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Enhancing Anticancer Efficacy through Nanoemulsion Formulation of Acetylsalicylic Acid and Black Seed Oil: A Comprehensive Characterization and Therapeutic Evaluation

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Abstract:
Nanoemulsions have gained significant attention as efficient drug delivery systems due to their improved solubility, stability, and potential for targeted therapy. This study presents the formulation, characterization, and evaluation of an oil-in-water nanoemulsion containing Acetylsalicylic Acid (ASA) and Black Seed Oil (BSO) for its potential as an innovative anticancer agent. The nanoemulsion was characterized for particle size, zeta potential, and polydispersity index. The formulation exhibited mean droplet sizes of 94.67 ± 5.4 nm and 157 ± 4.3 nm for plain BSO and ASA-BSO nanoemulsion, respectively. Zeta potential values were -15 ± 2.4 mV and -27.67 ± 1.24 mV, respectively. Drug content determination confirmed a high loading capacity of 98.43 ± 2.32% for ASA-loaded nanoemulsion. In vitro drug release studies demonstrated enhanced release from the nanoemulsion compared to non-formulated ASA. Stability investigations indicated retained physicochemical properties over three months. Biological assessments included cell viability assays, cell cycle analyses, and apoptosis assessments in MCF-7, HePG2, and HCT 116 cancer cell lines. The ASA-BSO nanoemulsion formulation exhibited potent cytotoxic effects, impacting cell viability and arresting cells at different cell cycle phases. Apoptosis assessment revealed a significant increase in apoptotic populations, particularly in MCF-7 cells (84.35 ± 2.5%). These findings suggest the ASA-BSO nanoemulsion's potential as an effective and multifaceted anticancer strategy. The study highlights the formulation's capacity to impact various aspects of cancer cell behavior, supporting its further exploration as a novel approach in cancer therapy.

Keywords: Nanoemulsion; Particle size; Cancer; Natural Oil

Introduction

Nanoemulsions are a class of colloidal systems composed of nanoscale droplets of one immiscible liquid dispersed within another, stabilized by surfactants. These unique structures have garnered substantial attention due to their remarkable properties and diverse applications, especially in the realm of drug delivery. Their ability to enhance solubility, stability, and bioavailability of hydrophobic compounds has positioned nanoemulsions as promising carriers for therapeutic agents in various fields[(1)(2)].

In the context of pharmaceuticals, nanoemulsions offer an innovative approach to overcome the challenges associated with traditional drug delivery systems. By providing a high surface area for interaction and encapsulation, nanoemulsions enable efficient transport of active compounds to target sites, contributing to enhanced therapeutic outcomes. Their versatility makes them ideal candidates for delivering poorly water-soluble drugs and addressing issues of poor bioavailability [(3)].

Black seed oil, derived from the Nigella sativa plant, has long been recognized for its potential health benefits. Black seed oil is rich in bioactive constituents and exhibits anti-inflammatory, antioxidant, and immunomodulatory properties. The exploration of nanoemulsions as delivery vehicles for black seed oil opens up new avenues to harness its therapeutic potential, enhancing its solubility and bioavailability, thus potentially amplifying its health-promoting effects [(4)(5)].

Acetylsalicylic acid, commonly known as aspirin, has been a cornerstone of pain relief and anti-inflammatory therapy for decades. Beyond its established pharmaceutical applications, recent research has unveiled its potential as an anticancer agent [(6) (7)]. Repurposing acetylsalicylic acid for its anticancer properties offers a cost-effective strategy that builds upon its well-established safety profile [(8)]. However, unlocking its full potential as an anticancer agent requires innovative delivery systems that ensure targeted and controlled drug delivery.

This research aims to formulate and characterize oil-in-water nanoemulsions encapsulating black seed oil and acetylsalicylic acid. By combining these two compounds, this study aims to develop a novel formulation that capitalizes on their respective therapeutic properties. Specifically, the study seeks to evaluate the physical properties of the nanoemulsions, analyze their drug release profiles, assess their stability over time, and investigate their potential anti-cancer activity. According to existing literature, the research focuses on breast, colon, and liver cancers, which are prevalent in Saudi Arabia [(9) (10)].

Through the successful formulation of these nanoemulsions and the comprehensive evaluation of their properties and potential applications, this research contributes to the advancement of drug delivery systems. If proven effective, these nanoemulsions could pave the way for an efficient, safe, and cost-effective strategy to repurpose acetylsalicylic acid as an anticancer agent, offering hope for improved cancer treatments while minimizing the need for entirely new drug entities.

