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Formulating co-loaded nanoliposomes with gallic acid and quercetin for enhanced cancer therapy

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ABSTRACT

Cancer is considered one of the top global causes of death. Natural products have been used in oncology medicine either in crude form or by utilizing isolated secondary metabolites. Biologically active phytomolecules such as gallic acid and quercetin have confirmed antioxidant, antibacterial, and neoplastic properties. There is an agreement that microorganisms could mediate oncogenesis or alter the immune system. This research project aims to develop a novel formulation of co-loaded gallic acid and quercetin into nanoliposomes and investigate the efficacy of the free and combined agents against multiple cancerous cell lines and bacterial strains. Thin-film hydration technique was adopted to synthesize the nanocarriers. Particle characteristics were measured using a Zetasizer. The morphology of nanoliposomes was examined by scanning electron microscopy, Encapsulation efficiency and drug loading were evaluated using High-Performance Liquid Chromatography. Cytotoxicity was determined against Breast Cancer Cells MCF-7, Human Carcinoma Cells HT-29, and A549 Lung Cancer Cells. The antibacterial activities were evaluated against *Acinetobacter baumannii*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Therapeutic formulas were categorized into groups: free gallic acid, free quercetin, free-mix, and their nano-counterparts. Findings revealed that drug loading capacity was 0.204 for the mix formula compared to 0.092 and 0.68 for free gallic acid and quercetin, respectively. Regarding the Zeta potential, the mix formula showed more amphiphilic charge than the free quercetin and free gallic acid formulas (*P*-values 0.003 and 0.002 receptively). On the contrary, no significant difference in polydispersity indices was reported. Lung cancerous cells were the most affected by the treatments. The best estimated IC50 values were observed in breast and lung cancer lines for the nano-gallic acid and co-loaded particles. The nano-quercetin formula exhibited the least cytotoxicity with an IC50 value of

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≥200 μg/mL in both breast (MCF-7) and colorectal adenocarcinoma cell lines (HT-29) with no activity against the lung. A remarkable improvement in the efficacy of quercetin was measured after mixing it with gallic acid against the breast and lungs. The tested therapeutic agents exhibited antimicrobial activity against gram-positive bacteria. Nano-liposomes can either enhance or reduce the cytotoxicity activity of active compounds depending on the physical and chemical properties of drug-loaded and type of cancer cells.

1. Introduction

According to a recently published population-based statistical report by the American Cancer Society, around two million new cancer cases are estimated to occur in 2023, with projected mortality of 31%. Fortunately, the cancer mortality rate has continuously declined since 1991, with an overall reduction rate of 33%. The recorded reduction rate is due to advanced treatments and enhanced healthy lifestyles [1]. Despite the encouraging reports, cancer is considered as one of the top global causes of death according to data published by the World Health Organization [2].

Extensive research efforts were devoted to discovering and synthesizing appropriate anticancer drugs with an acceptable margin of safety and high efficacy. Protocols that are based on combined therapeutic agents have less substantial adverse effects than conventional treatments based on high doses of a single chemotherapeutic agent [3]. Since the earliest known anticancer medication originated from natural resources such as Vinca alkaloids, which were extracted from the *Catharanthus roseus* plant, increasing attention was directed toward investigating the cytotoxicity of variable phytomedicines [4]. Favorable pharmacokinetics and wide therapeutic indices motivate the inspection of herbal medicines for their anticancer characteristics [5]. Moreover, plants with anticancer properties have diverse mechanisms of action, for instance, suppression of cancer proliferation, inhibiting tumor cell growth, inducing apoptosis, and inhibiting angiogenesis. The diversity of their mechanisms of action enables treating different types of cancers like breast, head, and neck cancer cases [6–8].

On the other hand, immunity status and inflammations have been linked to the development of cancerous tissues, after finding that up to 20% of all cancers are associated with microbial coinfection [9]. Certain bacterial strains play a role in mediating cancer as they can thrive inside the cancerous tissues. Coincides, pathogenic bacteria such as *Pseudomonas aeruginosa* recommence to threaten cancer patients due to their ability to deteriorate immune systems [10,11]. Latest studies outlined the relationship between cancer and bacteria; *Escherichia coli* load increased in patients with colon cancer [12], *Acinetobacter baumannii* mutated to be resistant to multiple drugs in cancer patients [13], patients infected with *Staphylococcus aureus* bacteremia (SAB) have higher mortality rate than the uninfected patients during long-term follow-up, the latest finding supports the concept that SAB could serve as an early indicator of cancer [9]. Therefore, natural products with dual anticancer and antibacterial activities should be thoroughly investigated as promising alternatives to the available chemical drugs.

Plants, in general, are rich in secondary metabolites, which possess various biological and pharmacological activities such as antimicrobial, anti-inflammatory, antioxidant, cardio-protective, hypoglycemic, and anticancer properties [14–18]. Gallic acid (3,4,5-trihydroxy benzoic acid) (GA) is a biologically active natural phenolic compound and abundant in tea leaves, grapes, berries, and many other fruits. GA has clinically proven antioxidant, anti-inflammatory, analgesic, neuroprotective, anticancer, and anti-diabetic properties [19,20]. The antitumor activity of GA was screened and explored in various cancerous cells including oral, lung, pancreatic, brain, gastric and cervical cells. The supposed anti-tumor mechanisms of action are inhibiting cell proliferation, inducing apoptosis, and protecting human cells against oxidative damage without negatively affecting normal cells [21].

