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Evaluation the therapeutic impact of propolis loaded noisome in HCT-116 colorectal cancer cells

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Abstract

We conducted a study to examine the anticancer properties of Propolis Loaded Noisome and 5-fluorouracil (5-FU) in HCT-116 Colorectal Cancer Cells. Approaches: The niosomes were prepared using the thin film approach and subsequently characterized in terms of their shape, size, and polydispersity index (PDI). We conducted additional assessments to examine the influence of Propolis on the viability of colorectal cancer cells, as well as their effects on RT-qPCR assays. The objective was to establish any potential connections between these factors and the anti-proliferative characteristics of Propolis. The *in vitro* investigation demonstrated that Propolis effectively suppressed the viability of cancer cells. In addition, they have increased the expression of protein 53 (P53), BCL2-Associated X Protein (Bax), caspase-9, while decreasing the expression of B-cell lymphoma 2 (Bcl2). The results of our investigation conclusively showed that Propolis had superior anti-cancer properties in HCT-116 Colorectal cancer cells.

Keywords: Colorectal cancer, Natural compounds, Propolis, Nanotechnology.

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1. Introduction

Colorectal cancer (CRC) ranks as the third most prevalent cancer globally. The occurrence and death rate of colon cancer have experienced a significant surge in some developing nations. By 2030, the incidence of this cancer is projected to rise by 60%. Colorectal cancer arises gradually due to a gradual buildup of genetic and epigenetic changes, leading to a significant amount of genomic instability. Colorectal cancer has been linked to the inactivation of tumor-suppressor genes such TP53 and TGF-β, as well as the activation of oncogene pathways [1]. The majority of patients (60%) diagnosed with colorectal cancer have no family history of the disease. In contrast, familial colorectal cancer (30%) is characterized by the presence of at least one blood relative who has been diagnosed with colorectal cancer or an adenoma. Hereditary colorectal cancer (10%) is caused by the inheritance of specific genetic mutations from one's parents [2]. Various natural chemicals are being employed to treat a range of different ailments, and they are receiving significant attention. Certain natural compounds used as anticancer therapies exhibit anti-inflammatory effects. The chemicals were synthesized into nanoparticles for the purpose of cancer treatment, but they have the potential to be employed for the treatment of other inflammatory conditions in the future. Nanoparticles can enhance the bioavailability of natural substances, hence improving their effectiveness in disease therapy and prevention. Propolis is a natural substance [3]. Honeybees produce propolis, which is a natural mixture of resin obtained from various plant sources such as plant parts, buds, and exudates. Propolis has been recognized and employed by humans for an extended period [4]. Propolis is presently employed for its antibacterial, anti-inflammatory, antiviral, antioxidant, antiprotozoal, anesthetic, antitumoral, and antihepatotoxic properties. Nanopropolis is a naturally occurring nanomaterial with medicinal and antioxidant characteristics. Moreover, a study by Rezk et al. has demonstrated that propolis in nanoform has superior efficacy compared to regular propolis in terms of its antibacterial and antifungal properties [5]. The aim of this

study was to assess the preventive and/or therapeutic impact of the Propolis Loaded Noisome in HCT-116 Colorectal Cancer Cells and compare it with the effects of treatment with 5-fluorouracil.

2. Methodology

2.1. Chemicals

Propolis and 5-fluorouracil (5-FU) was acquired from Sigma Aldrich, a company based in St. Louis, MO, USA. Propolis Loaded Noisome was synthesized at the Nanomaterials Research and Synthesis Unit, Animal Health Research Institute (ARC, Giza, Egypt). The cell lines were acquired from the National Research Centre in Giza, Egypt. The remaining chemicals used in the experiment were acquired from Sigma Aldrich (St. Louis, MO, USA).

2.2. Propolis Loaded Noisome Preparation

The niosome was created using the thin-film method. A lipid film was created by dissolving 100 mg of Span 60 and 20 mg of cholesterol in 10 mL of chloroform, and then evaporating the solvent using a Buchi R-3 rotary evaporator at 120 rpm and 60 °C for 1 hour. The resulting thin film was further hydrated using a solution, resulting in the production of Propolis Loaded Noisome. In order to attain the intended ultimate concentration, 10 ml of phosphate-buffered saline were introduced to distinct solutions of Propolis (50 mg/ml) at a pH of 7.4, while maintaining a temperature of 60 °C. The niosomal formulation was prepared by dispersing the lipid layer with the aqueous solution and subjecting it to sonication in an ultrasonic bath (Sonics & Materials Inc., USA) at a frequency of 60 HTz and at room temperature for a duration of 15 minutes [6].

