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RESEARCH PAPER

Improving the anticancer efficiency of doxorubicin by luteolin nanoemulsion: *In vitro* study

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ABSTRACT

Objective(s): Recently, medicinal plants have grabbed much attention in the prevention and treatment of cancer due to their ability to increase the efficiency of chemotherapy agents. Luteolin is a flavonoid widely studied for its antitumor effects. However, luteolin has low bioavailability and poor efficacy due to its hydrophobicity. This study aimed to prepare luteolin nanoemulsion (NE) and evaluate its physicochemical and anti-tumor properties in combination with doxorubicin (DOX) *in vitro*.

Materials and Methods: NE containing luteolin was prepared by the prob-sonicate method. The physicochemical properties of nanoparticles, including particle size, zeta potential, morphology, encapsulation efficiency, viscosity, pH, drug release profile, and thermal stability were investigated. Finally, the toxicity of free luteolin and luteolin NEs at different concentrations, with and without DOX, was assessed against normal L929 fibroblast and C26 colon cancer cells *in vitro.*

Results: Luteolin NE was found to mimic a non-newtonian fluid with pH: 5.5 and an average particle size of 38.72 nm. The encapsulation efficiency was obtained at 79.61%. No significant changes were observed in particle size, PDI, and zeta potential after three months of storage at 4 °C. Seventy-two-hour drug release from these nanoparticles was about 25% in a neutral environment and 85% in an acidic environment. The combination of DOX and luteolin NE showed synergistic antitumor effects, while neither free luteolin nor luteolin NE showed significant toxicity against normal cells up to the 50 µg/ml concentration.

Conclusion: The simultaneous administration of DOX and luteolin NE synergistically increased the cytotoxicity of DOX against the C26 cell line. Therefore, the novel formulation developed can be considered a suitable alternative to increase the anti-tumor efficiency of DOX.

Keywords: *Cancer, Doxorubicin, Luteolin, Nanoemulsion, Teucrium polium L*

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INTRODUCTION

Cancer is a big challenge across the globe that kills millions of people every year. In recent decades, various promising cancer therapeutic methods have been developed, but the treatment continues to remain among the most challenging issues in medicine. Anthracycline antibiotics comprise an important category of

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chemotherapeutics, among which doxorubicin (DOX) is a commonly used drug. Unfortunately, similar to many other chemotherapeutics, DOX also causes many side effects such as acute and chronic cardiotoxicity. Today, special attention has been directed toward the use of medicinal plants as preventive and therapeutic agents against cancer because of reasons such as low toxicity, high efficiency, the possibility of oral administration, low costs, and known mechanism of action. In this regard, the emergence of novel formulations has been proven to increase bioavailability and reduce the toxicity of anti-cancer drugs [1].

Luteolin (3, 4, 5 ,7-tetrahydroxyflavone) is a natural flavonoid found in many plant species such as thyme, parsley, carrot, pepper, olive oil, mint, celery, rosemary, chocolate, tea, turnip, lettuce, beet, broccoli, and spinach. Luteolin can form chelates with metal ions without being oxidized in this process and protects plants against extrinsic aggressors [2, 3]. This compound has been reported to have antimicrobial, anti-inflammatory, antioxidant, anti-cancer, anti-aging, anti-hypertensive, and wound-healing properties and plays an important role in the generation and protection of neurons and other body tissues [2]. It has been specified that luteolin can prevent cancer progression, cancer cell transformation, metastasis, and angiogenesis by employing multiple mechanisms such as suppressing kinases, modulating cell cycle progression, inducing apoptotic cell death, regulating the expression of transcription factors, etc. [4, 5]. Despite the many therapeutic uses of luteolin, it is a hydrophobic compound and presents low bioavailability, poor systemic delivery, and low efficacy, urging scientists to seek better formulations of these compounds.

Nanoparticles (NPs) provide a viable option to achieve safe and effective tumor eradication, which not only obviates the water solubility problem but also can specifically target cancer [1, 6, 7]. Nanoemulsion (NE) is an emulsion system containing nanometer-size droplets stabilized via oil or water droplets dispersed in the incompatible phase with the help of a suitable surfactant. The average droplet size generally ranges from 0.1 to 500 nm depending on the cargo drug, mechanical energy, and the composition and relative quantity of the surfactant [8].

Nanoemulsions offer numerous advantages compared with conventional emulsion systems [9]. Due to small particle sizes and the lack of gravitational isolation, NEs are kinetically stable and thermodynamically metastable [10], in which phase separation occurs gradually over time without being affected by changes in physical or chemical properties (e.g., temperature, pH, etc.) [8]. The small droplet sizes of NEs prevent the aggregation of particles, reducing the likelihood of sedimentation or creaming [9], and guarantee a uniform distribution and dispersion system. This can increase the bioavailability of water-insoluble drugs because small particles easily pass through the absorption membrane. In addition, nano-scale particles provide a large surface area and can easily penetrate the skin or epithelial layer. Also, NEs' formulations can be adjusted for delivering drugs in different ways, including infusion that is applicable due to their tiny droplets [11].

In this study, firstly, luteolin NE was prepared and its properties investigated. For this purpose, we used the oil extract of *T. polium* to formulate NEs containing luteolin.