Methodology

4.1 Formulation of nanoemulsion

The nanoemulsion formulation process was initiated by meticulously combining the necessary components. Initially, black seed oil (3ml) and Span 20 (1.5mg), a lipophilic surfactant, were measured and blended together to form the oil phase. Acetylsalicylic acid (3 mg) was also incorporated into this oil phase for the ASA-loaded nanoemulsion.

Subsequently, a hydrophilic surfactant, Tween 80 (3.5 mg) was added to the water phase (96ml). This step was pivotal, as it ensured the appropriate partitioning of surfactants based on their solubility characteristics – Span 20 in the oil phase and Tween 80 in the water phase.

The next crucial step involved thoroughly dissolving the hydrophilic surfactant, Tween 80, in the aqueous phase to create a uniform mixture. This step was paramount in ensuring the stability and homogeneity of the ensuing nanoemulsion.

The emulsification process was then executed by gradually introducing the prepared oil phase into a warm aqueous solution while maintaining continuous stirring. The primary objective here was to establish a stable and finely dispersed nanoemulsion. This was accomplished by employing a Vortix mixer, which vigorously agitated the mixture, promoting the proper amalgamation of the oil and aqueous phases.

An optimization step was employed to further enhance the stability and uniformity of the nanoemulsion. This optimization involved subjecting the resulting emulsion to heating and cooling cycles in an ultrasonicator. The ultrasonication process was conducted for 60 to 90 minutes, with alternating intervals of 25°C and 60°C. This optimization step played a crucial role in refining the nanoemulsion, ensuring its overall quality and consistency [(12) (13)].

4.2 Characterization of Acetylsalicylic Acid-Loaded Black Seed Oil-Based Nanoemulsion

4.2.1 Measurement of Particle Size, Zeta Potential, and Polydispersity Index (PDI): The characterization of the nanoemulsion involved the measurement of its mean droplet size, zeta potential, and Polydispersity Index (PDI), which were conducted at a controlled temperature of 25 ± 1˚C using a Malvern particle size analyzer known as the Zetasizer Nano ZS. To facilitate these measurements, the nanoemulsion was appropriately diluted with distilled water at a ratio of 1:200. For the assessment of particle size and PDI, the diluted nanoemulsion

was placed in disposable cuvettes, while zeta potential measurements were carried out using a glass electrode sample holder [11] [12].

4.2.2 Drug Content Determination: To assess the drug content in the ASA-loaded nanoemulsion, a 1ml portion of the nanoemulsion was added to a 10 ml volumetric flask. Ethanol was then added to the flask until it reached the mark, ensuring complete dissolution of the components and creating a homogeneous solution. Afterwards, the solution was filtered to remove any particulate matter or impurities. Then, 0.50 ml of the filtered solution was accurately measured and transferred to another 10 ml volumetric flask. This volume was then filled with distilled water, bringing it to the mark. This resulting solution was specifically prepared for the measurement of absorption at 227 nm. A reference solution consisting of distilled water was also used for calibration purposes [14].

4.3 In Vitro Drug Release

For the assessment of in vitro drug release, a Franz diffusion cell was employed, incorporating a dialysis membrane with a Molecular Weight Cutoff (MWCO) ranging between 12,000 and 14,000 Da to separate the receptor and donor compartments. Within the donor compartment, 1 mL of either the drug-loaded formulation or a drug suspension (at a concentration of 5 mg/mL) was placed, while the receptor compartment contained 20 mL of Phosphate-Buffered Saline (PBS) supplemented with 0.5% Tween 80. The experiments were conducted at a controlled temperature of 37 ± 1 ˚C with continuous agitation at 100 rpm. At predetermined intervals, samples were withdrawn, and the drug content was quantified spectrophotometrically at a wavelength of λmax 276 nm. Subsequently, cumulative drug release profiles were calculated, providing insights into the release kinetics of the tested formulations[12].

4.4 Stability Studies on Optimized Nanoemulsion

The stability of a nano-emulsion formulation containing acetylsalicylic acid was evaluated following the guidelines established by the International Conference on Harmonization (ICH) [15]. A set of samples were kept in controlled refrigeration conditions at 4 ± 0.7 °C, and were examined periodically at both 0 and 90-day intervals to assess the formulation's stability and integrity over time. The parameters evaluated included particle size, Zeta potential, and drug content.

4.5 Biological Study

4.5.1 Cell Lines: Culturing of human breast adenocarcinoma cells (MCF-7), liver carcinoma cells (HePG2), and colon adenocarcinoma cells (HCT 116) was performed using RPMI 1640 medium, which was further supplemented with 10% fetal bovine serum and 100 IU/mL penicillin/streptomycin for optimal growth and maintenance of these cell lines.

4.5.2 Cell Viability Assay: Cell viability was determined via the sulforhodamine B (SRB) assay, which involved subjecting cells to various concentrations of nanoemulsion formulations for a duration of 72 hours. Cells were stained after treatment, and their absorbance was measured at 540 nm. The calculation of cell viability was based on a provided formula [16].

