction also decreased some cells debris was observed in the medium. The cells left on the wall became rounder and smaller. In the CUR alone group, the original shape was gone, cells became rounder and bigger, and

the cytoplasm became rougher. While in combination group morphology of the majority of cells were floating and had irregular cell walls and there was some cell debris in the medium. The last group consider as a negative control group.

Apoptosis is one of the major processes that lead to cell death. A number of chemotherapeutic agents could induce apoptosis in malignant cell lines. artesunate and curcumin combination exerts potent to suppress the breast cancer cells. artesunate and curcumin can induce apoptosis in a broad range of human cancer cell lines while sparing most normal cell types. Thus this combination is a promising therapeutic agent against cancer because of its tumor selectivity. In this study, the data showed that human breast cancer MCF-7 cells were relatively resistant to artesunate but sensitive to curcumin. The mechanism of resistance is not clear, but it may be due to relatively low DR4 levels or relatively high DcR2 expression.

Apoptosis is controlled by multiple pathways that integrate both Intra and extracellular signals. ART controls one such apoptotic pathway by binding to its two death receptors DR4 and DR5 [9, 12]. The extent of apoptosis induced by TRAIL is tightly regulated by the expression of these receptors and by downstream signaling. curcumin, a DNA damaging chemotherapeutic agent, is accepted as a first-line chemotherapeutic agent for breast cancer. It has been demonstrated that DNA damaging chemotherapeutic drugs could sensitize tumor cells that are resistant to TRAIL by up-regulating the expression of DR4 and DR5 in tumor cells [13-15]. We observed that human breast cancer MCF-7 cells were relatively resistant to ART and sensitive to CUR. Treatment with ART plus CUR had a synergistic cytotoxic and apoptotic effect was mediated by up-regulation of DR4 and DR5 mRNA expression. The result showed that co-encapsulated artesunate and curcumin Liposome is a promising approach to improve the performance of medicines and functional foods used to prevent and treat human malignancy. Further, *in vivo* studies will be carried out to confirm these improved effects in the model of other critical cancer types.

## CONCLUSION

Although a combination of surgery and chemotherapy has noticeably improved the survival rate of breast cancer, the application of anti-cancer drugs is still associated with significant adverse reactions, for instance, acquisition of drug-resistant phenotypes, the necessity to develop new and safe chemotherapeutic agents. Recently herbal lead molecule becoming more effective to control and prevent cancers. Experimental data showed that ART or CUR alone leads to 10 or 18 % apoptosis respectively, while their combination effectively increased cell apoptosis to 37.6%. These results suggest that ART combined with CUR had a synergistic apoptotic effect on MCF-7 cells. In addition, these findings also demonstrated that synergy in cytotoxicity was paralleled by synergy in apoptosis. Therefore, we believe that the synergistic antitumor effect of the combination of ART and CUR on MCF-7 cells is due to the induction of cellular apoptosis.

## CONFLICT OF INTERESTS

Declared none

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