Teucrium polium L. is a plant from the *Lamiaceae* family and one of the wild flowering species of this family widely growing in Iran [12]. This herb has been reported to have a wide variety of biological activities, including anti-inflammatory, analgesic, antibacterial, antihypertensive, fat-lowering, anti-rheumatoid, and hypoglycemic effects. Recently, the insulinotropic and antihyperglycemic activities of the crude extract of *T. polium* have been evaluated, and some studies have confirmed its antioxidant effects as well [12].

This plant has grabbed the attention of pharmaceutical companies in recent years due to its anti-tumor effects which are mostly attributed to its terpenoid and flavonoid compounds [13]. For example, in a study by Guesmi *et al*. who investigated the anti-cancer effects of *T. alopecurus* extract against colon cancer cells via promoting the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L), a pathway that is known to selectively induce apoptosis in cancer cells, it was reported that the plant extract inhibited the growth of cancer cells via boosting TRAIL-related apoptosis in colon cancer cells *in vitro* [14, 15].

As mentioned, a strategy to mitigate the side effects of chemotherapy drugs is to concomitantly use natural compounds, including medicinal plants or their derivatives, along with them.

Therefore, in the next step of this research, the cytotoxicity of luteolin NEs with or without DOX was investigated against C26 cancer cells.

MATERIALS AND METHODS

Materials

Doxorubicin, DMSO, MTT, Tween 80, Ethanol 70%, and PBS were purchased from Sigma (Germany); PEN-STREP (100X), RPMI and FBS were obtained from Gibco (USA), and luteolin was procured from Golexir Pars (Iran). *Teucrium polium* essential oil was provided by Ferdowsi University of Mashhad.

Methods

Preparation and GC-Mass analysis of the plant oil

Teucrium polium was identified by botanists at the herbarium of the Plant Sciences Research Institute of Ferdowsi University of Mashhad, Iran. Then the essential oil of the aerial parts of the plant was obtained by water distillation following the instructions of the European Pharmacopoeia. The essential oil obtained was analyzed by a gas chromatography device connected to a mass spectrometer at the Faculty of Agriculture of Ferdowsi University of Mashhad.

Synthesis of NEs containing luteolin

The following compounds with the respective ratios were used to prepare luteolin NE (Table 1).

To prepare one milliliter of each formulation, 2 mg of luteolin powder was weighed and dissolved in ethanol on a stirrer containing a magnet. For better dissolution, a sonication bath was used for five minutes. While the solution was on the stirrer, essential oil and the surfactant were added, and the mixture was allowed for 15 min to transform into a homogenous oily phase. Then, the aqueous phase was placed on the stirrer, and the oily phase was slowly and dropwise added to it to obtain a concentrated and milky solution. The solution was then homogenized and cleared using the probe sonicate method, in which the solution was placed in a container containing ice for six min with an interval of 100 sec and a power of 80% to reach a completely transparent compound (Fig. 1).

The physicochemical properties of NEs

The size of the synthesized NPs was determined by the dynamic light scattering (DLS) method. For this purpose, 100 µl of the formulation was suspended in one milliliter of filtered deionized water and placed in a sonication bath for 15 min to become more homogenous. The size of the particles was then measured using a Zeta particle size analyzer (ZSA). The polydispersity index (PDI) obtained by this instrument confirmed the homogeneity of the particles in terms of size.

The shape and surface morphology of the NPs containing luteolin were also investigated by a transmission electron microscope (Model CM120, Philips Co.) [16]. To obtain the efficiency of luteolin encapsulation into NPs, firstly, one milligram of luteolin powder was dissolved in one milliliter of methanol, and then a serial dilution with the concentrations of 0.5, 0.25, 0.125, 0.625, 0.0312, 0.0156, 0.0078, 0.0039, and 0.00195 mg/ml was prepared. The absorbance of the samples was read at 348 nm by a spectrophotometer to

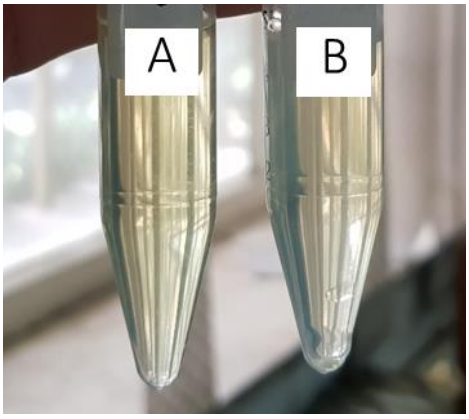

Fig. 1. A) Nanoemlusion containing luteolin and B) control

Table 1. Constituents of luteolin nanoemulsion

Formulation	Tween 80	Ethanol	*Teucrium polium* essential oil	Water
F1	36%	12%	5%	47%
F2	36%	9%	5%	50%
F3	36%	6%	5%	53%

draw a concentration vs absorbance graph and obtain the line equation.

