



Evaluation of the Biodistribution of Arginine, glycine, aspartic acid peptide-modified Nanoliposomes Containing Curcumin in Rats

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Background: Liposomes, as a biological membrane, is successfully used for drug delivery, reduces toxicity in normal cells and improves bio-accessibility of the drug to the target cells. Curcumin, as a bioactive substance with pleiotropic biological activities, is an anti-inflammatory compound and has several anticancer effects in different cancers such as pancreatic and breast cancer.

Objectives: This study was conducted to determine the bio-distribution of arginine-glycine-aspartic acid (RGD)-modified nanoliposomes containing curcumin in different tissues of rats.

Materials and Methods: The amount of curcumin in each tissue was examined by HPLC analysis. The distribution of liposomal Hoechst in the rats was evaluated by using fluorescence spectrophotometry, live animal imaging analyses and histological methods.

Results: HPLC analysis showed the mean of curcumin in the blood significantly increased in the liposomal curcumin modified with RGD compared to free curcumin. These results were confirmed by fluorescence measurement for RGD modified liposome containing Hoechst dye. There was negligible fluorescent intensity in the blood rats, which received Hoechst alone. Live animal imaging analysis showed the presence of fluorescent color in heart tissue for all groups. It was also detected in kidney tissue for liposomal Hoechst modified with RGD group.

Conclusions: The present study demonstrated that RGD-modified nano-liposomes can significantly improve drug retention time in the blood of rats.

Keywords: Biodistribution, Curcumin, Nanoliposome, Rats, RGD

1. Background

In recent years, nanotechnology emerged as a useful technique in medicine as a gene carrier, drug delivery, contrast agents and biosensors (1, 2). Different nanoparticles such as liposomes, micelles, polymeric

nanoparticles are used for smart drug delivery of efficient drugs with limitations for different diseases. A lipid vesicle such as liposome is a kind of nanoparticle made of cholesterol and phospholipids with hydrophobic and hydrophilic properties (3). This nanoparticle

has received more extensive attention because it can envelop the drugs and act as a carrier for drug delivery and macromolecules to cancerous cells or into the tumor tissue. Liposomal envelop technology is used for encapsulation of the drug or cosmetic materials and it can deliver the drug to the pre-determined cells (1). Liposomes have one or two lipid bilayer vesicles that surround a fluid space which makes them very suitable carriers for drug delivery (4,5). They have many proper features including amphipathic properties, low toxicity, biodegradability, and lack of immunogenicity (6). Liposomes can encapsulate different materials such as antioxidants, bioactive substance, cosmetic materials and pharmaceutical compounds. They can also support these materials from free radical and gastrointestinal enzymes such as alkaline and digestive juice (7, 8). Liposomes can enhance the stability of the materials by encapsulating and liberating the content in the cells without specificity. To select delivery, we use different specific ligands such as monoclonal antibodies, peptides, and aptamers in order to modify the surface of nanoparticles.

Arginine-glycine-aspartic acid (RGD) is the most common peptide motif responsible for cell adhesion to the extracellular matrix and mediated cell attachment and is used in targeted cell therapy (9). Ligand-targeted liposomes have more efficacies for the treatment of cancer disease and provide more effective treatment by controlling injury to the normal cells (3, 10). Transmembrane receptors such as integrin can be attached to the RGD motif and facilitate liposome and cell interaction and cause an increase in the uptake of liposomal content to the target cells (11). Liposome-containing bioactive compounds with anti-inflammatory and antioxidant property has also beneficial effects to prevent cardiovascular, cancer and arthritis diseases (12).

Curcumin is a bright yellow substance obtained from the turmeric (*Curcuma longa*) that is a natural phenol with useful properties for the treatment of tumorous cancer cells in different tissues such as breast, prostate and gastrointestinal tract (13,14). It is one of the ingredients used by liposome which is used to prevent the growth of cancer cells. *In vivo* or *in vitro* study indicated that curcumin-loaded liposomes have an efficacy to degenerate tumor cells in lung cancer (12). It has been shown that curcumin has anti-cancer, anti-inflammatory, antioxidant effects and is used as a

traditional chemotherapeutic medicine for the treatment of liver tumor (15).

In our previous study for evaluating the anticancer activity of RGD-modified liposome-containing curcumin showed that it has a high capability for the treatment of breast cancer (14). Despite intense research in the field, we have firstly analyzed RGD-modified liposomes bio-distribution, and thus little is known about them *in vivo* trafficking. In spite of beneficial effects of curcumin, it has low aqueous solubility and absorption which cause restrictions in its therapeutic effects (16). Nanoparticles (NPs) have several physical and chemical properties that encapsulate different substances such as curcumin to increase solubility and delivery to the cancerous cells (17).

