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Research Article

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Treatment for Colorectal Cancer: Ursolic Acid Loaded Poly (Lactic-Co-Glycolic Acid) Nanoparticles and Caffiene Loaded Poly (Lactic-Co-Glycolic Acid) Nanoparticles

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Abstract

Cancer is one of the leading causes of adult deaths worldwide. The aim of the present study was to develop UA-PLGA NPs and Caf-PLGA NPs to study its anticancer efficacy in colorectal cells in vitro. Synthesis the nanoparticles, Particle size, size distribution and zeta potential, Drug encapsulation efficiency and in vitro drug release are the method used for this study. In vitro studies was subjected in cancer cell lines and the parameters used were Drug treatment and dose fixation study, Apoptotic morphological changes. The results demonstrated the increased level of anticancer property of UA-PLGA NPs and Caf-PLGA NPs in cancer cells.

Keywords: *Nanoparticles; Anticancer; Ursolic acid (UA); Caffeine (Caf); Poly lactic-co-glycolic acid (PLGA)*

Introduction

Colorectal cancer (CRC) is a formidable health problem worldwide. It is the third most common cancer in men (663000 cases, 10.0% of all cancer cases) and the second most common in women (571000 cases, 9.4% of all cancer cases) [1]. Almost 60% of cases are encountered in developed countries. The number of CRC-related deaths is estimated to be approximately 608000 worldwide, accounting for 8% of all cancer deaths and making CRC the fourth most common cause of death due to cancer [2].

Chemotherapy involves the distribution of cancer-killing chemicals throughout a patient's body via a vein, or in a pill form by mouth, to destroy fast-growing cancer cells that may be lingering after surgery. Chemotherapy can also be used to shrink a rectal tumour before surgery, and treat advanced (Stage IV) disease [3].

Chemotherapy is the use of medicines or drugs to treat a disease, such as cancer. Many times this treatment is just called chemo. Surgery and radiation therapy remove, kill, or damage cancer cells in a certain area, but chemo can work throughout the whole body.

Chemotherapy, a major treatment for cancer patients primary for leukemia's and inoperable solid tumors, adjuvant and neo adjuvant for operable solid tumors often fails the patients due to inherent or acquired multidrug resistance (MDR) [4].

Nanotechnology has led to the development of drug delivery systems to achieve the targeted transport of anticancer drugs [5]. Incorporation of anticancer drugs into the nanoparticles, reduces the adverse reactions and increases the therapeutic efficacy due to

changes in the pharmacokinetics or tissue distribution [6]. Nanoparticles may be delivered to specific sites by size dependant passive targeting or by active targeting [7,8].

Poly (lactic-co-glycolic acid) (PLGA) is one of the most successfully used biodegradable polymers because its hydrolysis leads to metabolite monomers, lactic acid and glycolic acid. Because these two monomers are endogenous and easily metabolized by the body via the Krebs cycle, a minimal systemic toxicity is associated with the use of PLGA for drug delivery or biomaterial applications [9].

Ursolic acid, a pentacyclic triterpenoid carboxylic acid found in plants, has various biological properties, including anti-inflammatory, anticancer, anti-angiogenic, and antioxidative activities [10,11]. UA has antifungal [12], insecticidal [13], anti-HIV [14], diuretic [15], complement inhibitory [16], blood sugar depression [17] and gastrointestinal transit modulating [18] activities.

Caffeine (1, 3, 7-trimethylxanthine) is found in both coffee and tea, so a great number of people are exposed to various doses of caffeine. It acts as a stimulant for the central nervous, respiratory and cardiac system. Caffeine significantly reduces cancer risk caused by environmental and dietary carcinogens [19] and the protective action of caffeine against a variety of chemical carcinogens was established by several studies, carried out by Abraham [20]. The aim of the present study was to develop UA-PLGA NPs and Caf-PLGA NPs to study its anticancer efficacy in colorectal cells in vitro

Materials and Methods

Chemicals

Poly lactic-co-glycolic acid (PLGA) 65:35 (MW 40,000-75,000), poly vinyl alcohol (PVA) (MW 25,000), Ursolic acid (UA), Caffeine (Caf), thiobarbituric acid (TBA), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5, 5-dithiobis 2-nitrobenzoic acid (DTNB), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethidium bromide (EtBr), acridine orange (AO), fetal calf serum (FCS), DMEM medium, glutamine- penicillin-streptomycin solution, ficoll-histopaque 1077, trypsin-EDTA were purchased from Sigma Chemicals Co., St. Louis, USA.