For preparing a sample with an unknown concentration, 500 µl of the stock formulation, with a concentration of 2 mg/ml, was dissolved in 500 µl of methanol. For better solubility, bath sonication was used for five min, 15 min after which, the solution was centrifuged at 3500 rpm, and the upper layer was separated. Then 10 µl of this solution was harvested and dissolved in 990 µl of methanol under sonication. Considering an efficiency of 100%, the final concentration should have been 10 µg/ml (24). The following formula was used to calculate encapsulation efficiency (EE %) [16-18]:

Encapsulation efficiency (%) = weight of the drug loaded (g) / total weight of the drug in the formulation × 100

Assessment of viscosity

The viscosity of NE was measured separately by Brookfield R/S + rotational rheometer (Brookfield Co., USA) with SC4-25 spindle at a shear rate of 0-200 s-1.

Stability assessment

The stability of the formulations was assessed at temperatures of 4 °C and 25 °C for 90 days. The size and zeta potential of the particles were analyzed at different times (30, 60, and 90 days). Furthermore, the NEs were centrifuged at 3500 rpm for 30 min, and phase separation was inspected in the samples.

Luteolin release profile from NEs

A volume of 250 µl of the NE was placed into a dialysis bag with a cut-off of 10 kDa, which was then left afloat in falcon tubes with 50 ml of a phosphate buffer (pH = 7.5 as the body's) containing 0.1% Tween 80 or 50 ml of a citrate buffer (pH = 5.5 to mimic the environment around cancer cells) also containing 0.1% Tween 80. The falcon tubes were then placed in a shaker incubator at 37 °C and a speed of 100 rpm. At the time intervals of 1, 2, 3, 4, 24, 48, and 72 hr, 1 ml of the solution was collected and replaced with 1 ml of fresh buffer. Finally, the concentration of the luteolin released was measured using a standard curve. Cumulative drug release (%) was calculated using the following formula:

Cumulative drug release (%) = the amount of drug released / total amount of the drug × 100

The amount of drug released = $CnVT + \Sigma C (n-1) Vs$

Where Cn represents the concentration of the drug released in the solution at the time "n"; Cn-1 is the concentration of the drug released at the time "n-1"; VT shows the total volume of the solution received, and Vs is the volume of the sample collected.

In vitro toxicity assessment

The toxicity of DOX, luteolin, NEs containing luteolin, and a combination of these compounds was investigated against a mouse-derived colon carcinoma cell line (C26) and normal mouse fibroblast cells (L929) using the MTT method. These cell lines are usually used as a model in experimental studies *in vitro* [19, 20].

For this purpose, each well of a 96-well plate was seeded with 7000 cells. After 24 hr, when the cells were attached to the bottom of the wells, the culture medium on the cells was gently removed. The C26 cell line was treated with the following compounds: luteolin alone (1, 2, 4, 8, 12, 16, 24, and 32 µg/ml), luteolin-loaded NEs (the same concentrations as luteolin), NE alone, and DOX (0.125, 0.25, 0.5, 1, and 2 µg/ml).

In the next step, the C26 cell lines were treated by IC_{25} of DOX (0.17 µg/ml) along with the IC_{10} and IC_{25} of luteolin (6.32 and 15.06 µg/ml, respectively), the IC_{10} and IC_{25} of luteolin-loaded NEs (1.20 and 5.14 µg/ml, respectively) or the IC_{10} and IC_{25} of free NEs (and 3.8 and 7.98 µg/ml).

In addition, the L929 cell line was treated with 3.125, 6.25, 12.5, 25, and 50 µg/ml concentrations of luteolin alone and luteolin-loaded NEs, NE alone, as well as the same concentrations of DOX (0.125, 0.25, 0.5, 1, and 2 µg/ml) as the C26 cell line. The cells were then placed in an incubator at 37 °C for 48 hr.

The MTT reagent (5 mg) was dissolved in 1 ml of PBS (sonication was performed for further solubility). Next, 20 µl of freshly prepared MTT reagent was added to each well, and the plate was incubated at 37 °C for three hr. After that, the wells were completely emptied, 100 µl of DMSO was added to each well, and the plate was placed on a shaker incubator (600 rpm) for five min to dissolve formazan crystals. Finally, absorption at 570 and 630 nm was read in each well by an ELISA plate reader (Tecan), and the graph of absorption vs living cell percentage was prepared.

Drugs' combination index

Assessing the drug-drug interaction is an important topic in all medical fields, and the extent and nature of drug-drug interactions are usually determined *in vitro* by employing computational models to analyze experimental data and determine the nature of the interaction (e.g., synergistic, additive, or antagonistic).

The combination index (CI) is generally used to assess the results of chemotherapy with multiple drugs. The value of CI is calculated using the following equation:

$$CI=CAX/ICXA+CBX/ICXB$$

In this equation, CAX is the concentration of the first drug in the combination; CBX is the concentration of the second drug in the combination, and ICXA and ICXB represent the concentration of each of the first and second drugs alone equivalent to their effects in the drug combination, respectively. If the value of CI falls below 0.9, the two drugs have a synergistic relationship; if the value is between 0.9 and 1.1, the drugs show an additive relationship, and CI values greater than 1.1 are interpreted as an antagonistic relationship [21].

Statistical analysis and mathematical calculations

GraphPad Prism 8.4.3 software was used for statistical data analysis. Comparisons between the study groups were conducted using one-way ANOVA and Tukey's multiple comparisons test. In this study, a *P*-value smaller than 0.05 was considered statistically significant, and symbols *, **, ***, and **** were used to demonstrate *P*-values <0.05, <0.01, <0.001, and 0.0001, respectively. The results were presented using graphs and tables.