4.5.3 Cell Cycle Analysis: Treatment of cells involved exposing them to formulations at concentrations corresponding to their respective IC50 values over a 48-hour incubation period. Subsequently, cell cycle analysis was conducted, employing Propidium Iodide (PI) staining in combination with flow cytometry to assess and characterize the distribution of cells within different phases of the cell cycle.

4.5.4 Apoptosis Analysis: Apoptosis assessment was performed using Annexin V-FITC/propidium iodide staining, a method that evaluated apoptotic processes within the cells. Following treatment, the cells were subjected to staining and subsequently analyzed through flow cytometry, providing valuable insights into apoptotic events and cellular responses [17].

4.6 Statistical Analysis

All experiments were meticulously carried out in triplicate, and the results are presented as mean values accompanied by the corresponding standard deviation (mean ± SD). To assess statistical significance, both Student's t-test and one-way analysis of variance (ANOVA) were employed, with a significance level set at $p < 0.05$, indicating statistical significance.

Results

5.1 Particle Size, Zeta Potential, and Polydispersity Index (PDI) Analysis
The physical characteristics of the nanoemulsions were extensively analyzed through particle size, zeta potential, and polydispersity index measurements. The results are summarized in Table 1.

Formulation	Particle Size (nm)	Zeta Potential (mV)	Polydispersity Index (PDI)
Black Seed Oil Nanoemulsion	94.67 ± 5.4	-15 ± 2.4	0.481
Acetylsalicylic Acid - Black Seed Oil Nanoemulsion	157 ± 4.3	-27.67 ± 1.24	0.661

Table 1: Physicochemical characteristics of two nano-emulsion formulations: Black seed oil Nano emulsion and Acetylsalicylic acid- black seed oil Nano emulsion

The mean particle size of the plain black seed oil nanoemulsion was determined to be 94.67 ± 5.4 nm, while the acetylsalicylic acid-loaded black seed oil nanoemulsion exhibited a slightly larger particle size of 157 ± 4.3 nm. This observed increase in particle size can be attributed to the presence of acetylsalicylic acid within the nanoemulsion formulation.

Zeta potential, a crucial indicator of colloidal stability, was measured for both formulations. The plain black seed oil nanoemulsion demonstrated a zeta potential of -15 ± 2.4 mV, while the acetylsalicylic acid-loaded black seed oil nanoemulsion exhibited a more negative zeta potential of -27.67 ± 1.24 mV. This increase in negative zeta potential suggests improved stability and reduced likelihood of aggregation in the acetylsalicylic acid-loaded nanoemulsion.

The Polydispersity Index (PDI) provides insights into the size distribution uniformity within the nanoemulsion. A lower PDI value signifies a more uniform particle size distribution. The plain black seed oil nanoemulsion exhibited a PDI of 0.481, indicative of a relatively uniform particle size distribution. On the other hand, the acetylsalicylic acid-loaded black seed oil nanoemulsion had a slightly higher PDI of 0.661, suggesting a slightly broader size distribution, possibly due to the incorporation of acetylsalicylic acid.

These results collectively indicate that incorporating acetylsalicylic acid into the black seed oil-based nanoemulsion leads to a moderate increase in particle size and a notable increase in zeta potential, implying enhanced stability. The PDI values also suggest that while the size distribution remains relatively uniform, the presence of acetylsalicylic acid may contribute to a slightly broader distribution.

5.2 Drug Content Determination
One of the crucial parameters in any drug delivery system is the amount of drug effectively loaded into the carrier, as it directly influences the therapeutic efficacy. In our study, the drug content of the Acetylsalicylic acid-loaded nanoemulsion was assessed, revealing a drug content of 98.43 ± 2.32%. This result underscores the remarkable drug loading capacity of the nanoemulsion, meeting a fundamental requirement for efficient drug delivery systems. The high drug content indicates successful drug incorporation and is a promising feature for targeted therapy.

5.3 In Vitro Drug Release
The in vitro drug release profile from the Acetylsalicylic acid-loaded nanoemulsion was investigated using a Franz-type diffusion cell apparatus, and the results are depicted in Figure 1. Comparatively, the release profile of Acetylsalicylic acid from the nanoemulsion was significantly higher than that of non-formulated Acetylsalicylic acid.