2.3. Propolis Loaded Noisome Characterization

2.3.1. The zeta potential, PDI, and morphology

The size, PDI (PDI) testing, and zeta potential (ZP) values of the niosomes were determined by dynamic light scattering (DLS) analysis using the Malvern Zetasizer, Nano ZS model, from Malvern Instruments Ltd., U.K. Three separate analyses were conducted on each sample. The morphology of Propolis Loaded Noisome formulation was examined using a transmission electron microscope (TEM) (JEOL JEM-2100; JEOL Ltd, Tokyo, Japan) with the assistance of digital micrograph and soft image viewer software. A single droplet of drug-coated niosomal dispersion was diluted by a factor of 10 using deionized water. Subsequently, the diluted solution was applied onto a carbon-coated copper grid for a duration of one minute to facilitate adhesion of the niosomes. After the materials had dried at room temperature, they were analyzed by TEM without any staining [7].

2.3.2. Calculation of Entrapment Efficiency (EE)

The Propolis Loaded Noisome formulations underwent ultrafiltration using an Amicon Ultra-15 membrane (MWCO 30,000 Da) at a speed of 4000 g for a duration of 30 minutes. The niosomes containing the drugs were held in the upper chamber, awaiting filtration, while the unbound medications were let to pass through the filter membrane. The drug's concentration at its maximum absorption wavelength (420 nm) was determined using UV-visible spectroscopy (JASCO, V-530, Tokyo, Japan). Every drug concentration was compared to a reference curve. The efficiency of encapsulation was determined using the equation [8].

Efficiency of Enclosure (%) = [(A-B)/A] X 100

where A represents the initial concentration of the drug in the niosomal preparations and B represents the amount of free drug that diffuses across the membrane.

2.4. Investigation of Drug Release

A dialysis bag with a molecular weight cutoff (MWCO) of 12 kDa was utilized to conduct an in vitro comparison of drug release between 2 mL of free medicine, and Propolis Loaded Noisome. The bag was immersed in a 50 mL solution of phosphate buffered saline (PBS) at a concentration of 1X and a pH of 5.4. The solution was stirred gently at a speed of 50 revolutions per minute and maintained at a temperature of 37°C. Periodically, a fraction of the PBS solution was extracted and substituted with a fresh aliquot.

2.5. Investigation of Stability

In order to assess the stability of the Propolis Loaded Noisome, we did the following; the formulation was stored at both 25 ± 1 ºC and 4 ± 1 ºC for a duration of one month. Subsequently, the physical characteristics of the substance were assessed, including its average particle size (measured in nanometers) and entrapment efficiency (EE), at three time points: immediately, fourteen days later, and thirty days later.

2.6. Cultivation of cells

The HCT-116 cells were cultured at a sub-confluent state in a controlled environment of 37°C and 5% CO2. They were grown in complete Roswell Park Memorial Institute-1640 media supplemented with 10% fetal bovine serum, penicillin/streptomycin, and L-glutamine, all obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.7. Assessment of cytotoxicity

The MTT test is a colorimetric technique that involves the conversion of yellow MTT into purple formazan. The experiments were conducted in a controlled environment using a Laminar flow cabinet that meets the biosafety standard II (Baker, SG403INT, Sanford, ME, USA). A total of 104 cells per well were exposed to different concentrations of Propolis Loaded Noisome and 5-FU. Following a 48-hour period, we introduced a concentration of 2.5 µg/ml of MTT to each well, and subsequently placed the plates in an incubator at a temperature of 37°C for an additional 4 hours. Following the production of formazan crystals, a solution of 10% sodium dodecyl sulfate (200 µl per well) was employed to dissolve the crystals. We conducted absorbance measurements at a wavelength of 595 nm, and employed a positive control that induces complete mortality under identical circumstances. The change in viability was determined as a percentage using the following formula:

Cytotoxicity %= (Extract reading/Negative control reading) x100,

Viability %= 100- Cytotoxicity%,

The efficacy of each treatment is quantified by its IC50, which is the concentration at which 50% of the viability is suppressed.

2.8. Gene expression

The total RNA of the cells was extracted using the RNA Mini Kit from Ambion by Life Technologies, provided by Thermo Scientific (catalog number: 12183018A). In order to maintain the quality and reliability of the RNA samples, we employed a NanoDrop® ND-1000 Spectrophotometer manufactured by NanoDrop Technologies in Wilmington, Delaware, USA. The cDNA amplification was performed using the Thermo Scientific High Capacity cDNA Reverse Transcription Kit (4374966). The real-time PCR amplification was performed using the Maxima SYBR Green qPCR Master Mix (2X) kit from Thermo Scientific, specifically cataloged as #K0251. The housekeeping GADPH were employed as a reference or control. The levels of target gene expression were calculated using the $2^{-\Delta\Delta ct}$ method [9]. Table 1 comprises the sequences of the desired gene primers.

2.9. Statistical analysis

The statistical analysis was conducted using Graph Pad Prism 5 software developed by Graph Pad Software, located in La Jolla, CA, USA. One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to make multiple group comparisons and determine statistical significance. The provided numbers represent the mean standard error of a minimum of three independent measurements. The selected level of significance was below 0.05.