RESULTS

GC-Mass analysis of T. polium Essential Oil

The chemical components of *T. polium* essential oil were detected using GC-MS methods, as demonstrated in Table 2. β- Pinene was identified as the main component with a ratio of 36.37% of the total, followed by α-Pinene (14.40%).

Table 2. List of some biochemical components of *T. polium* essential oil

Compounds	Ratio%	Ret.Time (min)
β- Pinene	36.37	10.78
α- Pinene	14.40	9.18
Caryophyllene	8.15	26.56
Pinocarveol	7.15	16.92
β-Selinenol	5.00	33.79
Myrtenol	4.68	18.95
Caryophelen Oxide	3.53	31.67
β-Farnesene	3.44	27.58
Sabinone (PinoCarvon)	3.18	17.76
β-Cubebene	2.41	28.53
α-Cubebene	2.29	25.09
Trans-Calamene	1.76	31.56
Germacrene B	1.47	30.92
Naphthalene	1.39	29.66
Longiverbenone	1.27	34.36
Octen-3-yl-acetate-1	1.26	15.63
Bornyl acetate	1.25	22.05

Physicochemical properties of prepared NEs

In this study, the probe sonicate method was used to synthesize NEs containing luteolin. The second formulation (i.e., F2, Table 1) was subjected to analysis as it had a transparent appearance. The average size of the NPs was 38.72±0.88 nm, and according to the PDI, the particles had a uniform size distribution (PDI=0.283±0.21). The zeta potential of the formulation was obtained as -3.24±0.93mV.

The particles were photographed using a TEM microscope (model CM120, Philips Co.). As shown in Fig. 2, the particles had uniform and nano-scale spherical morphology. The size of the particles was estimated to be about 100 nm. The difference compared with the estimation made by the DLS method can be related to the method of sample loading under the microscope [22].

The efficiency of encapsulation was determined at about 79.61% using the standard curve (Fig. 3) [23]. The pH values of the drug formulation and the blank sample were measured as 5.5 and 5.4, respectively, by a pH meter.

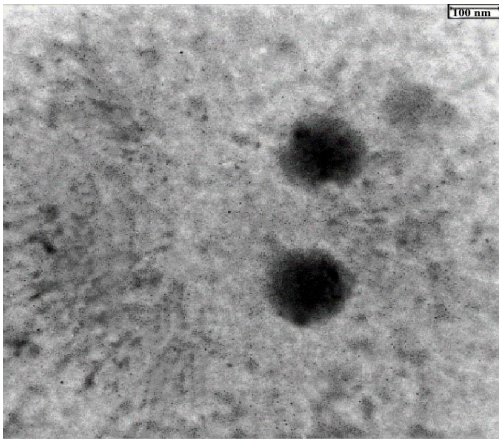

Fig. 2. Transmission electron microscopy image of luteolin nanoemulsion (scale: 100 nm)

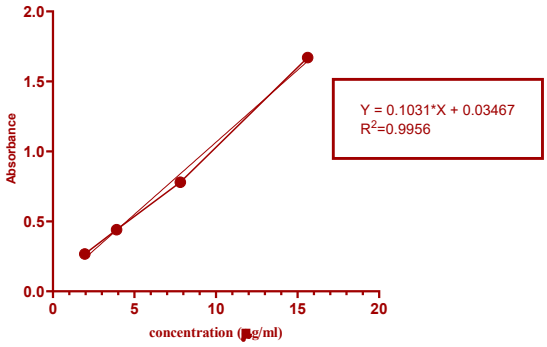

Fig. 3. Standard curve of light absorbance at 348 nm versus luteolin concentration

Viscosity assessment

As shown in Fig. 4, luteolin-loaded NE and drug-free NE had the properties of a non-Newtonian fluid because their viscosity changed with the application of force. Also, they showed the behavior of a pseudo-plastic substance, where an increase in the shear stress led to a reduction in viscosity. Notably, the viscosity of a pseudo-plastic fluid is inversely related to the magnitude of the force applied and shear stress.

Stability of the formulation

The results of stability assessment at ambient and refrigerator temperatures have been presented in Table 3. As shown, the size of NEs did not change considerably at 4 °C within three months. However, at 25 °C, the size and appearance of the formulation significantly altered over one month, and the solution became opaque. In addition, centrifugation at 3500 rpm for 30 min did not lead to phase separation in NEs (Fig. 5).

In vitro release of luteolin from NEs

The *in vitro* release of luteolin from NEs was assessed at pH 7.5 and 5.5 (Fig. 6). After 72 hr, the release reached its maximum at pH = 5.5 (85%) and pH = 7.5 (25%).