Preparation and characterization nanoparticles

Preparation of drug loaded nanoparticles

UA-PLGA NPs and Caf-PLGA NPs were prepared by nanoprecitation method as previously described by Brown [21]. 0.1% of PVA was prepared by dissolving 100 mg of PVA in 100 ml distilled water in magnetic stirrer at 60ºC. Organic solution of PLGA (100 mg) and UA/Caf (10 mg) in acetone (10 ml) was added to PVA solution (10 ml). The sample was sonicated at 25 watts for 2 min (Sonics VC-130, Sonics and Materials Inc. CT, USA) and kept the sample under magnetic stirrer at room temperature for 6h. To remove the non-incorporated drug, the obtained nanosuspension was centrifuged and washed with distilled water twice at 14,000 rpm for 30 min. The supernatant containing the free drug was discarded and the pellet was freeze dried at -50ºC.

Particle size, size distribution and zeta potential

DLS (Zetasizer Nano, Malvern Instruments Ltd. United Kingdom) was used to measure the average size and size distribution of the prepared nanoparticles. Three different batches were analyzed to give an average value and standard deviation for the particle diameter and zeta potential.

Scanning electron microscopy (SEM)

The morphological features of RSV-GNPs were examined by scanning electron microscopy (Quanta 200F, FEI, Hillsboro, OR, USA). The samples were sprinkled onto a double-sided tape and sputter-coated with a 5 nm thick gold layer. The inner-structure of nanoparticles was observed after fracturing by a razor blade.

Drug encapsulation efficiency

Drug encapsulation efficiency was described by the method of Mathew, 2010 [22]. UA-PLGA NPs and Caf-PLGA NPs were centrifuged at 14,000 rpm for 30 min. The supernatant containing unencapsulated drug was removed. The samples were washed with deionised water and the pellets obtained were re-suspended in deionised water and freeze dried for 48h to get powdered sample. Three mililitre of the supernatant obtained after centrifugation was taken in a cuvette and the absorbance value was recorded at 266 nm using a UV spectrophotometer.

Encapsulation efficiency (%) = [Drug] tot – [Drug] free/[Drug] tot × 100

In vitro drug release

Drug release profiles of nanoparticles were investigated in PBS and 10% FBS medium at pH 7.4 accordingly by the method of Dong, 2004 [23]. Five microgram of lyophilized UA-PLGA NPs and Caf-PLGA NPs were dispersed in 30 ml of 10% FBS/PBS and placed

in water bath shaker set at 37ºC with a shaking speed of 120 rpm. At 1h time intervals 3 ml of supernatant from the sample was taken for analysis and the same amount of fresh 10% FBS/PBS was replaced to the sample. Each time absorbance value at 266 nm was recorded using UV spectrophotometer.

$$\text{Drug release (\%)} = \frac{[UA/Caf]_{rel}}{[UA/Caf]_{tot}} \times 100$$

Anticancer efficacy of drug loaded nanoparticles

Cell lines and culture conditions

The present work was carried out in colon cancer cell lines (HT29). HT29 cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The present work has been approved by Institutional Ethical Committee (IEC), Annamalai University. The cells were grown as monolayer in DMEM medium supplemented with 10% FCS, 1mM sodium pyruvate, 10 mM HEPES, 1.5 g/L sodium bicarbonate, 2mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C in 5% CO_2 incubator.

Drug treatment and dose fixation study

Cells were treated with different concentration of UA-PLGA NPs and Caf-PLGA NPs (6.25, 12.5, 25, 50 and 100 µg) and incubated for 24 h at 5 % CO_2 incubator. Cytotoxicity was observed by MTT assay by the method of Mosmann, 1983 [24]. IC_{50} was calculated by (ED_{50} plus software V 1.0). IC_{50} values were calculated and the optimum dose was used for further study.

Experimental groups

The HT29 cells were divided into 4 experimental groups. Group 1: Untreated control cells, Group 2: UA-PLGA NPs treatment alone (88.10 µg), Group 3: Caf-PLGA NPs treatment alone (49.63 µg) and Group 4: UA-PLGA NPs (88.10 µg) + (after one hour) Caf-PLGA NPs (49.63 µg) treatment.