Likewise, quercetin (2-(3,4-dihydroxy phenyl)-3,5,7-trihydroxy4H-chromen-4-one) (Qu) is a polyphenolic flavonoid detected and isolated from a wide variety of natural sources such as tea, berries, buckwheat, broccoli, onions, apples, red grapes, honey, cherries, citrus fruits and green leafy vegetables [16,22]. Qu is a biologically active compound with proven antioxidant, anticancer, anti-diabetes, antiviral, anti-inflammation, and anti-proliferation pharmacological properties [7,23]. The presumed chemo-protective mechanisms are apoptosis, antioxidant activity, angiogenesis cell cycle and proliferation inhibitions, and prevention of tumor metastasis [24,25]. Furthermore, *in vitro* and *in vivo* studies confirmed Qu's ability to suppress tumor growth of various cancerous cell lines including breast, colorectal, stomach, head and neck, ovarian, gastric, colorectal, oral, liver, prostate, thyroid, pancreatic, lung, melanoma and leukemia cells [16]. This makes both GA and Qu strong candidates as potential anticancer agents. Fig. 1 (A & B) shows

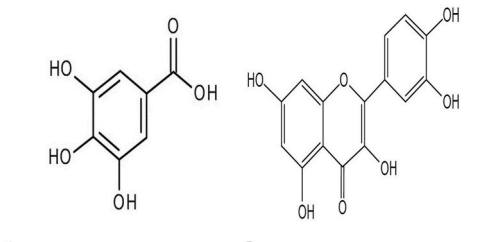

Fig. 1. Chemical structures of Gallic acid[5] (A) and Quercetin[7] (B).

the chemical structures of GA and Qu compounds, respectively.

As an integrated work, these efficient natural compounds need to be formulated into an appropriate delivery system to ensure and then enhance the anticancer activity. Nanocarriers represent an evolution in the delivery systems due to their preferable characteristics including the nanoscale size and high surface area to volume [26].

Liposomes are phospholipidic vehicles that serve as optimum delivery vehicles for their compatibility, safety, and fast elimination rate. Liposomes are categorized into multi-lamellar liposomes with more than one phospholipid bilayer in an onion-like structure and uni-lamellar liposomes with only one phospholipid bilayer. Liposomes supersede other nanocarriers due to their low toxicity, adaptability, low immunogenicity, readily regulated qualities, and acceptable pharmacological traits. On top of that, due to their capacity to boost the drug's bioavailability [27–29]. However, the limitations of using liposomes as delivery carriers include their short shelf-life and poor stability [30].

The current research study aims to develop a novel anticancer formulation based on mix-loaded nano-liposomes with GA and Qu for the synergistic treatment of cancerous cells and enhanced antibacterial activity.

2. Materials and methods

2.1. Materials and cell lines

Chemicals and solvents were purchased from Sigma-Aldrich (Germany), unless otherwise stated. Cell lines and the required media were obtained from the Pharmacological and Diagnostic Centre (PDRC) (Al Ahliyya Amman University, Jordan). *In vitro* tests were performed using the following cell lines: Breast Cancer Cell MCF-7 (ATCC number: HTB-22), Human Carcinoma Cells HT-29 (ATCC number ATCC HTB-38), and A549 lung Cancer Cell (ATCC number: CCL-185). Bacterial strains were obtained from PDRC (Al Ahliyya Amman University, Jordan). Antibacterial activities were evaluated against the following strains: *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (ATCC 8739), *Proteus mirabilis* (ATCC 12453), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 43300).

2.2. Preparation and characterization of nanoliposomes

The nanoliposome preparation method adopted the thin-film hydration techniques following the previously described specifications [31]. Where co-loading has been done as disciples in a paper by Gkionis and colleagues [32]. Three different formulae were prepared, to study the combined effect of Qu and GA on the characterization of nanoparticles: (HSPC: Cholesterol: DSPE/PEG2000), 65:30:5 with fixing loading F1: Qu loaded nanoliposomes (2 mg/mL), F2: GA loaded nanoliposomes (2 mg/mL), and F3: mix co-loaded nanoliposomes (2 mg/mL). Each prepared formula was tested for its maximum encapsulation efficiency with a lipid concentration of 0.51 mmoL.

2.3. Average particle size, zeta potential (charge), and morphology

Average particle size, zeta potential (charge), and polydispersity index (PDI) for free and loaded nanoliposomes were measured by dynamic light scattering (DLS) on a Zetasizer (Malvern Instruments Ltd., Malvern, UK). Samples were diluted 1:20 with distilled water. Where Surface morphologies of nanoliposomes were characterized using a Quanta scanning electron microscope Nanotechnology Institute (JUST/Jordan).

2.4. Encapsulation efficiency (%EE) and drug loading (%DL)

The encapsulation percentage of quercetin and gallic acid was determined by the degradation of nanoliposomes. This was performed by the addition of HPLC grade acetonitrile (800 μL) to the nanoliposomes (200 μL), followed by bath sonication (10 min, 35 °C). Then centrifugation (10 min, 12,000 rpm). Filtration step using 0.45 μm syringe filters. HPLC system was used to determine the concentration of encapsulated Qu and GA.