Toxicity studies in the C26 cell line

The C26 cell line was exposed to different

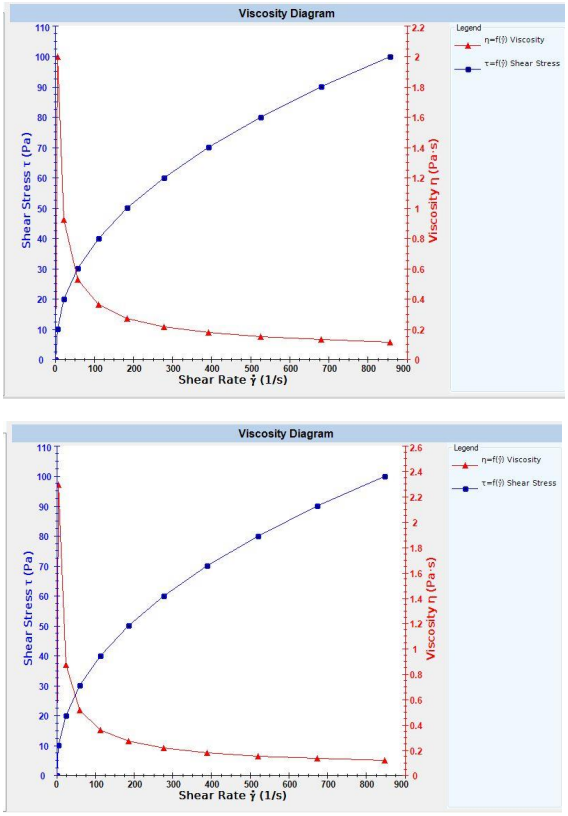

Fig. 4. Viscosity assessment. A) luteolin-loaded nanoemulsion and B) control drug-free nanoemulsion

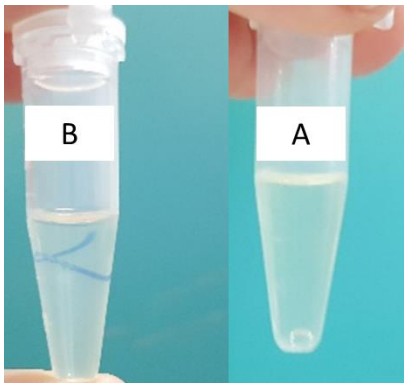

Fig. 5. Phase separation in luteolin-loaded nanoemulsion. A) before centrifugation and B) after centrifugation

Table 3. Results of stability assessment at 4 °C and 25 °C after 1, 2, and 3-months

Time/Temperature		Size Z-average (d. nm)	PDI	Zeta potential (mV)
1 month	4°C	33.71±1.24	0.289±0.99	-3.28±0.50
	25°C	124.1±2.73	0.081	-6.24±0.42
2 months	4°C	29.79±0.95	0.279±1.52	-4.22±0.90
	25°C	turbid	turbid	turbid
3 months	4°C	39.31±2.18	0.377±1.33	-2.67±0.44
	25°C	turbid	turbid	turbid

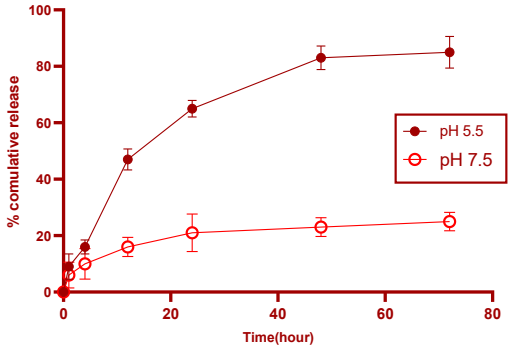

Fig. 6. Release process of luteolin from NPs

concentrations of free luteolin, DOX, luteolin-loaded NE, and free NE for 48 hr, and cytotoxicity was evaluated at different concentrations by the MTT assay (Fig. 7). The values of IC_{10}, IC_{25}, and IC_{50} indicated that luteolin-containing NEs had comparable toxicity against the C26 cell line at a lower concentration than free luteolin. Also, free NEs showed toxicity against the cancerous cells at a lower concentration than free luteolin.

Toxicity assessment against the L929 fibroblast cell line

The results showed that neither free luteolin nor luteolin conjugated with NEs had toxicity against L929 fibroblast cells up to the concentration of 50 µg/ml (Fig. 8).

Results of the Co-delivery of luteolin, DOX, and their NE formulations

The C26 cells were exposed to the combination of DOX (at the IC_{25} concentration) with either luteolin or NEs (IC_{25} or IC_{10}) for 48

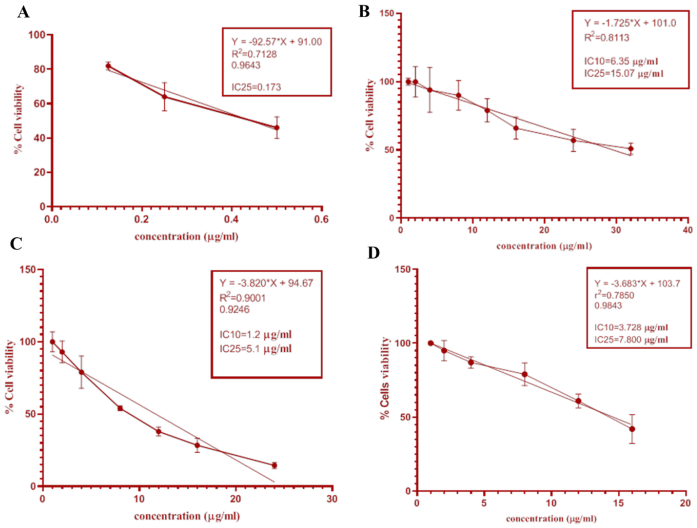

Fig. 7. Results of MTT assay against C26 mouse colon cancer cell line. The cells were treated with A) doxorubicin, B) free luteolin, C) luteolin-loaded nanoemulsion, and D) free nanoemulsion. The results have been presented as mean ± SD of three replications