3. Results

3.1. Characterization of Propolis Loaded Noisome

The mean particle diameter (Figure 1), PDI, and zeta potential were determined using dynamic light scattering analysis using the Malvern Zetasizer. The average size in Propolis Loaded Noisome was 78±0.34 nm. The PDI score of 0.11345±0.21 falls within the permitted range. The ZP value of Propolis Loaded Noisome was measured to be -17±0.13 mV. The drug release profiles of formulation including Propolis Loaded Noisome was analyzed for a duration of 48 hours at a pH of 5.4 and a temperature of 37°C in order to gain insights into the in vitro drug release process. As evident from the "Release" plot (Figure 2), the initial administration of the free drugs resulted in a significant surge in their concentration in the bloodstream (45% increase within the first 6 hours). However, after 24 hours, the rate of release stabilized, reaching a constant level. Based on the Propolis Loaded Noisome release profile monitoring, it was seen that after 6 hours, 23.1% of the drug had entered the cells at a pH of 5.4. it was found that niosomes undergo expansion in an acidic environment, leading to the release of 87% of the medicine into the bloodstream after 48 hours at pH 5.4, respectively. The change in acidity of niosomes is associated with electrophilic addition reactions. An acidic environment induces greater cytotoxicity due to alterations in propolis, as well as an elevation in osmotic pressure. Figure 2 displays the drug release patterns of Propolis Loaded Noisome from the dialysis bag under pH 5.4 conditions at a temperature of 37 °C. The results are expressed as the mean value plus or minus the standard deviation (n = 3). The statistical significance levels are denoted as $*P < 0.05$ and $**P < 0.001$.

3.2. Investigation on the physical stability of Propolis Loaded Noisome

In order to assess the physical stability and effectiveness Propolis Loaded Noisome, we assessed the size of the vesicles, the PDI, and the EE at temperatures of 4 °C and 25 °C. These measurements were taken on days 0, 14, and 30 after the manufacturing date. Surprisingly, the results revealed that the temperature had no effect on the particle size, PDI, or EE. The recently developed formulation had the smallest size, with an average of 78 nm, the highest PDI value of 0.113, and an EE of 81.12%. The stability curve (Figure 3) demonstrates that the temperature exerted an influence on all the parameters throughout the 30-day period, starting from day 0. Expanding the pores of the niosomes can have positive effects on the particle size and PDI, resulting in an increase in either of them and a decrease in the EE to a minimum level of 72%. This is because temperature can impact the rigidity and elasticity of the niosomes. By comparison, at a temperature of 25 °C, the formation of pores resulted in increased size, higher PDI, and decreased EE of 62.6%. This indicates that the niosomes exhibit more rigidity and elasticity at lower temperatures.

Figure 3 illustrates the comparison of the stability of the optimal formulation of Propolis Loaded Noisome at temperatures of 4 °C and 25 °C. The stability parameters investigated were the mean particle size (A), PDI (B), and encapsulation efficiency (C). The results are expressed as the mean value plus or minus the standard deviation (n=3). *Significant at a confidence level of less than 0.05, **Highly significant at a confidence level of less than 0.001.

3.3. Cytotoxic effects on HCT-116 cells

The data presented in Figure 4 demonstrate a dose-dependent relationship. The viability percentage of HCT-116 cells was dramatically decreased after incubating them with various concentrations of Propolis Loaded Noisome, and 5-FU for 48 hours. In addition, Propolis Loaded Noisome had a cytotoxic impact on both cell types, with an IC50 of 43.54 µM. Similarly, 5-FU showed a cytotoxic effect with IC50 concentrations of 21.9 µM. These results are illustrated in Figure 4. Figure 4 illustrates the effects of varying concentrations of Propolis Loaded Noisome, and 5-FU on the survival of HCT-116 cells. This was determined using the MTT test following a 48-hour incubation period. The data are shown as the mean ± standard error, and statistical significance was determined at a p-value of less than 0.05.

3.4. Evaluation of mRNA expression levels of marker genes in HCT-116 cells.

The findings demonstrated a notable increase in the mRNA expression levels of P53, Bax, caspase-9 in HCT-116 cells following treatment with Propolis Loaded Noisome, and 5-FU, as compared to untreated cancer cells. There were significant differences in the mRNA expression levels of P53, Bax, caspase-9, and Bcl2 between HCT-116 cells treated with Propolis Loaded Noisome and 5-FU (Figure 5). Unlike our previous findings, the levels of anti-apoptotic Bcl2 was notably elevated in untreated cancer

cells. However, when the cells were treated with Propolis Loaded Noisome, and 5-FU, there was a significant decrease in Bcl2 expression in HCT-116 cells compared to the untreated cancer cells. Figure 5 shows the effects of Propolis Loaded Noisome, and 5-FU on the expression levels of P53, Bax, caspase-9, Bcl2 genes in the HCT-116 cell lines. The gene mRNA expressions are measured relative to GADPH. * A statistically significant difference was observed between the control and treatment groups ($p < 0.05$).