Herein, we evaluated the biodistribution profile of RGD-modified nanoliposomes containing curcumin in different tissues, for example, liver, blood, kidney and spleen using various analyses such as HPLC analysis and fluorescent spectrophotometry. **Figure 1** depicts a schematic representation for RGD-modified nanoliposome-containing curcumin and Hoechst for the evaluation of their bio-distribution.

2. Objective

In this study, we evaluated the bio-distribution of liposomal curcumin modified with RGD compared to liposomal curcumin in different tissues by using HPLC analysis. Furthermore, the distribution of liposomal Hoechst modified with RGD was evaluated in blood, liver and kidney by using fluorescence spectrophotometry and live animal imaging analyses

3. Materials and Methods

3.1. Synthesis of Dipalmitoyl-RGD

Dipalmitoyl-RGD peptide was synthesized by solid phase technique utilizing (Fmoc)-chemistry (18). Dimethylformamide (DMF), N-methylpyrrolidone (NMP) solvents (Merck Chemical, Germany) were used for resin swelling and washing; and Piperidine 20% solution (SIGMA, Germany) was used to remove amine-protecting group (Fmoc group) from Rink Amide MBHA resin (GLS, China) surface. The activation and coupling of amino acids were performed using (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Hexafluorophosphate Benzotriazole Tetramethyl Uronium) (HBTU) (GLS, China) and N, N-diisopropylethylamine (DIEA) (Merck Chemical,

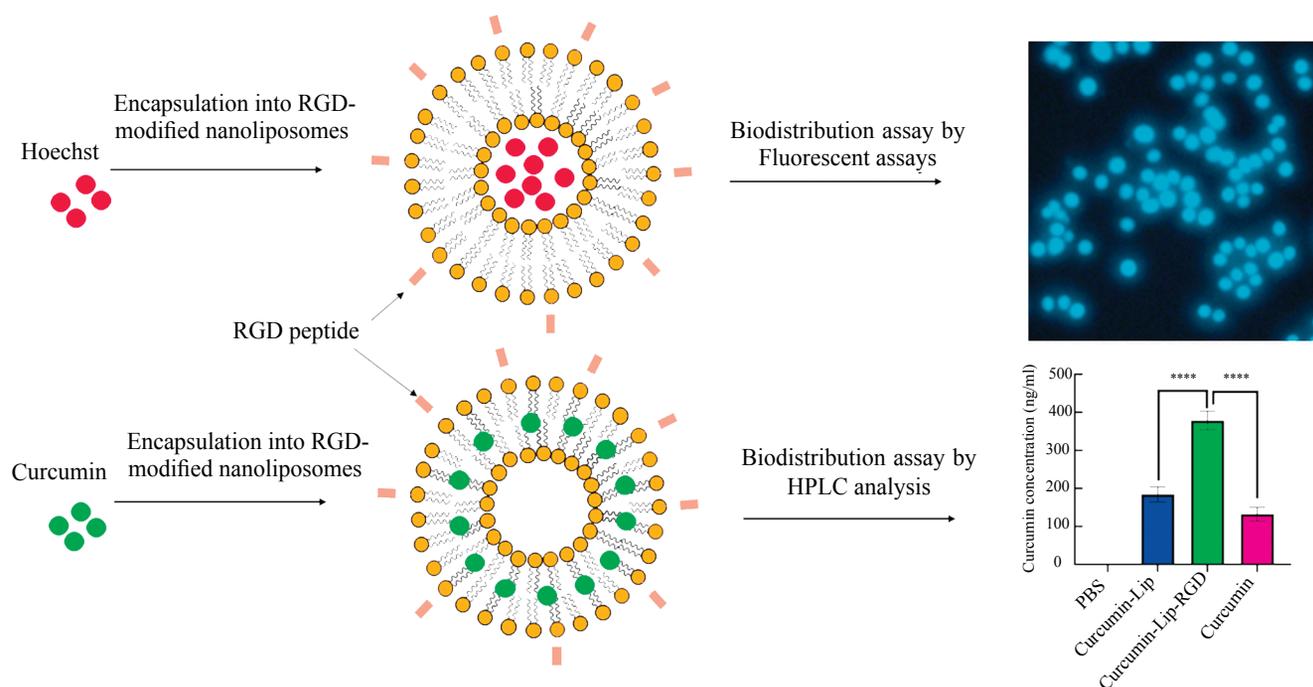


Figure 1. A schematic illustration of RGD-modified nanoliposome-containing curcumin and Hoechst for bio-distribution assays. RGD-modification can significantly change biodistribution of nano-liposomes.

Germany) solutions. Two palmitic acid groups were bonded to the synthesized peptide using HBTU and DIEA solutions as other amino acids. The synthesized peptide was removed from resin using Trifluoroacetic acid (TFA) (SIGMA, Germany) solution and was filtered from resin. Then, ether solution (SIGMA, Germany) was used to precipitate synthesized peptide and was finally dried by a freeze dryer (CHRIST, Germany). The final product was examined by LC-MS analysis and its data were published in our previous report (18).