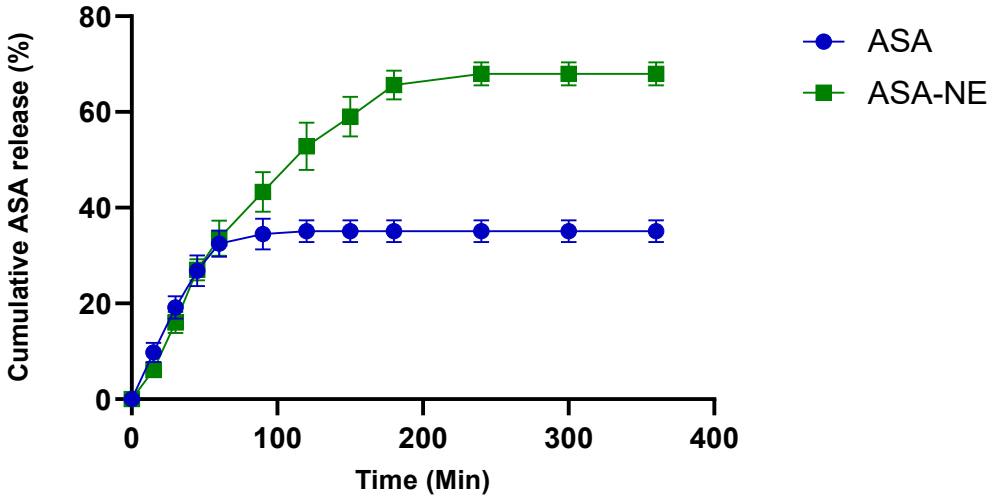

Figure 1: In vitro drug release profiles for Acetyl Salicylic Acid (ASA), comparing the non-formulated ASA to ASA-loaded black seed oil nano-emulsion formulations.

The in vitro release of Acetylsalicylic acid from the nanoemulsion reached 67.98 ± 1.95% after 360 minutes, whereas Acetylsalicylic acid alone exhibited a release of 35.1 ± 1.2%. This notable enhancement in drug release from the nanoemulsion can be attributed to the nano-sized droplets within the formulation. These small droplets provide a large interfacial area for drug release, facilitating quicker dissolution and diffusion of Acetylsalicylic acid from the nanoemulsion. This enhanced release profile is crucial for ensuring the timely and effective delivery of the drug to target tissues.

5.4 Stability Studies on Optimized Nanoemulsion

Stability studies are pivotal in evaluating the robustness of a formulation over time, especially when considering potential therapeutic applications. In our study, the stability of the Acetylsalicylic acid-loaded black seed oil nanoemulsion was examined and compared between freshly prepared and stored formulations. The results are summarized in Table 2.

Formulation	Particle Size (nm)	Zeta Potential (mV)	Drug Content (%)
Fresh Acetylsalicylic Acid-Black Seed Oil Nanoemulsion	157 ± 4.3	-27.67 ± 1.24	98.43 ± 2.32
Stored Acetylsalicylic Acid-Black Seed Oil Nanoemulsion	211 ± 3.21	-29 ± 1.2	95.275 ± 1.39

Table 2: Stability profile for the nano-emulsion formulation containing Acetylsalicylic Acid (ASA) loaded into black seed oil, assessed at 0 and 90 days period.

Upon comparing the fresh and stored Acetylsalicylic acid-loaded black seed oil nanoemulsions, some changes in physical characteristics were observed. The particle size increased from 157 ± 4.3 nm in the fresh formulation to 211 ± 3.21 nm in the stored formulation. Additionally, the zeta potential shifted from -27.67 ± 1.24 mV to -29 ± 1.2 mV. However, it's noteworthy that these particle size and zeta potential changes remained within acceptable limits for a stable nanoemulsion.

Furthermore, the drug content in the stored formulation was determined to be 95.275 ± 1.39%, indicating a slight decrease compared to the fresh formulation but still well within an acceptable range. These results suggest that the Acetylsalicylic acid-loaded black seed oil nanoemulsion maintains its stability over the storage period of 3 months, with only modest changes in physical characteristics and drug content.

5.5 In Vitro Cytotoxicity of Acetylsalicylic Acid-Black Seed Oil Nanoemulsion

The Acetylsalicylic acid-black seed oil nanoemulsion's ability to kill cells was tested on MCF-7, HePG2, and HCT 116 cells using the Sulforhodamine B (SRB) assay. The SRB assay is a widely used colorimetric method

for evaluating cell viability by measuring cellular protein content. Figures 2-4 show that Acetylsalicylic acid reduced the viability of all tested cell lines in a dose-dependent manner.