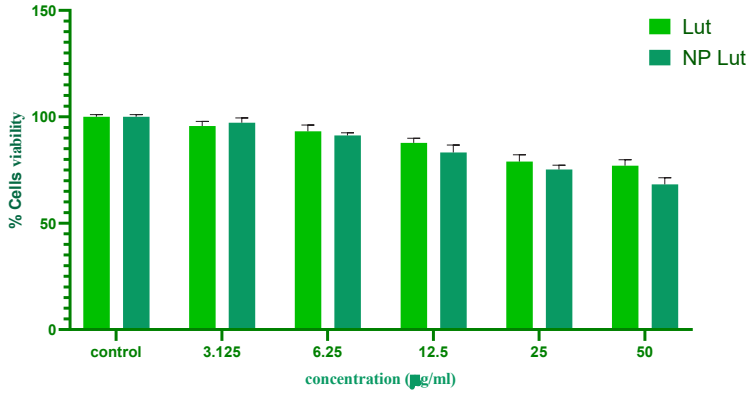

Fig. 8. Results of toxicity assessment by the MTT assay against the L929 fibroblast cell line

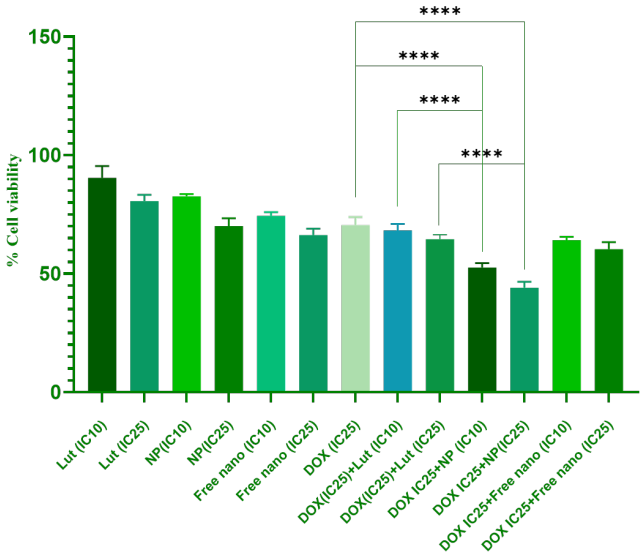

Fig. 9. Results of the co-delivery of DOX with either luteolin or NE against C26 mouse colon cancer cells. DOX was used at the fixed concentration of IC_{25}. Luteolin or NEs were used at either IC_{10} or IC_{25} concentrations. The values are mean ± SD of three replications. **:*P*<0.01, ****: *P*<0.0001

Table 4. Combination index values of the compounds studied in Co-deliveries to C26 cancer cells

Compounds	Combination index	The type of interaction
DOX IC_{25} + Lut (IC_{10})	1.055	Additive
DOX IC_{25} + Lut (IC_{25})	1.31	Antagonistic
DOX IC_{25} + NP (IC_{10})	0.52	Synergistic
DOX IC_{25} + NP(IC_{25})	0.72	Synergistic
DOX IC_{25} + Free nano (IC_{10})	0.96	Additive
DOX IC_{25} + Free nano (IC_{25})	1.19	Antagonistic

hr. As shown in Fig. 9, in the comparison of DOX (IC_{25}) (where 70.5 ± 3.4% cells were alive), the percentage of viable cells dropped significantly in the group treated with the combination of DOX (IC_{25}) + NE (IC_{25}) and DOX (IC_{25}) + NE (IC_{10}) (the values of living cells were 44.06±2.57% and 52.61 ± 1.86%, respectively).

Although the toxicity was not significantly different between DOX alone and the DOX (IC_{25}) + Lut (IC_{25}) or DOX (IC_{25}) + Lut (IC_{25}) groups, there was a significant difference in the percentage of viable cells between the combination treatment with free luteolin and luteolin-loaded NEs.

In addition, there was no significant difference in the toxicity of DOX alone (IC_{25}) and its combination with free NEs (IC_{25}) containing *T. polium* essential oil, with an average difference of 10.16%.

Results of CI

Using the formula mentioned in the method section, the types of interactions between drugs in the cells receiving co-deliveries were determined (Table 4). As can be observed, most CI values calculated were less than 0.9 for the C26 cell line, indicating the synergistic effects between DOX and luteolin-containing NEs (IC_{10} and IC_{25}). In combination with luteolin and free NEs (IC_{10}) and DOX, an additive interaction was observed. However, at the IC_{25} concentration of luteolin and free NEs, the respective CI values were greater than 1.1, suggesting a dose-dependent decline in the synergistic effect.

DISCUSSION

Anthracycline antibiotics constitute an important group of chemotherapy drugs, among which DOX is of special importance. Similar to other anti-cancer drugs, DOX is associated with numerous side effects such as acute and chronic cardiotoxicity. On the other hand, due to the complexity of cancer treatment, single-drug regimens usually do not work successfully,

directing attention towards the simultaneous use of medications, leading to a reduction in the dose of the main drug due to synergistic effects between these compounds.