3.2. Preparation of Liposomes and Drug Encapsulation

Two types of nanoliposomes consisting of dipalmitoylphosphatidylcholine (DPPC) (AVANTI polar, USA): cholesterol (SIGMA, Germany): curcumin (BIO BASIC INC, CANADA) and DPPC: cholesterol: Hoechst dye (SIGMA, Germany) (with 10:1:1: and 10:1:1 molar ratios, respectively) were formulated by thin film hydration method (19, 20). Firstly, lyophilized phospholipids (cholesterol and DPPC) and hydrophobic drugs were weighed and dissolved in 1 mL of chloroform. Then, the solution was transferred to a round-bottomed flask and dried under a vacuum in a rotary evaporator with 150 rpm to form a lipid film. After that, the lipid film was hydrated

by using PBS at 55 °C and 150 rpm for approximately 2 h. Finally, the liposomes were sonicated and stored at 4 °C. Encapsulation efficiency (EE) of curcumin was evaluated by using equation 1.

$$\text{Equation 1: (EE)} = \frac{\text{Cur}_{\text{initial}} - \text{Cur}_{\text{free}}}{\text{Cur}_{\text{initial}}}$$

where $\text{Cur}_{\text{initial}}$ is initial curcumin and Cur_{free} stands for non-encapsulated curcumin in the supernatant after centrifugation.

3.3. Characterization of Liposomes

Dynamic light scattering (DLS) (Malvern Zetasizer 3000, Malvern Instruments, UK) was used to evaluate the particle size at a detection angle of 90° at 25 °C.

The size and morphology of nanoliposomes were examined by transmission electron microscopy (TEM) analysis. One drop of the diluted liposomal solution was placed onto a carbon-coated copper grid (400 mesh, Agar Scientific, UK) and air-dried for 10 min at room temperature. Then, digital micrographs were taken using JEM-1010; JEOL (Tokyo, Japan).

3.4. Animals

Thirty-eight Male Wister rats with weights of 180–200 gr was purchased from Pasteur Institute of Iran and housed in the animal laboratory of Yasuj University of Medical Sciences. All animal experimental procedures

were carried out in accordance with the guidelines of the Iranian Ministry of Agriculture. This study was approved by the biomedical research ethics committee of Yasuj University of Medical Sciences (Code: IR.YUMS.REC. 1397.127).

The animals were divided into 7 groups of 6 rats (Group 1: receiving PBS; Group 2: receiving liposomes with curcumin; Group 3: receiving RGD-modified liposomes with curcumin; Group 4: receiving curcumin alone; Group 5: receiving liposomal Hoechst; Group 6: receiving liposomal Hoechst modified with RGD; Group 7: receiving Hoechst alone) and were used for this study. Each group was then injected with 500 ml of samples via intraperitoneal injection (IP).

3.5. Assessment of Biodistribution

3.5.1. HPLC Analysis

The rats were sacrificed 24 hours after injection (0.5 ml of samples) and seven organs including blood, liver, kidney, spleen, lung, heart, brain was isolated from each group. Then, the tissues were homogenized and centrifuged at 10,000 rpm for 5 min. Finally, the amount of curcumin in each tissue was analyzed by HPLC analysis in groups 1, 2, 3 and 4. In the case of blood tissue, serum was separated by centrifugation at 15,000 rpm for 15 minutes. Then, 300 ML of methanol was added to the serum to separate proteins, and they were finally evaluated by using HPLC analysis

A Knauer HPLC system (Berlin, Zehlendorf, Germany) was used in this work. The system was composed of a Knauer (2500 basic model) UV detector, operating at a wavelength of 420 nm. A Knauer column (4.6 mm diameter × 250 mm length, particle size of 5 μm, Eurospher 100-5 C18) with a pre-column (Eurospher 100-5 C18) was employed for separation. The mobile phase consisted of 1.0% acetic acid in water and Acetonitrile (Merck Chemical, Germany) (70:30 v/v). The flow rate was set at 1.15 mL. min⁻¹. The chromatographic conditions were optimized, and all the experiments were carried out at room temperature. The volume of all injections into the column was 20 μL which took place in triplicate.

3.5.2. Fluorescence Measurement

In groups 1, 5, 6 and 7, half of the blood, heart tissue, kidney, liver, brain, lungs and spleen organs were isolated and homogenized. Then, the fluorescent content of Hoechst dye in the tissues related to

each group was analyzed by using a Hitachi F-2500 spectrofluorophotometer (Tokyo, Japan). The fluorescence intensity of Hoechst dye was quantified at $\lambda_{em} = 497$ nm following excitation at $\lambda_{ex} = 361$ nm.