4. Discussion

Propolis has found extensive application in the fields of food and drinks, medicine, and cosmetic items [10]. The active compounds of propolis possess a range of pharmacological properties, including cytotoxic, antiherpes, free radical scavenging, antimicrobial, anti-HIV, antiprotozoal, antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, anti-tumor, immunomodulatory, hepato-protective, antidiabetic, and healing properties [11]. Propolis has varying compositions according on geographical areas, including Europe, North America, New Zealand, temperate zones of Asia, and Brazil. Propolis contains phenolic acids, flavonoids, terpenes, aromatic aldehydes and alcohols, fatty acids, stilbenes, and b-steroids [12]. Nanoparticular systems refer to colloidal drug delivery systems that have gained significant attention in cancer treatment. This is mostly owing to their small particle size, surface characteristics, structure, and stability [13]. Nanoparticular systems encompass a range of forms including liposomes, niosomes, solid lipid nanoparticles, dendrimers, silicon or carbon compounds, and magnetic nanoparticles [14]. Niosomes are a type of drug delivery systems where the drug is enclosed within a vesicle. The vesicle consists of a bilayer of nonionic surfactants. Niosomes have the ability to alter the metabolism of a drug, as well as extend its circulation and half-life. Niosomes mitigate the adverse effects of medication [15]. Niosomes play a crucial role in cancer treatment by serving as a drug delivery mechanism. They offer several advantages, such as controlled release of the active component and the ability to encapsulate various medications [15]. Multiple conservative therapies exist for cancer. Traditional treatment modalities, such as surgical intervention and chemotherapy, possess several drawbacks that contribute to the ineffectiveness of cancer treatment [16]. A significant amount of research has been dedicated to the development of innovative medication delivery and targeting systems in order to reduce the adverse effects of pharmaceuticals in cancer treatment. The treatment's effectiveness is hindered by many side effects such as low bioavailability, toxicity, nonspecificity, rapid clearance, and restrictions in metastasis [17]. Medicinal plants and their preparations have been utilized since ancient times. Natural compounds exhibit non-toxicity towards normal cells and are also better tolerated. Phytochemicals and their metabolites, including alkaloids, flavonoids, phenolics, tannins, glycosides, gums, resins, and oils, are the causative components. These elements or their modified derivatives have demonstrated substantial anticancer efficacy. The investigation of drugs' anticancer efficacy involves assessing their cytotoxicity against cancer cell lines. A large number of chemicals can be rapidly screened to assess the viability of cancer cells. Various phytochemicals have been discovered from plants and nutritional supplements. Herbal extracts additionally inhibit the vitality of cancer cells. Potent bioactive phytochemicals and formulations hold promise for the creation of more secure anticancer medications [18].

Table 1: Primers sequences used in this study

Gene	Primers	Gene bank accession number
P_{53}	F 5′- CCTCAGCATCTTATCCGAGTGG-3′ R 5'-TGGATGGTGGTACAGTCAGAGC-3′	NM_000546
Bax	F 5′- TCAGGATGCGTCCACCAAGAAG -3′ R 5'- TGTGTCCACGGCGGCAATCATC -3′	NM_001291428
Bcl2	F 5′- ATCGCCCTGTGGATGACTGAGT-3′ R 5'- GCCAGGAGAAATCAAACAGAGGC-3′	NM_000633
Caspase-9	F 5′-GTTTGAGGACCTTCGACCAGCT-3′ R 5'-CAACGTACCAGGAGCCACTCTT-3′	NM_001229
GAPDH	F 5′- GTCTCCTCTGACTTCAACAGCG -3′ R 5'- ACCACCCTGTTGCTGTAGCCAA -3′	NM_001256799