Today, special attention has been dedicated to the use of medicinal plants and nanotechnology in the prevention and treatment of cancer to enhance the efficiency of chemotherapy agents. Due to their high surface-to-volume ratio, nanocarriers have the ability to modify the essential properties and bioactivity of drugs. Other advantages of NPs include protecting the drug via encapsulation, improving drugs' pharmacokinetics and biodistribution, reducing drug toxicity, augmenting solubility and stability, and improved controlled-release and targeted delivery [24]. For example, in a study, PEG-DOX-Cur NPs were designed for the co-delivery of DOX and curcumin, which increased the drug's antitumor activity, plasma stability, and intra-tumor accumulation compared with free DOX [25].

As mentioned earlier, luteolin is a flavonoid that has been widely studied because of its antitumor effects. However, like many other herbal compounds, luteolin is hydrophobic, has low bioavailability, and may deliver low efficacy, encouraging researchers to seek better formulations to resolve these problems. So far, researchers have developed various formulations of luteolin (e.g., phytosome, SLN, etc.) to enhance its function [23, 26].

This study aimed to prepare luteolin-loaded NEs using *T. polium* essential oil and investigate their biological characteristics and anticancer effects in combination with DOX *in vitro*.

Nanoemulsion is considered a novel advanced technology for formulating biological agents and can be an enthralling approach for increasing the bioavailability of luteolin and improving its cellular uptake [27].

Studies show that NEs efficiently penetrate tumor cells and reduce tumor growth and metastasis without exposing much toxicity against healthy cells. Because cancer cells are surrounded by vascular tissues, NEs can easily pass through these barriers (3). For example, NEs of vitamin E containing paclitaxel modulated the expression of BCL-2 (a drug resistance marker) and promoted anti-proliferative effects in a paclitaxel-resistant ovarian cancer cell line (A2780), suggesting a promising way to overcome drug resistance in tumors [1, 27]. Also, another study supported the therapeutic potential of NEs containing curcumin (Cur), a compound with poor water solubility and low bioavailability, reporting that the formulation increased the oral absorption of curcumin and its effectiveness against prostate cancer cells [28]. Also, several clinical trials are evaluating the role of curcumin NEs in reducing the risk of breast cancer [1].

Another study investigated the clinical effects of the lipid NEs of methotrexate able to bind to LDL receptors, designed to increase the accumulation of the drug in the tumor tissues overexpressing the LDL receptor. The results of the recent study indicated that NEs containing the drug had significantly higher absorption and toxicity against tumor cells compared with the free drug formulation [1]. In another study, miriplatin was encapsulated in NEs bearing a peptide with the ability to bind to epidermal growth factor receptor (EGFR), reporting that this formulation enhanced cytotoxicity against cisplatin-resistant ovarian cancer cells overexpressing EGFR (SKOV3, A2780, and A2780CP cells) [1].

In the present study, a transparent solution of luteolin NE was obtained using 80% tween (36%), *T. polium* essential oil (5%), ethanol (9%), and water using the sonicate probe method. Next, the physicochemical properties of the novel formulation and its toxicity against the C26 mouse colon cancer cells were studied. Using this method, NEs with an average diameter of about 38.72 nm were synthesized, showing a uniform size distribution (PDI= 0.283). One of the features of NPs with a size of less than 200 nm is that they have a higher rate of cell uptake and longer retention time in the tumor tissue due to a phenomenon known as enhanced permeability and retention (EPR) [29, 30].

The dispersion index is an indicator for measuring the uniformity of NEs, with a value greater than 0.4 indicating the non-homogeneity of particles. In the present study, the zeta potential of the formulation synthesized was obtained as -3.24 mV.

Studies have shown that electrostatic interactions between charged nanocarriers with the cell membrane are important for the cellular uptake of the carrier. Positively-charged nanocarriers usually have better internalization

and higher cell uptake efficiency than negatively-charged carriers. Nevertheless, positive surface charges accelerate the clearance of NPs from circulation. The electrostatic attraction between albumin (a negatively charged protein) and positively-charged particles is the main reason for the clearance of cationic nanocarriers. It is generally accepted that the normal pH of blood ranges from 7.35 to 7.45, while the isoelectric point of most blood proteins falls below 7. This means that most blood proteins are negatively charged, allowing them to form strong bonds with positively-charged nanocarriers, which can affect their interactions with and uptake by cells, as well as the biodistribution of these NPs. On the other hand, neutral or slightly negatively-charged NPs can somewhat resist being absorbed into and cleared by proteins. Studies have shown that negatively-charged NPs can be efficiently absorbed by cancer cells, which may be attributable to a structure named the protein corona. It is noteworthy that when NPs come into contact with biological environments, the absorption of biological molecules on their surface forms a layer called protein corona that alters various physicochemical properties of NPs, such as surface charge, size, aggregation behavior, etc. These modifications, directly or indirectly, affect the endocytosis of NPs by cells, as well as their pharmacokinetics and therapeutic efficacy [31].