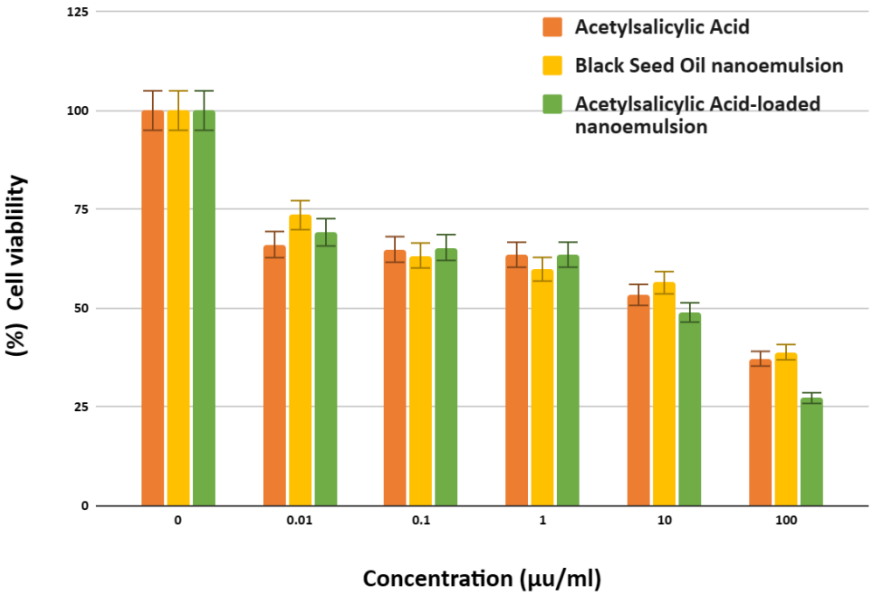

Figure 2: Cell viability of HePG2 cells after 72-hour treatment with non-formulated acetylsalicylic acid, black seed oil Nano emulsion, or acetylsalicylic acid-loaded black seed oil Nano emulsion (0–100 µg/mL). Mean ± SD.

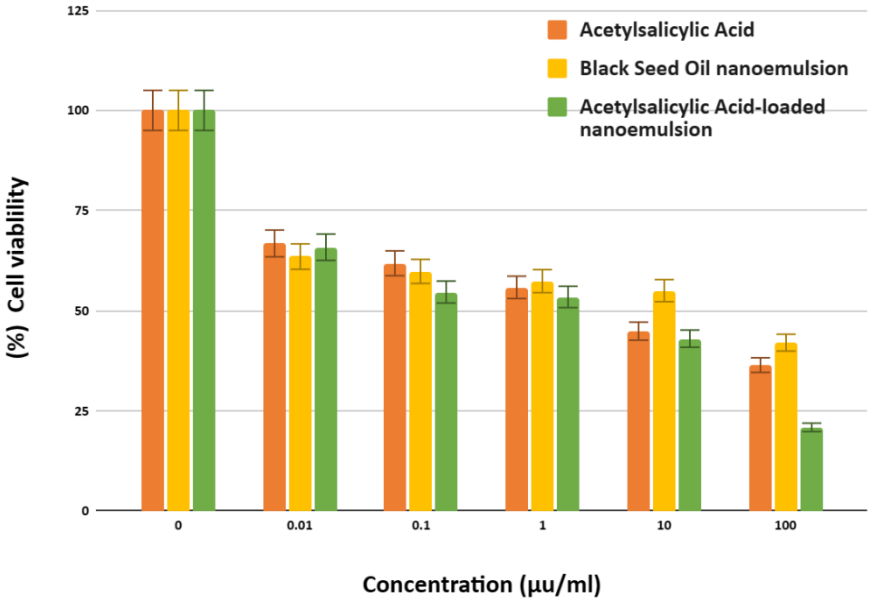

Figure 3: Cell viability of MCF-7 cells after 72-hour treatment with non-formulated acetylsalicylic acid, black seed oil Nano emulsion, or acetylsalicylic acid-loaded black seed oil Nano emulsion (0–100 µg/mL). Mean ± SD.