Apoptotic morphological changes

Apoptotic morphological changes during drug loaded nanoparticles treatment were analyzed by AO/EtBr staining. This dual staining method differentiate condensed chromatin of dead apoptotic cells from the intact normal cell nuclei (Lakshmi, 2008) [25]

Alkaline single cell gel electrophoresis

DNA damage was estimated by comet assay according to the method of Singh., *et al*. 1988 [26]. For analysis of the comet images, the extent of DNA damage was estimated by fluorescence microscopy using the digital camera and analyzed the image by Tritek comet scoring software [27]. DNA damage was quantified by the tail moment, tail length and Olive tail moment [28].

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by DMRT taking $p < 0.05$ to test the significant difference between groups.

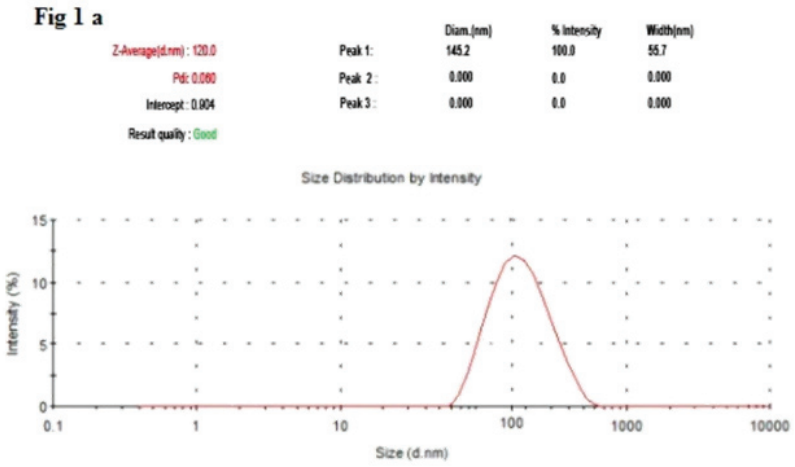
Results

Physicochemical characterization of drug loaded nanoparticles

It has been noticed that the prepared UA-PLGA NPs and Caf-PLGA NPs possess average size of 120 nm and 100 nm and polydispersity index (PI) of 0.060 and 0.054 (Figure 1a and b). Further, the prepared UA-PLGA NPs and Caf-PLGA NPs had zeta potential of −24.8 mV and −14.3 mV. It has been found that 69% of UA and 78% of Caf were encapsulated in PLGA NPs (Table 1). SEM images of the UA-PLGA NPs and Caf-PLGA NPs are shown in Figure 2. The prepared UA-PLGA NPs and Caf-PLGA NPs had smooth surface but with some irregular small particles.

Fabrications variables		Encapsulation Efficiency (%)	Mean Diameter (nm)	Polydispersity index	Size (nm)	Zeta potential (mV)
Polymer	Drug					
PLGA	UA	69 ± 0.21	145.2 ± 80.3	0.060 ± 0.11	120 ± 4.8	-24.8 ± 0.4
PLGA	Caf	78 ± 4.36	133.6 ± 39.5	0.054 ± 0.09	100 ± 4.3	-14.3 ± 0.5

Table 1: *The drug encapsulation efficiency, polydispersity index, size and surface charge of the UA-PLGA nanoparticles and Caf-PLGA nanoparticles.*