2.5. High performance liquid chromatography (HPLC)

Prepared samples were analyzed using HPLC in accordance and modified from Alam and colleagues [33]. Namely, using a gradient HPLC system (Prominence-i LC-2030C plus® HPLC system) consisting of a solvent delivery system pump, DGU-20A degasser, SIL- 20A auto-sampler, UV-VIS Plus detector and a CBM-20A communication bus module (All from Shimadzu, Kyoto, Japan). The signals were captured using LC-solution version 1.25 (2009–2010) workstation (Shimadzu, Japan) operating under Microsoft Windows XP, analyzed via Chrom Quest® software 4.2.34. Chromatographic separation was achieved on EC HPLC analytical column (NUCLEODUR® 100-5 C_{18} ec, 5 μm, 250 mm × 4.6 mm; MACHEREY-NAGEL, Germany). A gradient elution method was adopted using the following mobile system: methanol (A), and acetonitrile (B). Other variables were set as follows: the temperature at 40 °C, the injected volume of 2 μL, and the flow rate was set to 1.0 mL/min. HPLC-UV at $\lambda_{max} = 272$ and 370 nm for gallic acid and quercetin, respectively.

Table 1
Linear regression data for the constructed calibration curves.

Parameters	Gallic Acid (GA)	Quercetin (Qu)
Linearity range (mg/mL)	0.05–0.50	0.05–0.80
Regression equation	Y = 67586X + 93990	Y = 70000000X + 2000000
Correlation coefficient (R^2)	1.000	0.9950
Slope	67586	7000000
Intercept	93990	2000000
LOD (mg/mL)	0.002	0.00002
LOQ (mg/mL)	0.010	0.0001

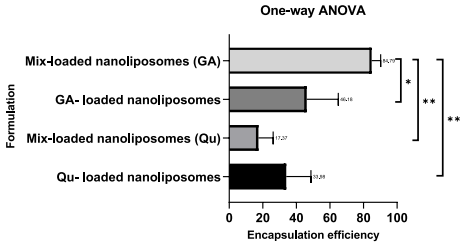

Fig. 2. Effect of drug loading on the encapsulation efficiency.

2.6. Standard solution preparation

A 25.0 mg of each standard, weighed separately, GA and Qu were weighed using an electronic analytical balance, dissolved in acetonitrile in a 25 mL volumetric flask, and stored at 4 °C for later use. Furthermore, the standards solution was diluted to different concentrations to produce standard curves (GA (0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL)), and Qu (0.05, 0.1, 0.3, 0.5, and 0.8 mg/mL). The detection was carried out under the same chromatographic conditions as previously described [27].

% EE and % DL were calculated as:

$$EE\ \% = \frac{\text{Entrapped drug}}{\text{Total drug added}}\ \text{x}\ 100\ \% \tag{1}$$

$$DL\ \% = \frac{\text{weight of entrapped drug}}{\text{Total weight of lipids}}\ \text{x}\ 100\ \% \tag{2}$$

2.7. Cell viability assay (MTT)

Cell viability assay test was performed in accordance with a previously published protocol using the reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [34]. Cell lines (MCF-7, HT-29, and A549; 5 × 103 cells per well) were seeded in 96-well plates. Groups were tested separately; Qu-treated, GA-treated, Qu-loaded nanoliposomes, GA-loaded nanoliposomes, and mix-loaded nanoliposomes in a range of selected concentrations, and the negative control (no treatment) The absorbance was measured at λ max 570 nm using a Glomax® microplate reader (Promega®, Madison, WI, USA).

2.8. Bacterial strains and growth conditions

The selected bacterial strains were cultured onto Mueller-Hinton agar (Biolab, Budapest, Hungary) and incubated at 37 °C for 24 h. The strains were kept in Mueller-Hinton nutrient broth supplemented with 15% glycerol and stored at −70 °C for later use [35].

2.9. Determination of minimum inhibitory concentration (MIC)

The MIC of gallic acid, quercetin, and co-loaded liposomes in a range of concentrations were determined separately using a microdilution method [36]. Tests followed the National Center for Clinical Laboratory Standards recommendations [37]. Ethanol (95%) was used as a negative control. The MIC values were visually determined after 24 h of incubation, with MIC expressed as the minimum concentration that led to bacterial growth inhibition.

2.10. Bacterial strains and growth conditions

This study was conducted on the various types of bacteria, such as *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 8739, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 43300. Bacterial strains were