Some rats related to groups 1, 5, 6 and 7 were selected and examined by live animal imaging. Animal imaging spectrophotometry is one of the noninvasive methods in medicine used to diagnose various diseases. The rat's abdomen and chest were shaved and anesthetized with 50 μL of 10% animal ketamine and 10 μL of xylazine 2% intraperitoneally. Then, the rats were transferred to a device for imaging (Kodak Molecular Imaging Systems, 4 Science Park, New Haven, Connecticut 06511 USA).

3.5.3. Histological Examination by Fluorescent Microscopy

Twenty-four hours after injections, blood samples were collected by cardiac puncture under mild ether anesthesia. Then, the rats were anesthetized with a combination of doses of ketamine (110 mg.kg⁻¹) and xylazine (10 mg.kg⁻¹). Then, they underwent laparotomy; the kidney and liver were removed and preserved in 30% sucrose overnight in refrigerator in a dark box. Serial transverse sections (5 μm -thick) were made of the samples by using a freezing microtome (Leica cryostat). Every third section of the kidney and liver embedded samples was mounted onto gelatin coated glass slides and searched for labeled Hoechst fluorescence using fluorescent microscopy (Olympus Ax70).

4. Results

4.1. Characterization of Nanoliposomes

Encapsulation efficiency of curcumin into RGD-modified nanoliposomes was more than 98 %. The morphology and size of the liposomes were examined by DLS and TEM analyses. The results of TEM analysis showed that the prepared nano-liposomes are spherical and have a nanometer size in the range of 140 ± 15 nm (**Fig. 2A**). In addition, DLS results showed that the nanoparticles were about 177 nm in size (**Fig. 2B**). The difference in liposome size measured by DLS and TEM analyses is because of the fact that DLS measures hydrodynamic diameter.

4.2. Distribution of Curcumin

The bio-distribution of liposomal curcumin was investigated using HPLC analysis by measuring the amount of curcumin in different tissues of treated rats.

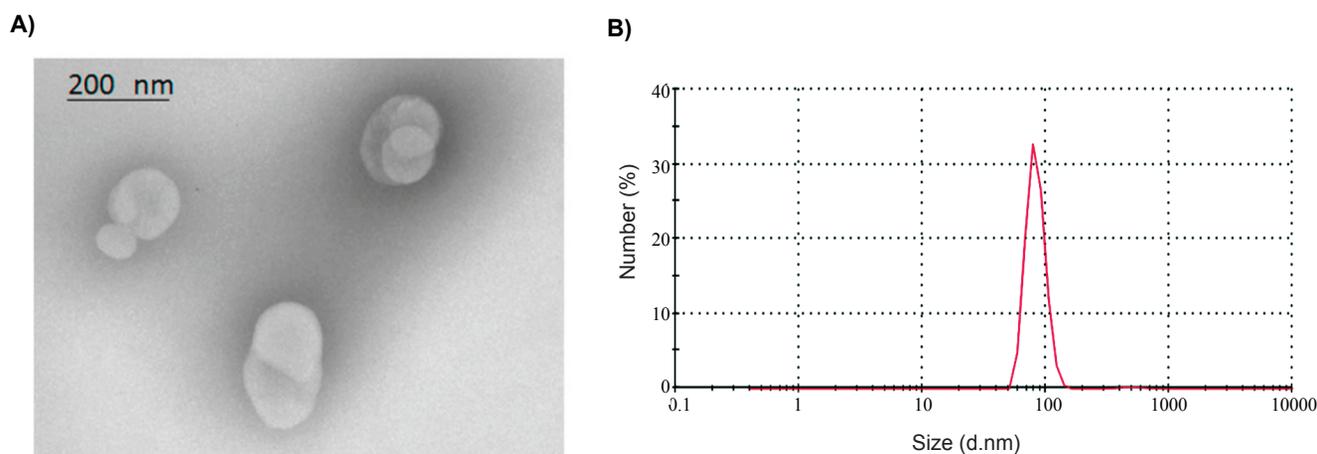


Figure 2. Size, morphology and distribution of liposomal curcumin modified by RGD peptide
A) TEM analysis of RGD- nano-liposomes on carbon-coated copper grid
B) The hydrodynamic diameter of RGD- nano-liposomes was evaluated using DLS analysis.

Limit of detection (LOD) was defined as the signal-to-noise ratio of 3, while for limits of quantification (LOQ), it was defined as 10. The LOD of 10.15 and LOQ of 33.84 ng.mL⁻¹ revealed that the sensitivity and precision of the method were satisfactory.