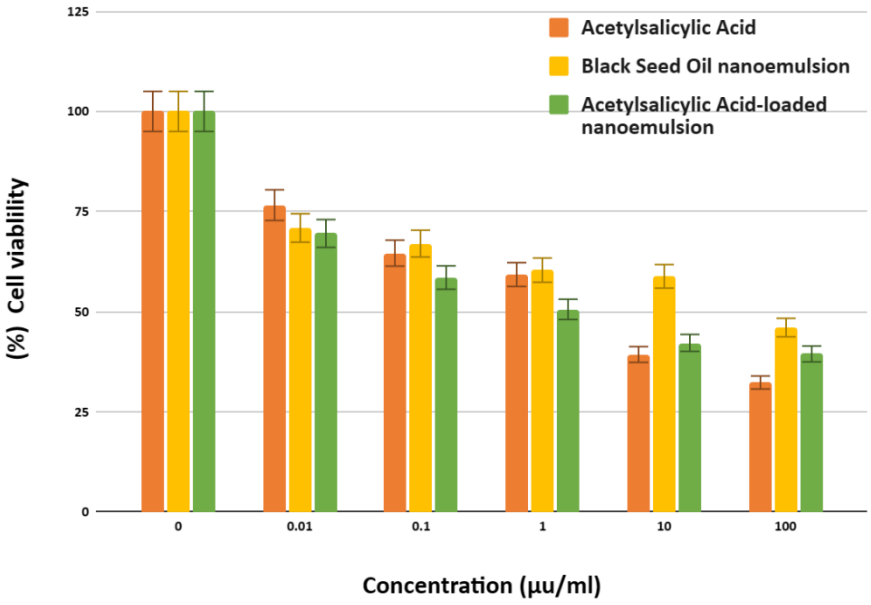

Figure 4: Cell viability of HCT 116 cells after 72-hour treatment with non-formulated acetylsalicylic acid, black seed oil Nano emulsion, or acetylsalicylic acid-loaded black seed oil Nano emulsion (0–100 µg/mL). Mean ± SD."

Table 3 provides a summary of the calculated IC50 values for different cell lines.

Cell Line	IC50 (µg/mL)
MCF-7	2.36 ± 0.24
HePG2	7.96 ± 1.16
HCT 116	2.314 ± 0.322

Table 3: IC50 Values of Acetylsalicylic Acid-Black Seed Oil Nanoemulsion on Cancer Cell Lines.

The results indicated that Acetylsalicylic acid exhibited potent cytotoxic effects against all tested cell lines in a dose-dependent manner. MCF-7 cancer cells were particularly susceptible to Acetylsalicylic acid treatment, with an IC50 of 2.36 ± 0.24 µg/mL. The effects on HePG2 and HCT 116 cells were also promising, with IC50 values of 7.96 ± 1.16 and 2.314 ± 0.322 µg/mL, respectively.

Furthermore, plain black seed oil nanoemulsion displayed significant cytotoxic effects against all the tested cell lines, with IC50 values of 15.316 ± 0.89 µg/mL for MCF-7, 15.29 ± 1.2 µg/mL for HePG2, and 34.156 ± 1.9 µg/mL for HCT 116 cells.

5.6 Effect of Compounds on Cell Cycle Distribution of Solid Tumor Cells

The impact of ASA-loaded Nanoemulsion on cell cycle distribution of MCF-7, HePG2, and HCT 116 cells was analyzed using flow cytometry after 48 hours of treatment to gain insights into potential anticancer mechanisms