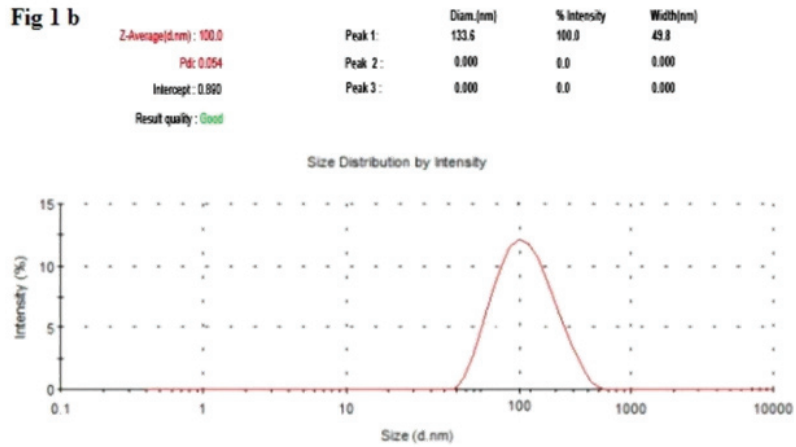


Figure 1A: *The size distribution of UA-PLGA nanoparticles by DLS.*

Figure 1B: *The size distribution of Caf-PLGA nanoparticles by DLS.*

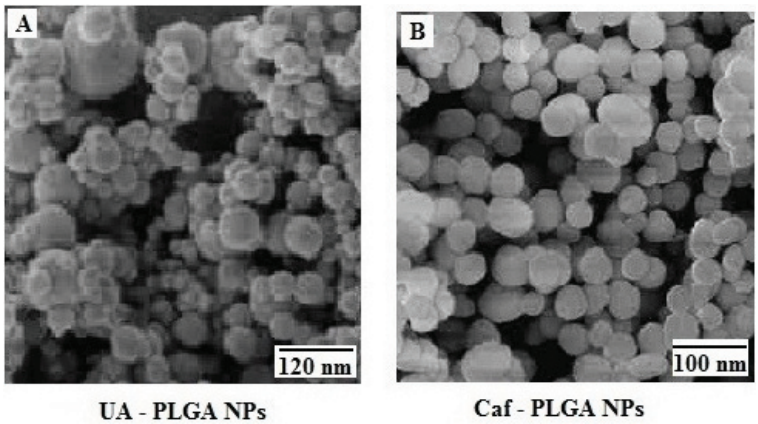


Figure 2: *The morphology of UA-PLGA nanoparticles and Caf-PLGA nanoparticles by SEM.*

Drug encapsulation efficiency and in vitro drug release

Table 1 shows the amount of drug encapsulated in PLGA nanosystem. The encapsulation efficiencies of UA-PLGA NPs and Caf-PLGA NPs are in the range of 69% and 78%. Figure 3 shows the % drug release in PBS during different time interval. After 3h incubation, 7% of UA and 9% of Caf was released in the PBS and the maximum of 69% of UA and 82% of Caf release were observed upon 30h incubation. Upon incubation with 10% FBS, 7% and 69% UA was released in 3h and 30h and 9% and 82% Caf was released in 3h and 30h respectively.

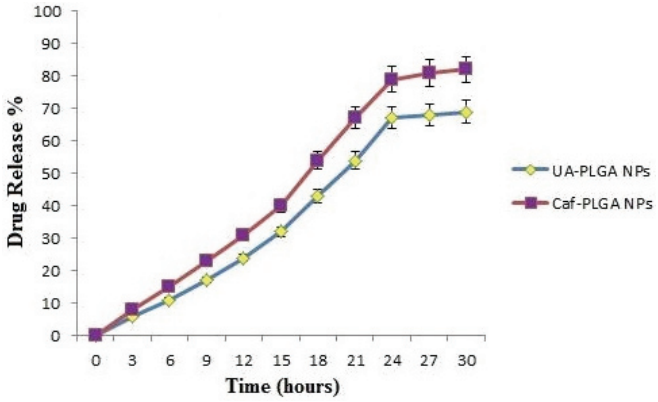


Figure 3: *The percentage of UA and Caf released from PLGA nanoparticles during different time intervals in 10% FBS/PBS.*

Drug treatment and dose fixation study

Figure 4 shows the % cytotoxicity of UA-PLGA NPs, UA, Caf-PLGA NPs and Caf (6.25, 12.5, 25, 50 and 100 µg) in HT29 cells. Inhibitory concentration 50 (IC_{50}) value for UA-PLGA NPs, UA, Caf-PLGA NPs and Caf were found to be 88.10 µg, 79.28 µg, 49.63 µg and 43.29 µg respectively.