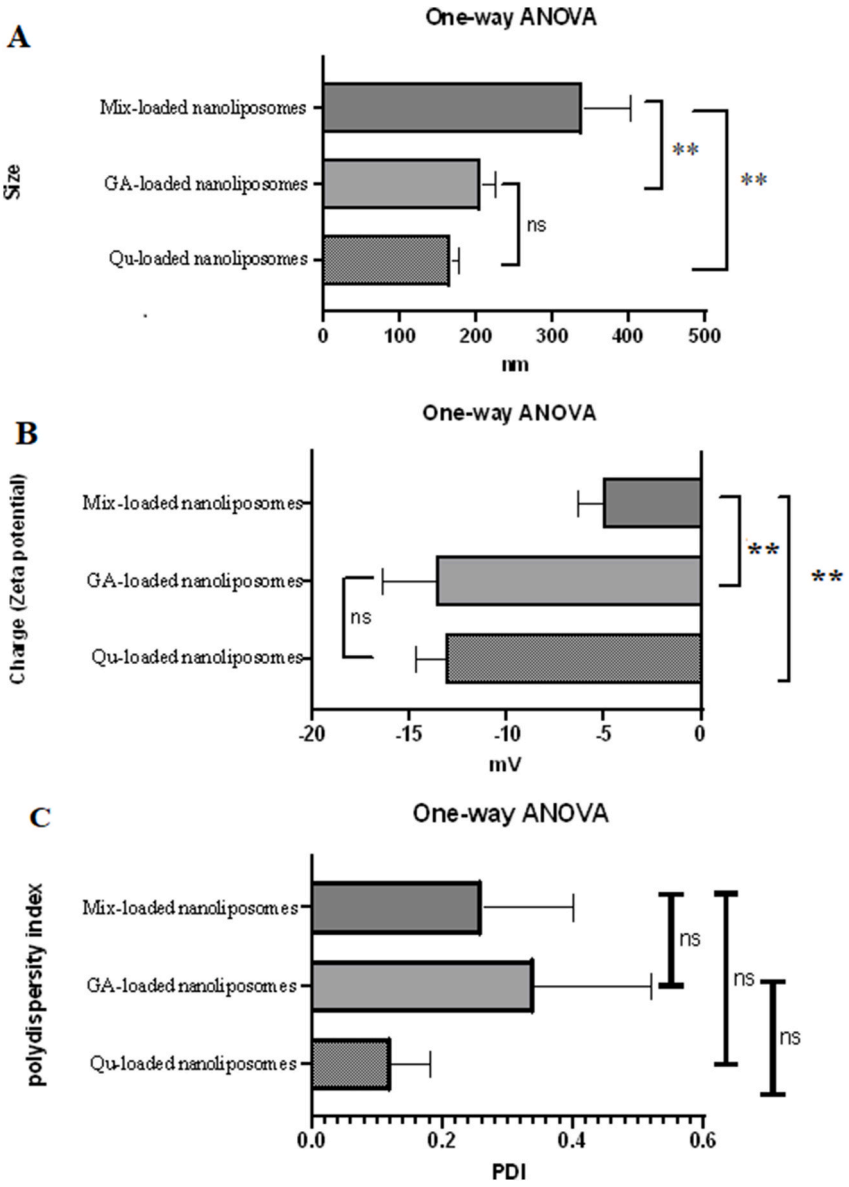

Fig. 3. Characterization Analysis of loaded nanoliposomes: size (A), charge (B), and polydispersity indexed (PDI) (C).

cultured onto Mueller-Hinton agar (Biolab, Budapest, Hungary) and incubated at 37 °C for 24 h. The strains were kept in Mueller-Hinton nutrient broth supplemented with 15% glycerol and stored at −70 °C for later use [38].

2.11. Determination of minimum inhibitory concentration (MIC)

The nanoliposome formulations of gallic acid, quercetin, and a mixture of both were evaluated using a microdilution method [39]. The tests were conducted in triplicate using 96-well microplates, following the recommendations of the National Centre for Clinical Laboratory Standards [40]. Negative control using ethanol (95%) was included.

The first well of each plate was filled with 180 μL of Mueller-Hinton broth (MHB), while 100 μL of MHB was added to the 2nd-10th wells. A 20 μL aliquot of each tested stock solution (GA-loaded nanoliposome at 0.6 mg/mL, Qu-loaded nanoliposome at 0.23 mg/mL and mix loaded nanoliposomes with 0.33 and 0.053 mg/mL of GA and Qu respectively) was added to the first row of the plate. Serial dilutions were then performed using a micropipette.

The wells were inoculated with 1 McFarland standard of bacteria, resulting in final concentrations of 30–0.015 μg/mL for the gallic acid-loaded nanoliposome, 11.5–0.0056 μg/mL for the quercetin-loaded nanoliposome, and 16.5–0.008 μg/mL for the mix loaded nanoliposomes containing a mixture of GA and Qu (2.65–0.0013 μg/mL for Qu). The bacterial inoculum was prepared from overnight

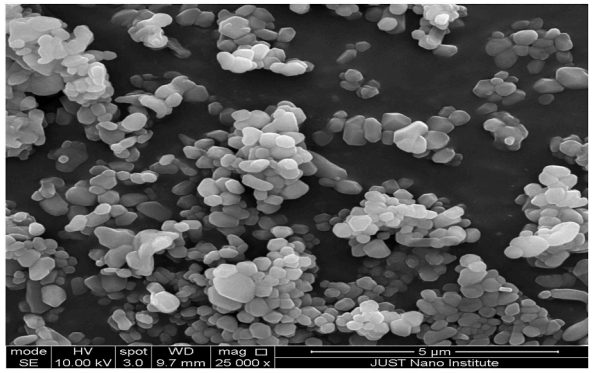

Fig. 4. Scanning electron microscopy (SEM) photograph of nanoliposomes. Three-dimensional spheroids with smooth surface and uniform size vesicles were found in all images.

cultures of each strain diluted in broth.