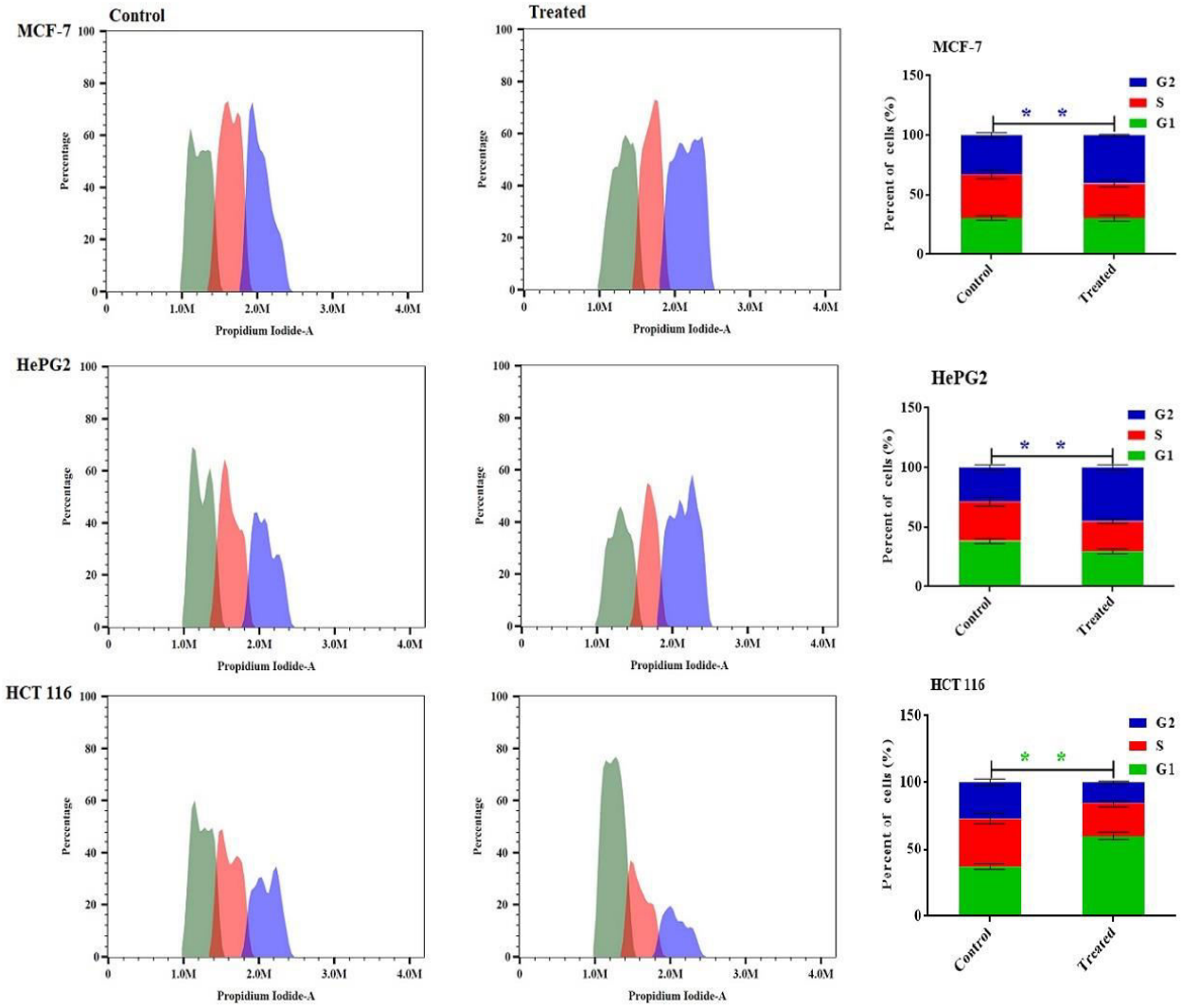

Figure 5: Effect of ASA-loaded Nano emulsion on Cell Cycle Distribution

The ASA-loaded Nanoemulsion significantly influenced the cell cycle distribution of MCF-7, HePG2, and HCT 116 cells. MCF-7 and HePG2 cancer cells were arrested in the G2 phase with a cell cycle distribution of 40.9 ± 1.9% and 45.3 ± 1.9%, respectively. Conversely, the same formulation showed a significant effect on HCT 116 cells by arresting them in the G1 phase, resulting in a 59.8 ± 2.2% distribution compared to the control cells.

5.7 Evaluation of Cell Apoptosis using Annexin V-FITC

Annexin V-FITC/PI staining, coupled with flow cytometry analysis, was employed to discern between apoptotic (programmed cell death) and necrotic (non-programmed cell death) cells in MCF-7, HePG2, and HCT 116 cell lines (Figure 6).

Before delving into the effects of the formulation on cancer cells, an elevation in the population of apoptotic cells in MCF-7 cells was observed following treatment with Acetylsalicylic acid-black seed oil nanoemulsion, with a percentage of 84.35 ± 2.5%. In comparison, the apoptotic populations in HePG2 and HCT 116 cells were 74.5 ± 2.3% and 74.1 ± 1.9%, respectively, following treatment with the same combination formulation, as compared to the cell control.