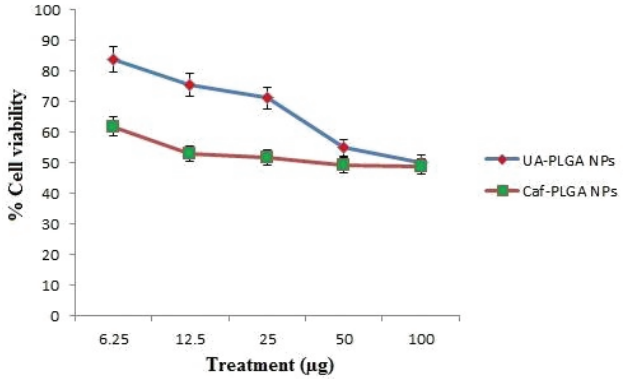


Figure 4: *Optimum dose fixation study by MTT assay. (IC_{50}) value for UA-PLGA nanoparticles and Caf-PLGA nanoparticles was found to be 88.10 µg and 69.63 µg, respectively.*

Apoptotic morphological changes

Figure 5A shows the photomicrographs of apoptotic morphological changes in UA-PLGA nanoparticles, Caf-PLGA nanoparticles and UA-PLGA nanoparticles + Caf-PLGA nanoparticles treated cells. The % apoptotic cell death was increased during UA-PLGA nanoparticles, Caf-PLGA nanoparticles and UA-PLGA nanoparticles + Caf-PLGA nanoparticles treatment. It was found that treated cells showed 64%, 87 % and 93% of apoptotic cells, respectively (Figure 5B).

Fig 5A

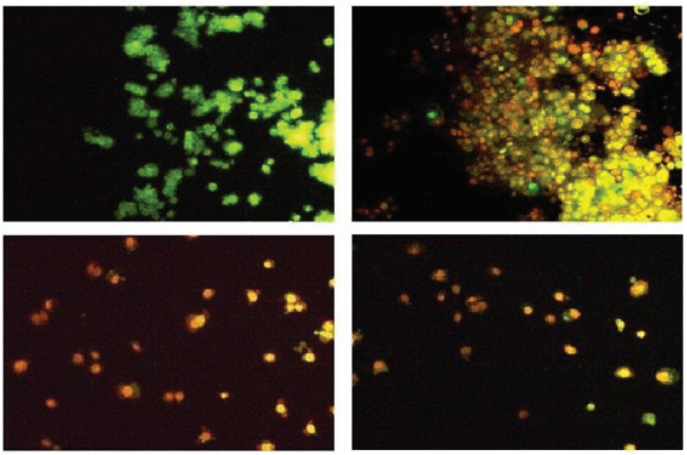


Fig 5B

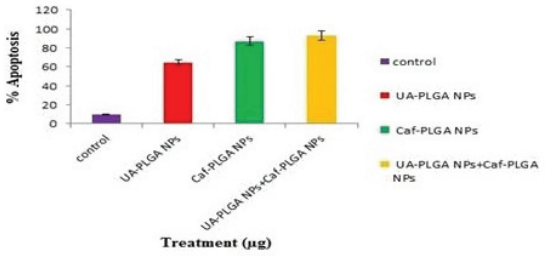


Figure 5A: *Orange-red color indicates the occurrence of apoptosis, while green color indicates the absence of apoptosis in HT29 cells.*

Figure 5B: *Percentage apoptotic cell death was increased in UA-PLGA nanoparticles, Caf-PLGA nanoparticlesand UA-PLGA nanoparticles + Caf-PLGA nanoparticles treated cells.*

Alkaline single cell gel electrophoresis

The changes in the levels of DNA damage in nanoparticles treated HT-29 cells were shown (Figure 6A and B). Significant increase in the levels of DNA damage was observed in nanoparticles treated cells when compared to control. Among the three groups, UA-PLGA

nanoparticles+Caf-PLGA nanoparticles treated cells showed maximal DNA damage when compared to UA/Caf-PLGA nanoparticles treatment alone. UA-PLGA nanoparticles+Caf-PLGA nanoparticles treated cells showed significantly increased % tail DNA (28%), % tail length (54%) and % tail moment (17%) in HT-29 cells (Figure 6C).