The inoculated plates were incubated at 37 °C for 24 h, and the MIC values were visually determined as the minimum concentration that inhibited bacterial growth.

2.12. Statistical analysis

The results were presented as the mean ± standard deviation of at least three independent experiments. Statistical significance was determined by using different statistical tests (normality test, Pearson correlation, paired *t*-test, and one-way ANOVA). A value of $p < 0.05$ was considered to assign a statistically significant difference. SPSS software, Version 21, GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA), and Microsoft Office Excel 2010 (Microsoft, Washington, DC, USA) were used.

3. Results and discussion

3.1. HPLC analysis and encapsulation efficiency

The analysis was run based on the obtained variations of the chromatograms after injecting Qu, GA, and co-loaded nanoliposomes separately by comparing the retention time (t_R) of each peak sample. Quantifying the amount of Qu and GA loaded in the formulas were computed based on the constructed calibration curve; concentration vs peak area). The regression equations were $Y = 67586X + 93990$, and $Y = 70000000X + 2000000$ for GA and Qu, respectively.

The computed parameters were demonstrated in Table 1. Results indicated that the proposed method exhibits a good sensitivity for quantification of the compounds of interest, GA and Qu. The method of validation was carried out following the ICH's guidelines for linearity range, LOD, and LOQ (ICH 2005). Based on the standard deviation of the response (SD) and the slope (S) of the calibration curves, the determination of LOD and LOQ was performed using the following formulas: LOD = 3.3(SD/S) and LOQ = 10(SD/S), respectively.

The Reversed-Phase High-Performance Liquid Chromatography (RP- HPLC) was adopted for separation. Adjusting the parameters; the separation temperature, the mobile system, and the analytical column were the main focuses of the optimization process.

The impact of Qu and GA on the Encapsulation Efficiency (EE %) was demonstrated in Fig. 2. The average EE % was found to be different across the tested formulas, $F(3, 4) = 9.747$, p 0.026. The LSD multiple comparisons, performed at the 0.05 significance level, found that the mean formula for the Mix (GA) 84.7 ± 5.3% was significantly higher than that for the GA, Qu and Mix with *P*-values 0.017, 0.007, and 0.041 respectively. Both formulations F2 and F3 presented high entrapment efficiency of GA 46.18 and 84.79%, respectively. These results matched the expectations because GA is an aqueous-soluble substance and has hydrophilic characteristics. This explained its encapsulation in the core of liposomes in the presence of Qu, a hydrophobic compound [41,42]. The improvement of the Gallic acid encapsulation efficiency (from 46.18 to 84.79) placed the concentrated GA in the core of liposomes. Moreover, an overall significant improvement in drug load ability in the mix formula was observed (*P*-value ≤0.001).

3.2. Particle characterization

Results obtained from the descriptive analysis of the average particle size, charge, and PDI characteristics are demonstrated in Fig. 3 (size (A), charge (B), and polydispersity indexed (PDI) (C)). And Fig. S The average particle size and charge were found to be different across formulas ($F(2, 6)$ 17.191, and 17.254, *P*-value = 0.003). LSD multiple comparisons, performed at the 0.05 significance level, revealed that the mean size of the Qu formula (166.57 ± 10.47) was significantly lower than that for the mix formula. There were no significant differences between GA formulas (*P*-values 0.001 and 0.241, respectively). Regarding the particle size characteristic, the GA formula had a significantly lower particle size than the mix formula (*P*-value 0.005). By co-loading two compounds having different