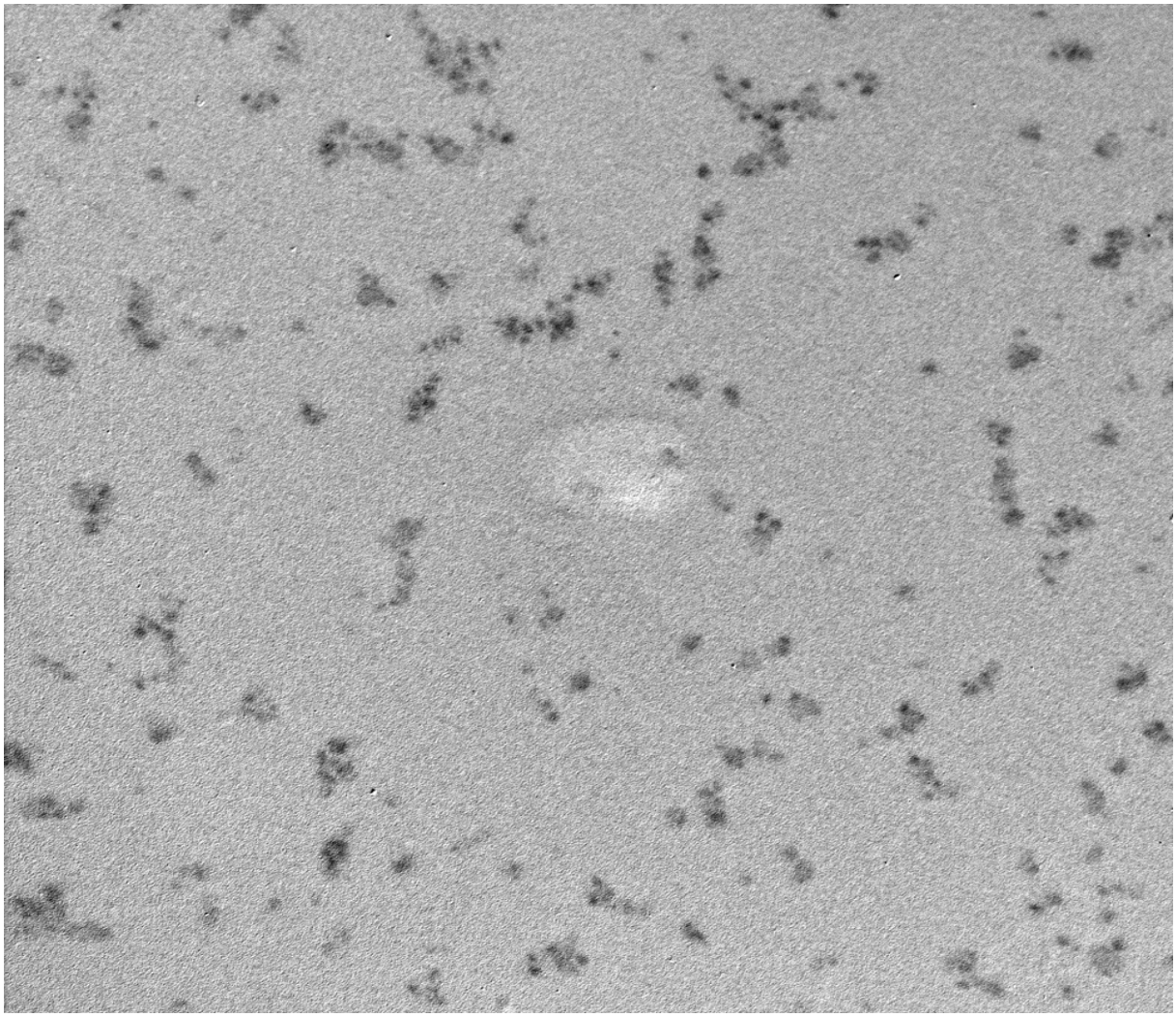

Figure 1: Transmission electron microscopy image of the Propolis Loaded Noisome.

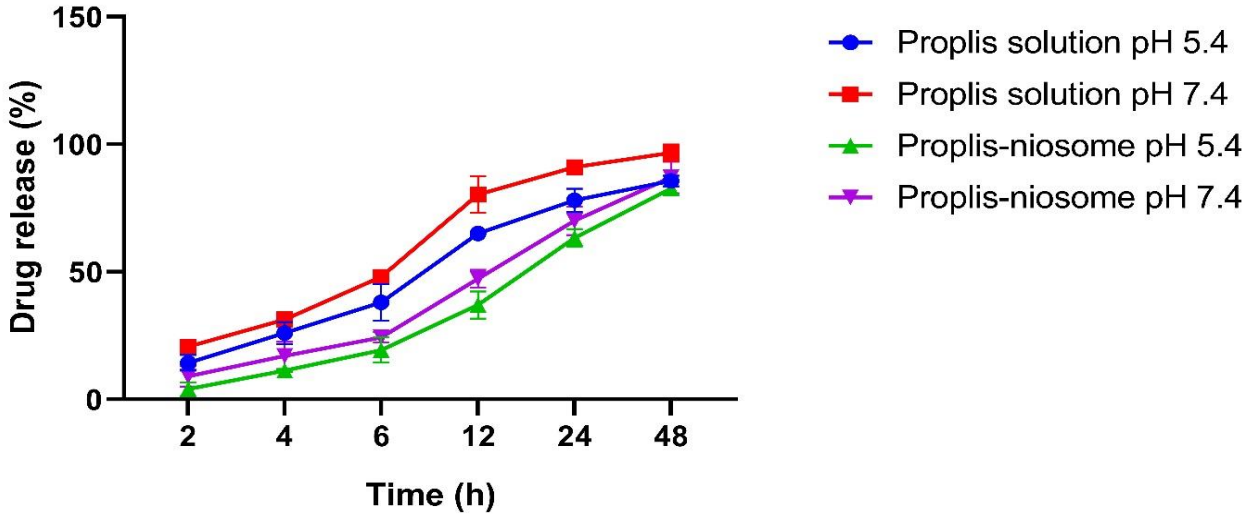

Figure 2: Displays the drug release patterns of propolis-loaded noisome from the dialysis bag under pH 5.4 conditions at a temperature of 37 °C. The results are expressed as the mean value plus or minus the standard deviation (n = 3). The statistical significance levels are denoted as *P < 0.05 and **P < 0.001.