Fig 6A

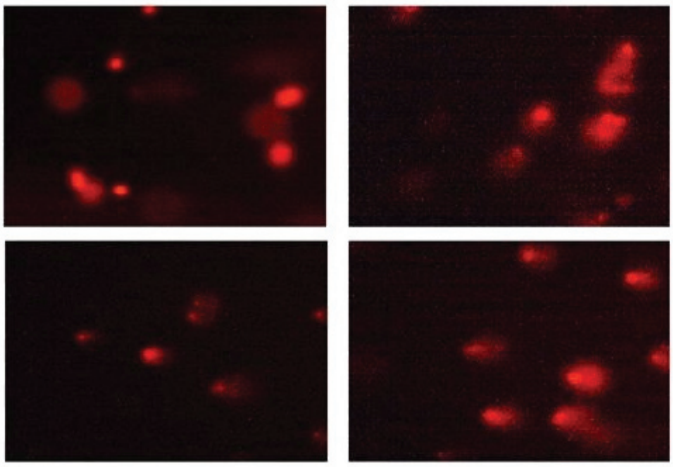


Fig 6B

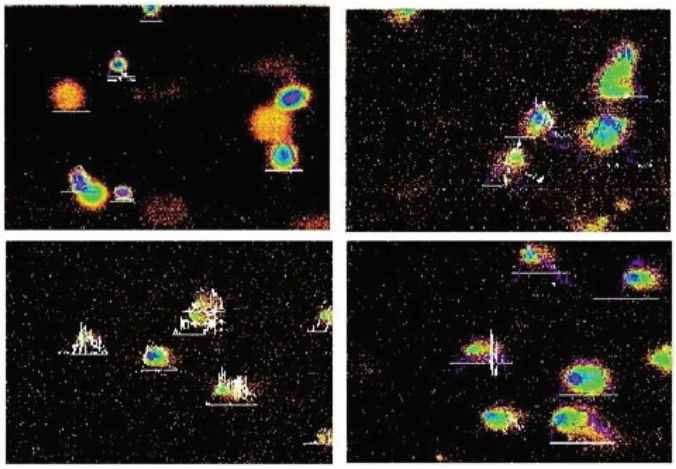


Figure 6A: *Shows the photomicrographs of DNA damage (comet assay). The control cell shows largely non-fragmented intact DNA.*

Figure 6B: *Shows the photomicrographs of DNA damage (comet assay). Comets were scored using Tritek comet scoring software.*

Fig 6C

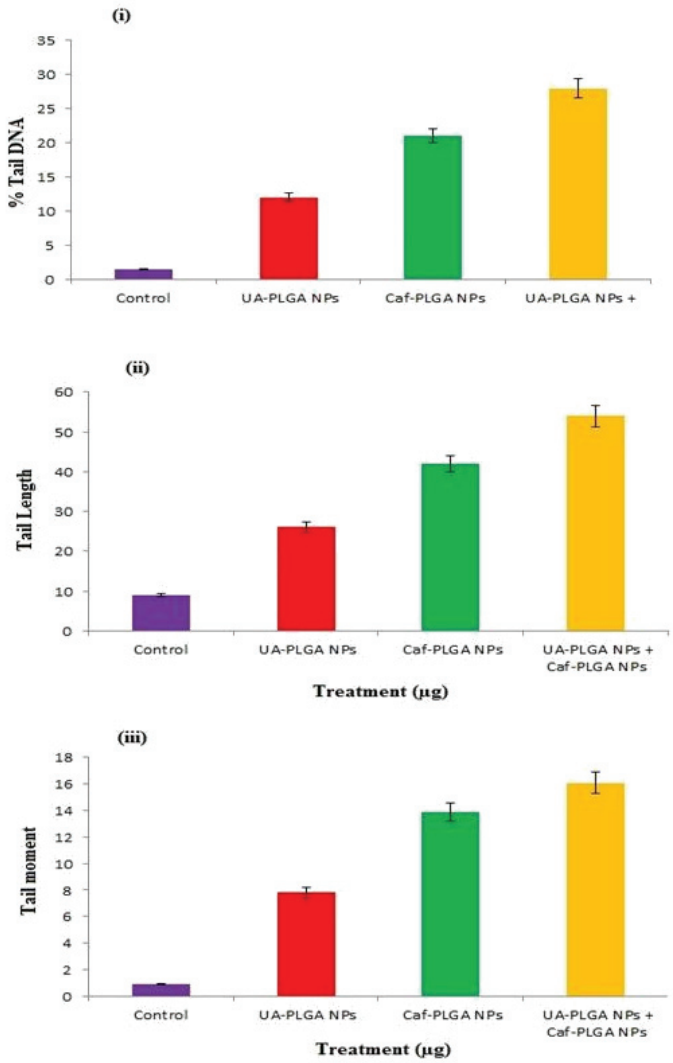


Figure 6C: *(i), (ii) and (iii) show the percentage of tail DNA, tail length and tail moment in HT-29.*