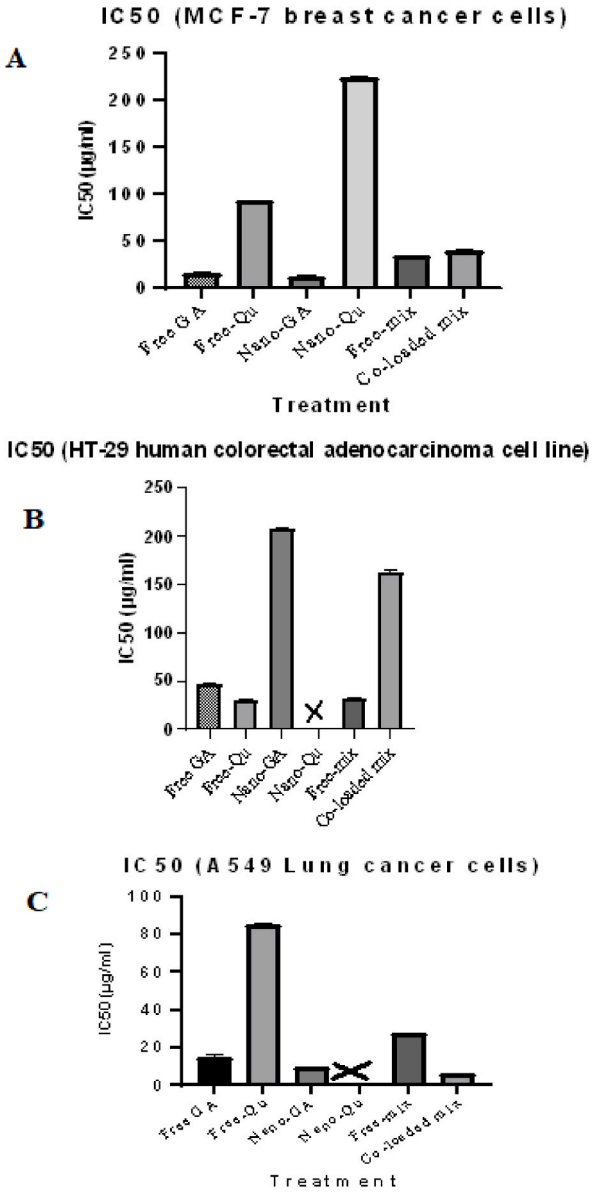

Fig. 5. IC_{50} values (µg/ml) of *in vitro* antiproliferative activity of free-Qu, nano-Qu, free GA, nano-GA, free-mix, and co-loaded mix in cancerous breast cells (A), cancerous colorectal cells (B), and cancerous lung cells (C). Results are mean ± SD (n = 3–4 independent replicates). IC_{50} values (concentration at which 50% inhibition of cell proliferation took place in comparison to non-induced basal 72 h incubations). X is non-inhibitory.

polarities, the lipophilic compound was encapsulated in a bilayer while the other, with hydrophilic properties, caused an increase in particle size [43]. Concerning the Zeta potential, significant differences between formulas were reported. Mix formula showed more amphiphilic charge than Qu and GA formulas (*P*-values = 0.003 and 0.002, respectively). On the contrary, no significant difference in PDI was reported (*P*-value = 0.202). Images of SEM demonstrated that nanoliposomes were three-dimensional spheroids with smooth surfaces and uniform size Fig. 4.

3.3. *In vitro cytotoxicity assay*

To determine the cytotoxicity of the compounds of interest, Qu and GA were prepared into different formulations and then tested against three cancerous cell lines: the breast, colorectal, and lung. Formulations were divided into groups as follows: free GA, free Qu, free GA and Quercetin, GA-loaded nanoliposomes, Qu-loaded nanoliposomes, and mix-loaded nanoliposomes. The cytotoxicity of each group was measured over a range of concentrations (3.0–100 µg/mL). The results were demonstrated in Fig. 5 (cancerous breast cells (A), cancerous colorectal cells (B), and cancerous lung cells (C)) and Fig. 6 (The percentage of cytotoxicity over a range of

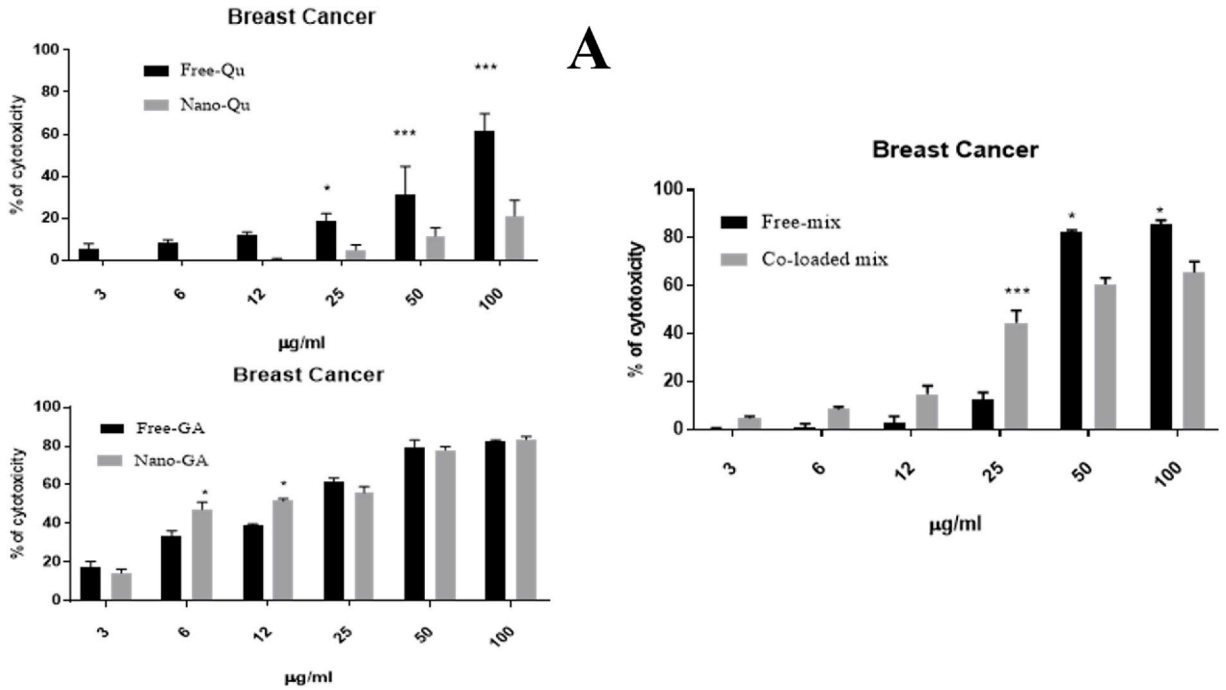

Fig. 6. The percentage of cytotoxicity over a range of concentrations of free-Qu, nano-Qu, free GA, nano-GA, free-mix, and co-loaded mix in cancerous breast cells (A), cancerous colorectal cells (B), and cancerous lung cells (C).

concentrations of free-Qu, nano-Qu, free GA, nano-GA, free-mix, and co-loaded mix in cancerous breast cells (A), cancerous colorectal cells (B), and cancerous lung cells (C)).