The results showed that the amount of curcumin in blood in the group receiving liposomal curcumin modified with RGD was the highest compared to other groups (**Fig. 3A**). In addition, the amount of curcumin in the blood in the group receiving liposomal curcumin was more than group receiving curcumin alone (**Fig. S1**). The curcumin was not detected in the blood of groups receiving PBS which did not contain any curcumin (**Fig. 3A**). The amount of curcumin in liver tissue in the group receiving liposomal curcumin modified with RGD was higher than that in other groups receiving liposomal curcumin and curcumin alone (**Fig. 3B**). Moreover, the amount of curcumin in the liver tissue in the group receiving curcumin alone was more than that in the group receiving liposomal curcumin (**Fig. S2**). The curcumin was not detected in the liver of the group receiving PBS which did not contain any curcumin (**Fig. 3B**). In addition, the concentration of curcumin detected in kidney tissue in the group receiving curcumin alone was more than other groups (**Fig. S3**). The curcumin was not detected in the kidney of the groups receiving PBS which did not contain any curcumin (**Fig. 3C**). No curcumin was detected in other tissues (including spleen, heart, lung and brain) in all groups (Data not shown).

4.3. Fluorescence Spectrophotometric Measurement

The distribution of liposomal fluorescent in the rats was evaluated by using fluorescence spectroscopy and small animal imaging analyses. The results of fluorescence spectroscopy showed that the most fluorescent intensity in the groups liposomal fluorescent and liposomal fluorescent modified with RGD was observed in the blood tissue (**Fig. 4A**). However, there was negligible fluorescent intensity for group liposomal fluorescent dye alone in the blood (**Fig. 4A**). Fluorescent intensity in the kidney and liver tissues was only detected in group liposomal fluorescent modified with RGD) (**Fig. 4B and C**). Injection of saline bisphosphate did not show color distribution in any of the tissues (**Fig. 4 and S4-S10**).

Live animal imaging showed the presence of fluorescent signal in heart tissue for groups (**Fig. 5**), but the fluorescent signal was also detected in spleen tissue for group 6 (liposomal Hoechst modified with RGD) and kidney tissue for group 7 (Hoechst alone). The fluorescent signal was not detected in other tissues of all groups. These results confirmed our previous results. **Figure 5A** revealed that in group 6 which received phosphate-saline buffer, no dye distribution was detected in any tissue. In group 7, as shown in (**Fig. 5B**), which received liposomal Hoechst, the distribution was only confirmed and detected in cardiac tissue. **Fig. 5C** shows the group which received RGD modified liposome-containing Hoechst was only confirmed and detected in cardiac and splenic tissues, but in group 9 (**Fig. 5D**) that received Hoechst alone, the dye distributions were only confirmed and detected in the tissues of the hearts and kidneys.

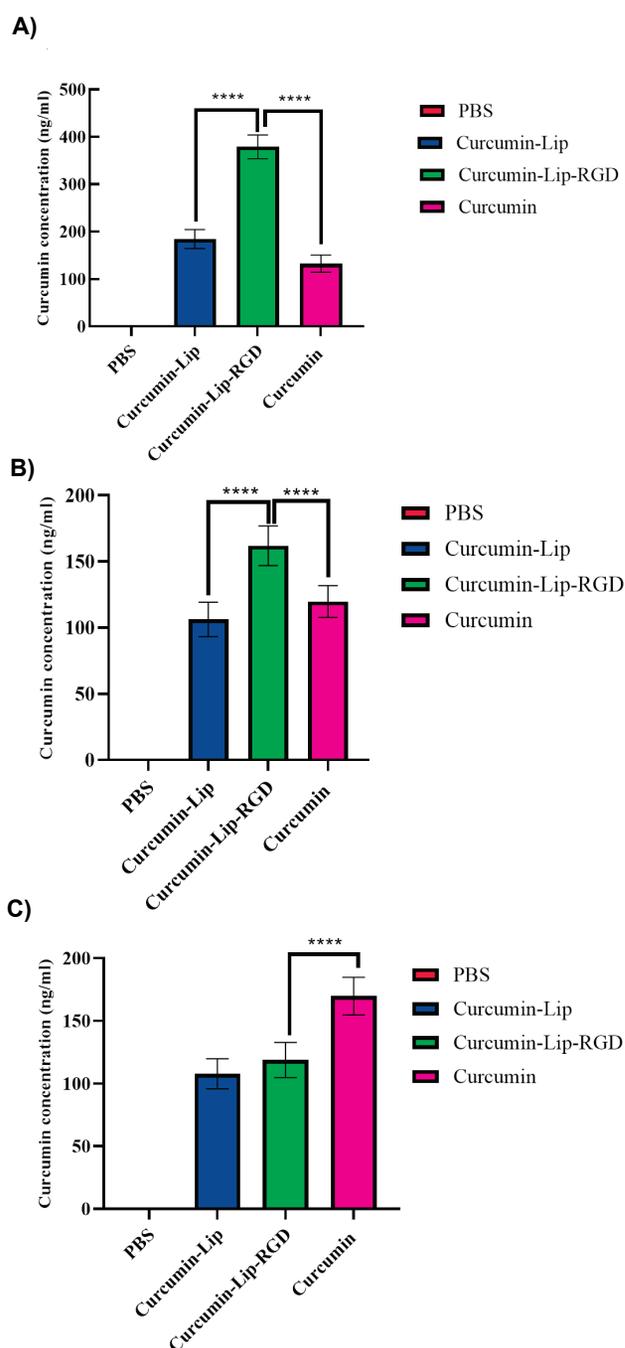


Figure 3. Biodistribution analysis of free curcumin, non-modified-nano liposome-containing curcumin and RGD-modified nano liposome-containing curcumin using HPLC

A) The concentration of curcumin ($\text{ng}\cdot\text{mL}^{-1}$) in the blood of rats was evaluated using HPLC 24 hours after injection of nano-liposomal curcumin and free curcumin.