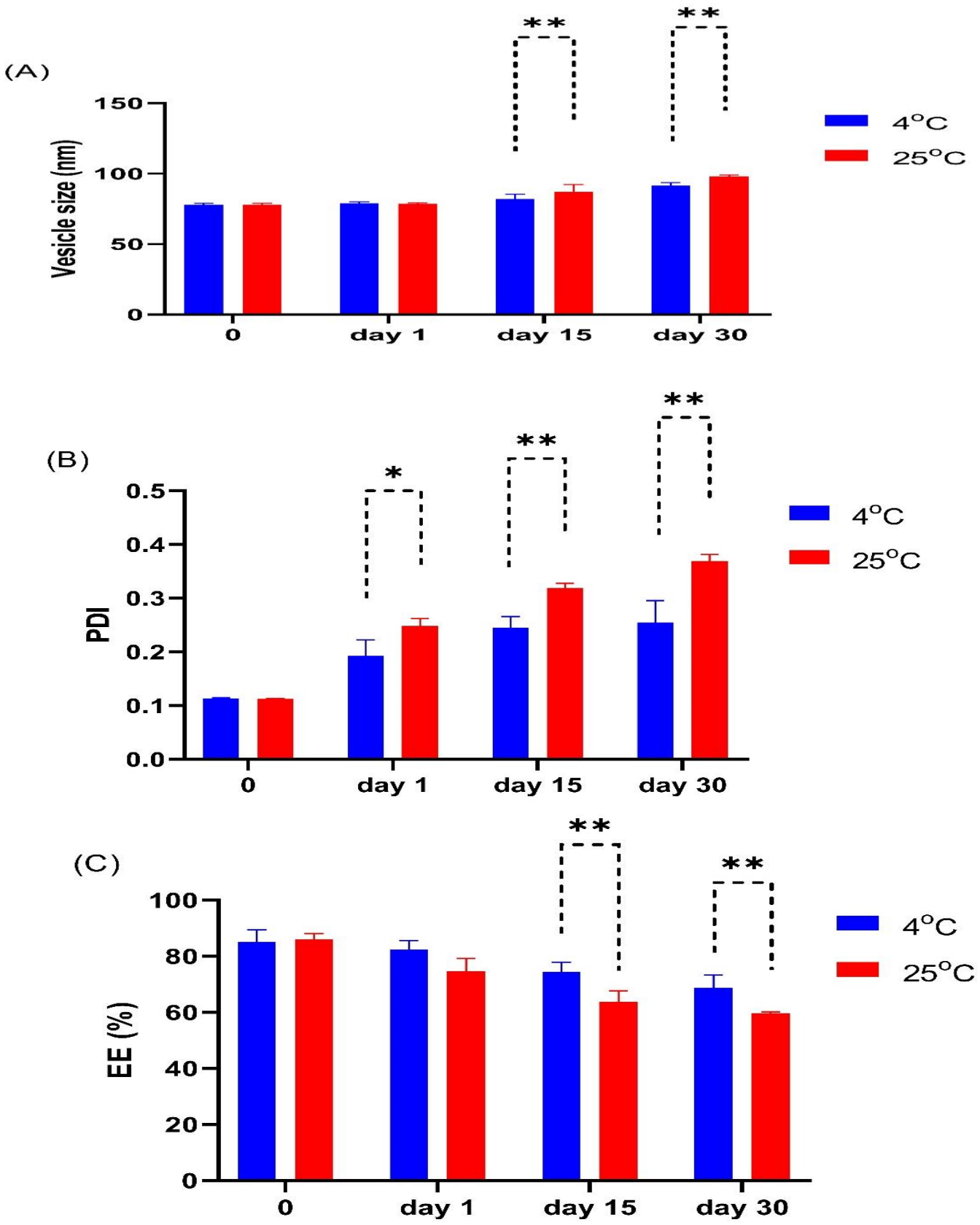

Figure 3: . illustrates the comparison of the stability of the optimal formulation of Propolis Loaded Noisome at temperatures of 4 °C and 25 °C. The stability parameters investigated were the mean particle size (A), PDI (B), and encapsulation efficiency (C). The results are expressed as the mean value plus or minus the standard deviation (n=3). *Significant at a confidence level of less than 0.05, **Highly significant at a confidence level of less than 0.001.