In this study, the encapsulation efficiency (EE %) of the NEs was calculated to be 79.61%, indicating the suitability of the synthetic method employed. In similar studies, the encapsulation efficiency of luteolin NEs has been reported to range from 74% to 84% [22, 32, 33]. In the present research, we noticed that luteolin NEs had a plastic-like behavior. Viscosity, as a parameter reflecting the material's resistance to flow, is an important feature in semi-solid pharmaceutical products that are packed in plastic tubes or ready-to-inject syringes. The product inside the tube has a high viscosity at rest, but when pressure is applied, its viscosity declines, leading to its easy expulsion from the container.

The results of the present study showed that drug release at pH = 5.5 reached a maximum of 85% and a peak of 25% at pH = 7.5 after 72 hr. The greater release rate of the drug in an acidic environment, mimicking the tumor milieu, suggests the NEs synthesized as a suitable carrier to deliver luteolin to cancer tissues.

Regarding the thermal stability of the NE formulation, we observed no significant changes in particle size, PDI, and zeta potential after three months of storage at 4 °C. However, at 37 °C, the formulation became opaque and unstable after one month. In the toxicity assay, it was observed that luteolin NEs had higher toxicity compared with free luteolin at the same concentration, which can be related to the higher bioavailability of luteolin in the NE form.

In a 2014 study, luteolin nano-phytosomes were designed to increase their bioavailability and passive targeting toward breast cancer cells. The results showed that the concomitant treatment of cells with the NPs containing luteolin and DOX significantly increased the ratio of apoptotic MDA-MB 231 cells, which was accompanied by a reduction in the expression of the Nrf2 gene, a contributor to drug resistance, at the mRNA level [23].

We here observed that control NEs had higher toxicity effects than free luteolin, indicating the anti-cancer effects of *T. polium* essential oil. The IC50 values of the control NEs and free luteolin were obtained as 14.58 and 29.55 µg/ml, respectively, showing that the control NEs required a lower concentration than free luteolin to cause the same toxicity. The comparison of the cells treated with either DOX alone or DOX + free NE (IC_{25}), showed that the ratios of living cells were 70.5±3.4% and 60.34±2.96%, respectively. This observation demonstrated that the NEs of *T. polium* essential oil could significantly boost the anti-tumor effects of DOX (P=0.0057), suggesting an additive interaction.

In line, a study reported that the methanolic extract of *T. polium* presented strong synergistic anti-cancer effects in combination with vinblastine, vincristine, and DOX [34]. Also, a novel nano-scale Cirsiliol polymer obtained from Jordanian *T. polium* was reported to exert significant toxicity against breast cancer cells [35].

In the present study, the cytotoxicity of the IC_{10} and IC_{25} concentrations of luteolin and its NEs were assessed in combination with the IC_{25} dose of DOX against C26 mouse colon cancer cells. Among all co-administration strategies in this study, the combination of luteolin NEs (IC_{25})

and DOX offered the highest toxicity against the C26 cell line, with a cellular survival rate of 44.06 ± 2.57%.

The codelivery of luteolin NEs with DOX significantly increased toxicity against the cancerous cells compared with DOX alone (a difference of 26.44% in the ratio of viable cells), showing a synergistic effect between the two compounds. When the IC_{10} concentration of luteolin NEs was used, the difference in the rate of viable cells reached 17.90%. In conclusion, the simultaneous delivery of luteolin NEs and DOX could significantly increase the toxicity of DOX against C26 cancer cells concomitant with a reduction in its dosage.

In association with DOX, the NE form of luteolin at the IC_{25} concentration significantly increased the rate of apoptotic C26 cells by 20.47% compared with free luteolin, indicating significantly higher toxicity in the NE form. The increase in the rate of apoptotic cells was 15.69% at the IC_{10} concentration. Furthermore, in combination with DOX, luteolin NE at the IC_{10} concentration delivered an 11.92% higher rate of apoptosis compared with the group treated with DOX, and the IC_{25} concertation of free luteolin, reflecting that despite a lower concentration, luteolin had higher toxicity in the NE form probably due to its better bioavailability.

CONCLUSION

According to the findings of the present study, the combination of DOX and luteolin NEs synergistically increased antitumor effects against C26 colon cancer cells. Particularly, the use of luteolin NEs at the IC_{25} concentration could significantly reduce the ratio of living C26 cancer cells from 70.5±3.4% to 44.06±2.57% compared with free DOX. It is recommended to investigate the efficiency of the NEs prepared in this study in an animal model and introduce structural changes to this formulation to improve its physicochemical properties and increase its thermal stability in future studies. Nanoemulsions are potent drug carriers that can be used to treat complex diseases. One of the main benefits of using NEs respective to conventional nanocarriers includes the capability of the former group to be tailored to specifically eradicate drug-resistant tumor cells. Luteolin, a natural flavonoid, is widely available in a variety of plant species. Here, C26 cancer cells treated with luteolin were found to show increased apoptosis. We observed that the combination of the free and NE forms of luteolin with DOX decreased the ratio of viable C26 cancer cells from 70.5 ± 3.4% to 64.53± 1.95% and 44.06±2.57%, respectively. Also, *T. polium* essential oil, which was used as the oily phase in the NE formulation, showed toxicity against the cancerous cells. Overall, the formulation developed here can be considered a suitable option to increase the anti-tumor efficiency of DOX.

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CONFLICTS OF INTERESTS

None to declare.

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