B) The concentration of curcumin ($\text{ng}\cdot\text{mL}^{-1}$) in the homogenized liver tissue of rats was evaluated using HPLC 24 hours after injection

C) The concentration of curcumin ($\text{ng}\cdot\text{mL}^{-1}$) in the homogenized kidney of rats was evaluated using HPLC 24 hours after injection liver

(PBS: phosphate buffered saline, Lip: liposome); (****= $P<0.0001$)

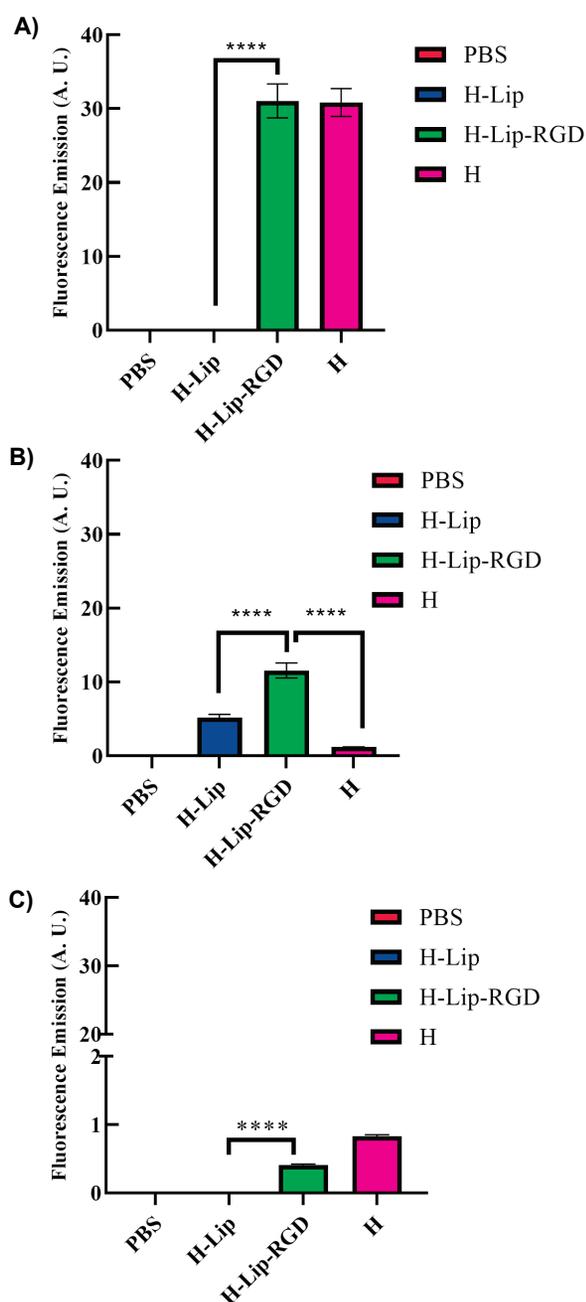


Figure 4. Biodistribution analysis of free Hoechst dye, non-modified-nano liposome-containing Hoechst dye and RGD-modified nano liposome-containing Hoechst dye using fluorescence spectroscopy

A) The fluorescent emission of Hoechst dye (497 nm) in the blood of rats after measuring the injection of nano-liposomal Hoechst and free Hoechst (PBS: phosphate buffered saline, H: Hoechst, Lip: liposome)

B) The fluorescent emission of Hoechst dye (497 nm) in the liver of rats after measuring the injection of nano-liposomal Hoechst and free Hoechst

C) The fluorescent emission of Hoechst dye (497 nm) in the kidney of rats after measuring the injection of nano-liposomal Hoechst and free Hoechst

(PBS: phosphate buffered saline, H: Hoechst, Lip: liposome); (****= $P<0.0001$)