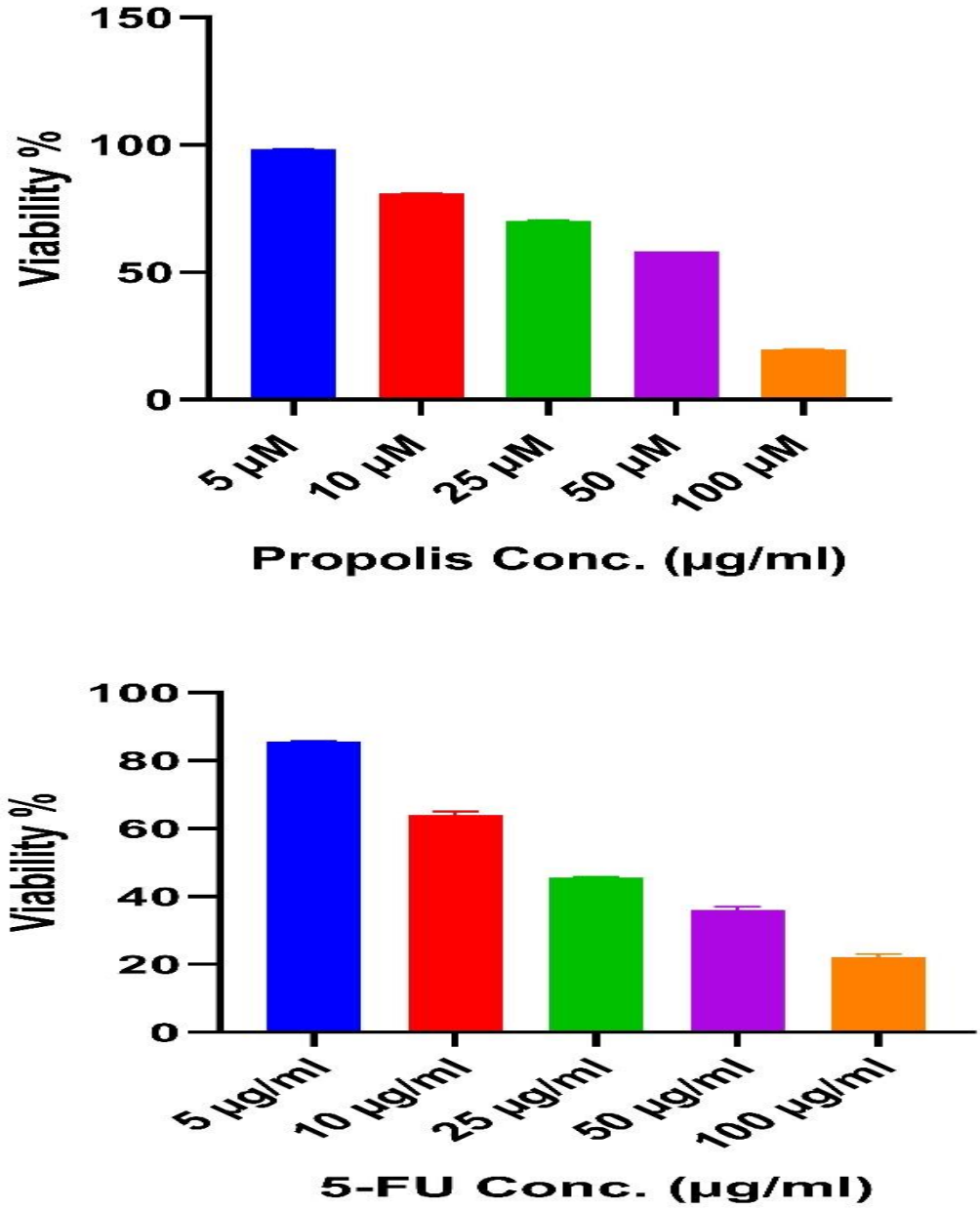

Figure 4: illustrates the effects of varying concentrations of Propolis Loaded Noisome, and 5-FU on the survival of HCT-116 cells. This was determined using the MTT test following a 48-hour incubation period. The data are shown as the mean ± standard error, and statistical significance was determined at a p-value of less than 0.05.