Lung cancerous cells were the most affected by the treatments by those loaded into nanoliposomes. For instance, significant toxicity was reported when treating lung cells with GA-loaded nanoliposomes, and mix-loaded nanoliposomes of 9.7, and 5.87 µg/mL, respectively.

In a comparative study between treating the cancerous lung cells with the free and GA-loaded nanoliposome formulas at a concentration of 25 µg/mL, the percentage cytotoxicity was computed to be 72.8 and 56.8%, respectively. On the other hand, the recorded percentages were 38.7 and 72.3% for the formulas containing free mix and mix-loaded liposomes, respectively.

The best estimated IC_{50} values in breast and lung cell lines were for the formula's GA-loaded nanoliposomes particles (12.9 and 9.7 µg/mL, respectively). On the contrary, the Qu–loaded nanoliposomes formula exhibited the worst cytotoxicity with an IC_{50} value of $\geq$200 µg/mL in both the breast and colorectal cell lines. The response was remarkably improved after reformulating the Qu-loaded nanoliposomes to be mixed with GA-loaded nanoliposomes; the IC_{50} values dropped to 40.4 and 161.9 in breast and colorectal cells, respectively.

Collectively, lung cancerous cells, the most sensitive type to the medications, were found to respond better to therapeutic formulas on a nanoscale. GA has a better profile on nano-formula against breast cancer. Surprisingly, the free form of Qu is much better with lower IC_{50} values than the loaded form. Regarding the colorectal line, relatively higher concentrations of medications (100 µg/mL) were needed to obtain ~80% of cytotoxicity with no exceptional benefits from encapsulating therapeutic agents into nanoliposomes.

GA was found to be an efficient cytotoxic agent regardless of the formulation type. Its enhanced activity could be due to the acidity of the compound, which stimulates the apoptosis process [44]. The obtained results agreed with previous findings, where the anti-cancer effects of GA were predicted to be mediated through inducing the apoptosis reactions and via ROS-dependent mitochondrial apoptosis in previous research performed against lung, breast, and colorectal cancerous cells. Lung cancer responded better as per reported [45].

Besides, combined therapy showed efficacy against cancerous cells with higher potency in either the free or the encapsulated mic forms. This matches the expectations because the nanoliposomes carries can improve the physiochemical properties, mask undesirable features, optimize water solubility, and increase drug bioavailability [46].

3.4. Antibacterial activity

Scientists have agreed on the role of microbiota in mediating immune and epithelial oncogenic processes [47]. Also, the direct impact of selective bacterial infection on cancer progression, such as colorectal cancer, was confirmed [48]. Therefore, the antimicrobial activities of the selected therapeutic agents have been investigated.