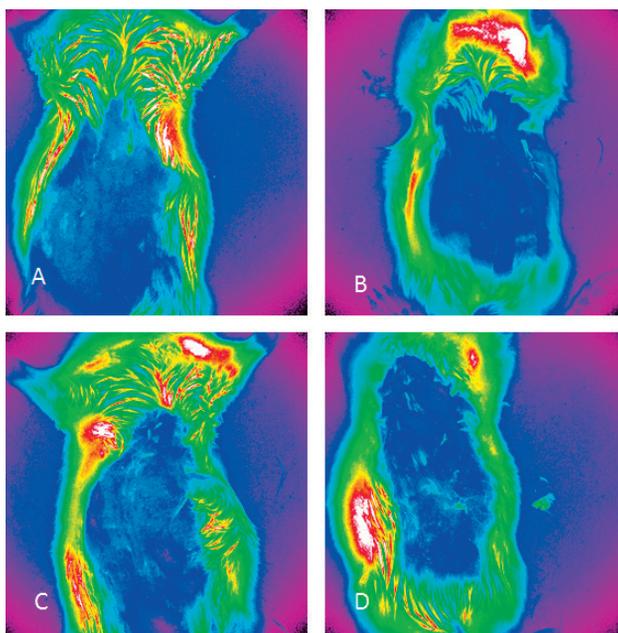


Figure 5. In vivo fluorescence imaging of live rats after IP injection of nano-liposomal Hoechst and free Hoechst
A) Fluorescence imaging of live rats was captured after IP injection of Phosphate buffer saline
B) Fluorescence imaging of live rats was captured after IP injection of Liposomal Hoechst
C) Fluorescence imaging of live rats was captured after IP injection of Liposomal Hoechst modified with RGD
D) Fluorescence imaging of live rats was captured after IP injection of Hoechst.

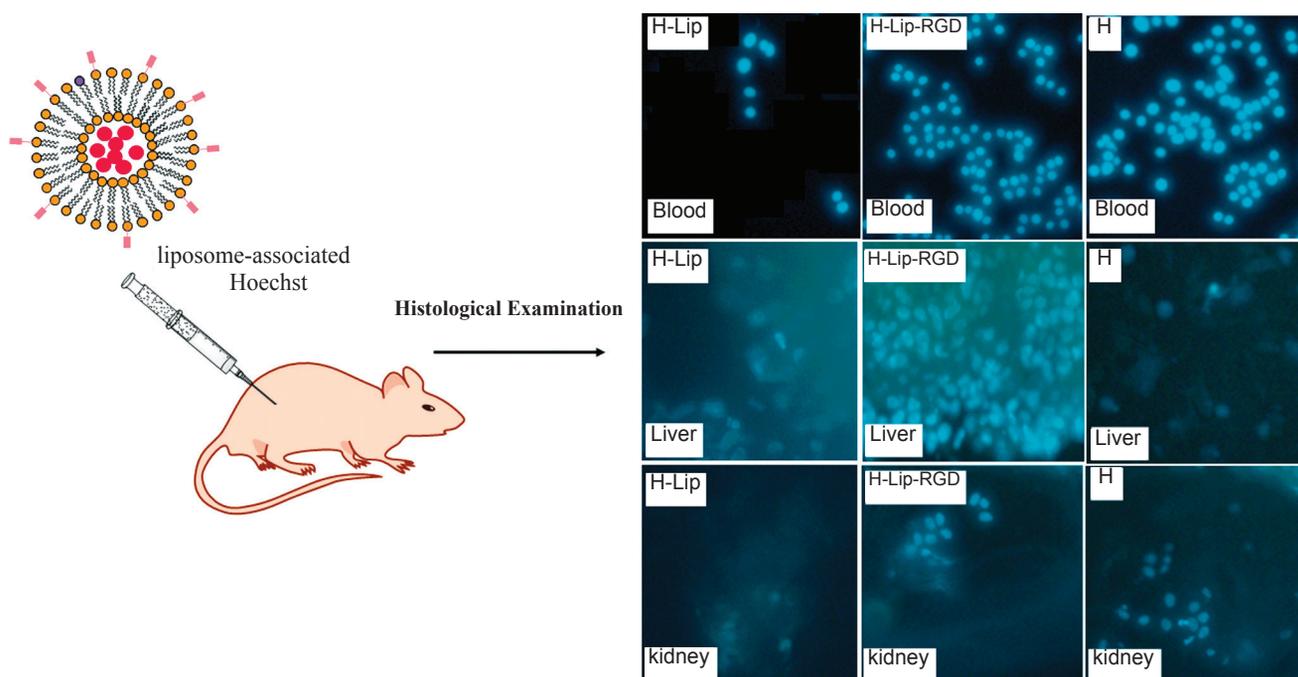


Figure 6. Fluorescence microscopy images of rat tissue specimens. The fluorescent image of different tissues of rat 24h post- injection of fluorescent free Hoechst and liposomal Hoechst using fluorescent microscopy (H: Hoechst, Lip: liposome; LiP-RGD; arginine-glycine-aspartic acid (RGD)-modified nano-liposome); Scale bar: 100 μ m for all image

4.5. Hoechst Fluorescent Staining

As shown in **Figure 6**, the Hoechst fluorescent staining has not been seen in the blood of the PBS group whereas the Hoechst staining diffused through the liposomes and increased in the blood considerably in the H-lip- RGD group compared to the H-Lip group (**Fig. 6**). Results demonstrated the fluorescence of Hoechst in liposomes diffused and they were binding to the liver cells. As shown in **Figure 2**, the numbers of tracer cells in the H-lip- RGD group increased compared to the H-Lip and H groups in the liver cells (**Fig. 6**). The Hoechst tracer cell in kidney cells decreased compared to the liver or blood cells, but as shown in this Figure, the number of kidney tracer cells in the H-lip- RGD group increased compared to the H-Lip.