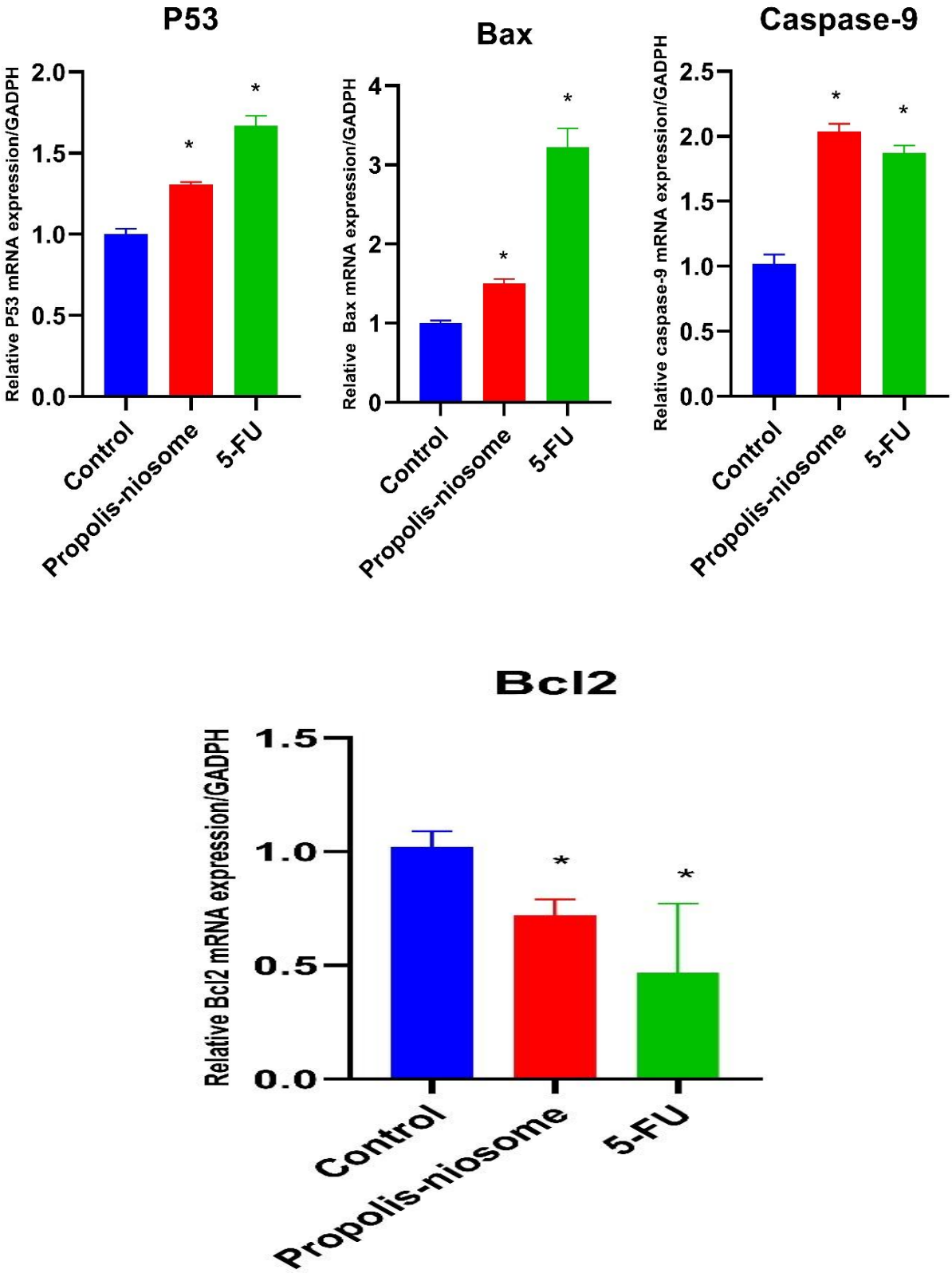

Figure 5: shows the effects of Propolis Loaded Noisome, and 5-FU on the expression levels of P53, Bax, caspase-9, Bcl2 genes in the HCT-116 cell lines. The gene mRNA expressions are measured relative to GADPH. * A statistically significant difference was observed between the control and treatment groups ($p < 0.05$).

Propolis was encapsulated within niosomes in our investigation for the purpose of cancer treatment. In contrast to previous investigations, our study utilized the thin film hydration approach to create niosomes [15]. Observation revealed a uniform particle size distribution of placebo and propolis-loaded niosomes. The placebo formulation had a particle size of 208 nm, while the propolis loaded formulation had a particle size of 232 nm. The zeta potential of the placebo formulation was 44.9 mV, while the propolis loaded formulation had a zeta potential of 44 mV [19]. The PDI quantifies the uniformity of the size distribution of the particles. The placebo niosome formulation had a polydispersity value of 0.184, while the propolis-loaded formulation had a PDI of 0.152. The niosome formulation is made stable due to its high zeta potential [20]. Nanoparticulate drug delivery systems exhibit significant characteristics due to their ability to modulate pharmacokinetics and biodistribution. The size of nanoparticular systems is directly associated to the cellular uptake ratio. The optimal diameter range for achieving the highest uptake ratio is between 200 and 300 nm [21]. Multiple studies have reported the antiproliferative effect of propolis and its different bioactive components. The potential of inducing apoptosis or cell cycle arrest in cancer cells is examined to determine the anticancer effect. The primary mechanisms behind the anti-cancer effects of propolis are the activation of apoptosis and cell cycle arrest [22]. Another investigation found that the spheroid form of L929 cells did not show significant changes after 24 hours of examination, following the application of EEP and PLN (at 192 hours). In the wells treated with PLN, the L929 cells maintained their spherical shape, however there was a little reduction in the size of the spheroids. The MCF7 cells were cultured and expanded using clone forms, resulting in the presence of many clones. The size of the spheroids and the number of clones were both reduced in the wells treated with PLN. Furthermore, the spheroid shape was impaired in wells treated with EEP. The size of spheroids in A549 cells was dramatically reduced in wells treated with PLN, while there was no significant change observed in wells treated with EEP [23].

5. Conclusion

Our investigation revealed that Propolis Loaded Noisome effectively suppressed the growth of HCT-116 cancer cell lines. Our research uncovered other effects of Propolis Loaded Noisome which resulted in an increase in the levels of P53, Bax, caspase-9, and a decrease in the expression of Bcl2. This also led to a reduction in MDA levels. The aforementioned implications suggest that Propolis Loaded Noisome possess anti-proliferative capabilities that could be beneficial in inhibiting the growth of HCT-116 cancer cells.

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