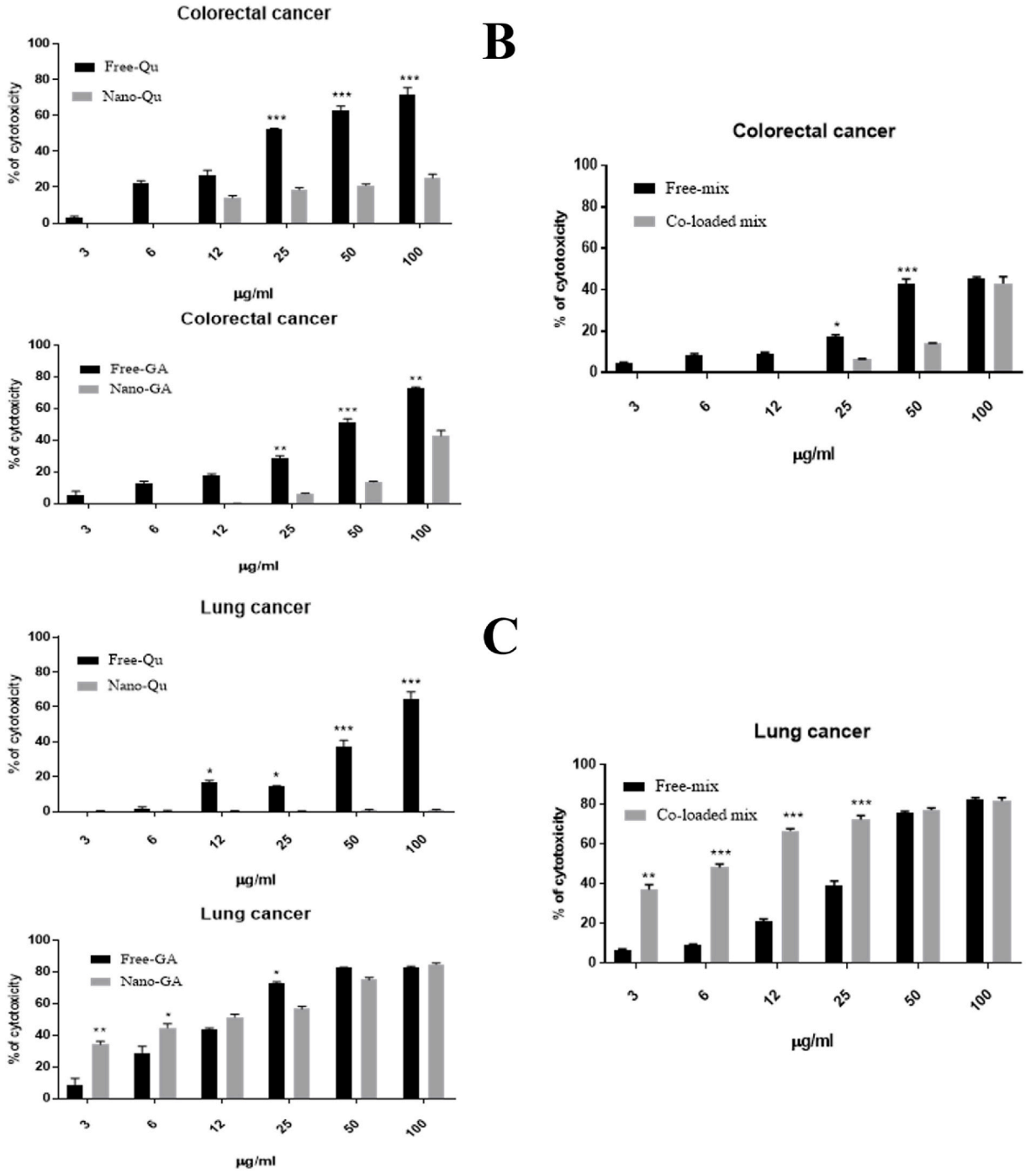

Fig. 6. (*continued*).

This study investigates the antimicrobial effects of nanoliposome formulations against *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 8739, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 43300. The three formulations tested were nanoliposomes loaded with GA, Qu, or a combination of both. The first and second formulations, containing GA and Qu, showed inhibitory activity against the gram-positive bacterium *S. aureus*, with MICs of 7.5 µg/mL and 2.9 µg/mL, respectively. The third formulation, containing a GA and Qu mixture, also displayed inhibitory activity against *S. aureus*, with MICs of 2.1 µg/mL and 0.331 µg/mL, respectively. However, none of the formulations or the combination showed any inhibitory activity against the tested gram-negative bacteria.

GA exhibits its bactericidal effect by disrupting the cell membrane [49]. While Qu activity might have attributed to several

mechanisms, including cell perforation, disruption of the membrane integrity, interference with the bacterial metabolic pathways, disruption of bacterial metabolism, or altering nucleic acid synthesis [50]. Considering that GA acts by disrupting the bacterial membrane, and that Qu has roles after microbial penetration; a synergistic activity of the combined formula was expected, as the combined action enhances access to intracellular targets and promotes bactericidal activity [51].

Furthermore, the acidic nature of both molecules contributes to producing cytoplasmic acidification of the bacteria. Once an interaction occurs between the hydrophobic moieties of the therapeutic compounds and the bacterial cytoplasmic membrane, the antimicrobial effect takes place [52].

4. Conclusion

Nano-liposomes Loaded with GA, Qu and mix loaded have been developed with the optimum nanoliposome characterization of size, charge and PDI as showed in zeta sizer, with spherical shape and smooth surface as represented in SEM.

Loaded nanoliposomes with GA, Qu and mix were successfully done and GA showed better encapsulation efficiency than Qu. *In vitro*, cytotoxicity results against Breast, Colorectal, and Lung cancer cells showed that Mix and Qu did not produce any improvement in the cytotoxicity after loading in Nano-liposomes, while there was a slight improvement in GA after loading. On the other hand, nanoliposomes-loaded GA, and mix showed remarkable improvement in cytotoxicity against Lung cancer cells. This information suggests that nano-liposomes can either enhance or reduce the cytotoxic activity of active compounds depending on the physical and chemical properties of the drug being loaded and the type of cancer cells. Moreover, loading GA and Qu into nano-liposomes can significantly improve the antibacterial activity against gram-positive bacteria.

The information obtained in this study could help to increase understanding of nano-liposomes loaded with phytochemical compounds and help in optimizing the new generation of phytosomes that could have potential pharmacological activity.

Author contribution statement

Ali Al-Samydai: conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; wrote the paper. Moath Al Qaraleh: conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data. Khaldun M. Al Azzam; Amal Mayyas; Nehaya Al-Karablieh; Fatima Alshaik: performed the experiments. Hamdi Nsairat; Amal Akour; Walhan Alshaer: contributed reagents, materials, analysis tools or data. Maha N. Abu Hajleh: analyzed and interpreted the data; wrote the paper. Lidia K. Al-Halaseh: contributed reagents, materials, analysis tools or data; wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

We confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Abbreviations

A549	Lung cancer cells
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DSPE-PEG2000	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-NPEG2000
EE	Encapsulation efficiency
HPLC	High-performance liquid chromatography
HT-29	human colorectal adenocarcinoma cell line
HSPC	Hydro Soy PC
GA	Gallic acid
IC50	Half-maximal inhibitory concentration

MCF-7	Breast Cancer Cells
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide solution
NP	Nanoparticle
nm	Nanometer
NO	Nitric oxide
NS	No significant differences
PDI	Polydispersity index
Qu	Quercetin
SEM	Scan electron microscopy
X	non-inhibitory
Z	Zeta

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