5. Discussion

Results of this study demonstrated that the concentration of curcumin in blood and liver in the animals receiving the liposomal curcumin was higher than the group receiving curcumin alone. Further, our result showed RGD modified curcumin-loaded liposomes could increase the uptake efficacy of curcumin in the liver, kidney and blood compared to the other groups. In this regard, uptake of liposomes in the liver was improved by labeling with asialofetuin (21). Although chemotherapy developed as a common way for treatment of cancers, most of the drug damage the normal cells and tissue, and it has weak bio-availability to the tumor cells (22). Therefore, nanotechnology emerges as a successful strategy for drug delivery to the tumor tissue by controlling damage to the normal cells (23). For cancer therapy, liposomes have enormous therapeutic capacities for transfer and delivery of drug and macromolecules to the targeted cells (24). Drug delivery by liposomes reduced general toxicity and improved the efficacy of releasing in the target tumor tissue cells (25). The surface of the liposomes can be loaded by different receptors, and they are specifically attached to the target organs (26). Our result demonstrated that the mean of the curcumin in the group with liposomal modified with RGD increased compared to the rats which received liposomes without ligand. RGD is an adhesion ligand that can be attached to the $\alpha\beta3$ receptor of the cancerous cells and enhance cellular penetration (27). It has been shown that RGD can modify the nanoliposomes for different purposes such as gene delivery, drug delivery, tumor treatment and chemotherapy (28). Different

nanoparticles such as biopolymer nanoparticles (NPs) and albumin NPs are extensively used to encapsulate curcumin to enhance its solubility and absorption to the cancerous cells (29). Contrary to our study, these nanocarriers have controlled release of their contents (29). Albumin NPs with biodegradability and biocompatibility is an appropriate platform for drug delivery (30). Further, encapsulation of curcumin with human serum albumin demonstrated more increase in cellular absorption compared to the free curcumin (31). It has been demonstrated that curcumin encapsulated with lipid nanoparticles has beneficial effects in bioavailability and stability of curcumin (32).

Liposomes have an effective encapsulation and delivery system for different drugs and molecules such as antigens, hormones, enzymes and antibodies. The result of this study showed that the targeting co-loaded liposomes to the determined tissue improved uptake with possible applications in experimental studies. The surface of the liposomes can be modified with ligand, antibody or in an engineering manner for different purposes such as enhancement in accumulation of the drug and flow inside the cytoplasm of the cancer cells (33). It has been demonstrated that that RGD-modified paclitaxel and curcumin co-loaded liposomes is a promising drug delivery candidate for the treatment of lung cancer (34).

Turmeric (come from *C. longa*) is a spice with yellow color containing 77% curcumin (with lower dimethoxy curcumin and bisdemethoxycurcumin), and it has been used in herbal medicine for many years (35). Curcumin, as a polyphenolic compound, has anticancer and polyphenol property; and daily consumption of the curcumin is effective in reducing gastrointestinal cancer (36). Further, it has been demonstrated that it has anti-atherosclerotic, antidiabetic and antiaging property (37). Previous studies reported that curcumin has significant efficacy to treatment of different carcinomas such as breast and pancreas (38). Moreover, curcumin has the ability to stop the proliferation of cancer cells in many tissues such as the liver, stomach and colon (39). Improvement in encapsulation of curcumin in liposomes increases delivery of curcumin in the liver cells. Further, galactosylated-liposomes can bind to the asialoglyco protein receptor (ASGPR) of the liver cells (40). Liposomes with aqueous content have various capacities to integrate with different hydrophilic, hydrophobic and amphiphilic materials (41, 42).

6. Conclusion

In the present study, the bio-distribution of RGD modified nanoliposomes in normal rats was evaluated by different analyses. The results showed that modified liposomal nano-carrier with RGD peptide-containing curcumin was accumulated in very small amounts in various tissues such as brain, heart, lung, spleen, liver and kidney. This nano-system had high potential for retention in blood 24 h after injection which is significant for drug delivery. Therefore, the long shelf life of this nano-system in the blood can greatly increase the effectiveness of the drugs.

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Conflict of interest

The authors report no conflicts of interest in this work.

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