Discussion

The method used in this work allowed the instantaneous and reproducible formation of UA-PLGA NPs exhibiting diameters below 120 nm and low polydispersity indexes, indicating an homogeneous size distribution. The mean zeta potential of UA-PLGA NPs exhibited a negative value of 24.8 ± 0.4 mV and Caf-PLGA NPs exhibiting diameters below 100 nm and low polydispersity indexes, indicating an homogeneous size distribution. The mean zeta potential of Caf-PLGA NPs exhibited a negative value of 14.3 ± 0.5 mV. The method used in this work allowed the instantaneous

and reproducible formation of nanoparticles exhibiting diameter below 120 nm and low polydispersity indexes, indicating an homogeneous size distribution. As mentioned above, these findings were confirmed by the SEM data which also showed that nanospheres were spherical in shape. Nanoparticles were shown to exhibit a negative surface charge which can be attributed to the type of polymer used and more specifically to the presence of polymeric carboxylic groups on the nanoparticle surface [29,30].

Further, we noticed that the PLGA degraded gradually and released the drug in a sustained manner. 7% UA was released in 3h and 69% UA was released in 30 h incubation in the PBS and 9% Caf was released in 3h and 82% Caf was released in 30 h incubation in the PBS. This result indicates that the prepared nanoparticles are useful for controlled delivery system for cancer treatment [31].

We evaluated the anticancer activity of UA-PLGA NPs and Caf-PLGA NPs in HT29 cell line. It was found that UA-PLGA NPs and Caf-PLGA NPs could greatly inhibit the HT29 cell growth. The reason for increased cytotoxicity observed in the UA-PLGA NPs and Caf-PLGA NPs group might be due to increased cellular uptake and sustained drug delivery. Enhanced cytotoxicity during UA-PLGA NPs and Caf-PLGA NPs treatment indicates that PLGA has the potency to transport more UA and Caf into the cells, thus achieving greater cytotoxicity. IC_{50} values for UA-PLGA NPs and Caf-PLGA NPs in our study were 88.10 µg and 49.63 µg. Previously 88.69 µM value reported before for PTX [32]. We have observed UA-PLGA nanoparticles + Caf-PLGA nanoparticles pretreatment significantly increased apoptotic morphological changes in HT29 cells than UA-PLGA nanoparticles and Caf-PLGA nanoparticles treatment alone. Apoptosis has been shown to be a significant mode of cell death after cytotoxic drug treatment [33]. Further studies warrants to explore the merits of UA-PLGA NPs + Caf-PLGA NPs for cancer chemotherapy.

Comet assay, with the advantage of high sensitivity to single strand break, is suitable for assessing the effect of induced DNA damage. The induction of DNA single strand breaks is often used to predict the tumor cells. The extent of DNA damage was greater in the UA-PLGA nanoparticles+Caf-PLGA nanoparticles treated group compared to UA/Caf-PLGA nanoparticles treated group. Phytochemicals have been reported to induce DNA damage in cancer cells by reactive oxygen species generation [34].

Conclusion

This is the first time that, UA/Caf-PLGA nanoparticle formulation was fabricated by a modified solvent displacement method to overcome multidrug resistance in colorectal cancer cells. The data showed that there was an increased level of uptake of UA-PLGA nanoparticles+Caf-PLGA nanoparticles in colorectal cancer cell lines HT-29 in comparison with UA/Caf-PLGA nanoparticles. The cytotoxicity of UA-PLGA nanoparticles+Caf-PLGA nanoparticles was higher than UA/Caf-PLGA nanoparticle in the HT-29 cell culture. However, the UA-PLGA nanoparticles+Caf-PLGA nanoparticles achieved a significantly higher level of cytotoxicity than both of UA/Caf-PLGA nanoparticles, indicating that UA-PLGA nanoparticles+Caf-PLGA nanoparticles could overcome multidrug resistance in colorectal cancer cells and therefore have considerable therapeutic potential for colorectal cancer.

Disclousure of interest

The authors declare that they have no conflicts of interest concerning this article.

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