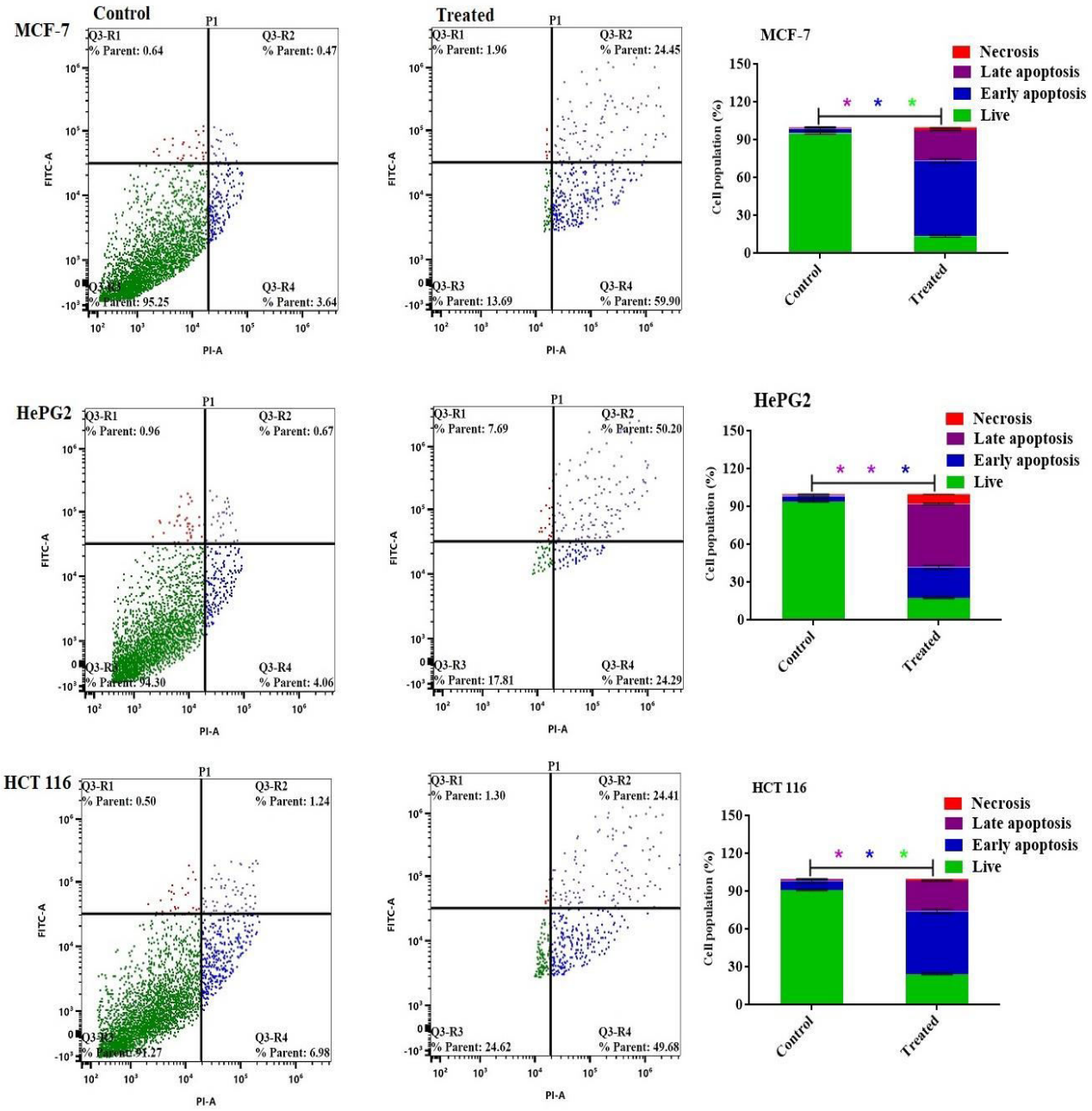

Figure 6: Evaluation of Cell Apoptosis using Annexin V-FITC/PI Staining

Discussion

Nanoemulsions are a promising drug delivery system, offering improved solubility, stability, and targeted delivery. Our study aimed to create and analyze an oil-in-water nanoemulsion containing black seed oil and acetylsalicylic acid for potential anticancer applications.

The physical analysis of the nanoemulsion included measuring particle size, zeta potential, and Polydispersity Index (PDI). The average particle size of a nanoemulsion significantly influences its behavior and efficacy[18]. Dynamic Light Scattering (DLS) analysis revealed that the black seed oil nanoemulsion had a particle size of 94.67 ± 5.4 nm, falling within the desirable nanoparticulate range[19]. In contrast, the acetylsalicylic acid-loaded black seed oil nanoemulsion exhibited a larger size, measuring 157 ± 4.3 nm. This increase in size is attributed to the incorporation of acetylsalicylic acid, which alters interfacial tension and particle interactions within the nanoemulsion. The Polydispersity Index (PDI) provides insight into particle size distribution. A PDI value

below 0.8 indicates a relatively uniform size distribution[20]. The black seed oil nanoemulsion displayed a PDI of 0.481, indicating homogeneity. Conversely, the acetylsalicylic acid-loaded nanoemulsion had a higher PDI of 0.661, suggesting a broader size distribution due to interactions with acetylsalicylic acid.

The acetylsalicylic acid-loaded nanoemulsion had a drug content of 98.43 ± 2.32%. This outcome illustrates that the nanoemulsion exhibited a favorable drug-loading capacity, a fundamental prerequisite for the formulation of nanoemulsions[21].

In vitro drug release tests showed that the acetylsalicylic acid-loaded nanoemulsion had significantly higher drug release than non-formulated acetylsalicylic acid. This may be associated with the diminutive droplet size within the nanoemulsion, offering an extensive surface area for drug release[22]. Additionally, it was observed that factors like drug-oil and surfactant-oil interactions and drug solubility in the oil phase significantly influenced drug release from nanoemulsions[21]. Stability studies confirmed that the formulation retained its physicochemical properties over three months.

The Sulforhodamine B (SRB) assay stands as a widely employed colorimetric method for assessing cell viability by quantifying cellular protein content[23]. The SRB assay demonstrated that the acetylsalicylic acid-black seed oil nanoemulsion formulation had potent cytotoxic effects on MCF-7, HePG2, and HCT 116 cancer cell lines. The IC50 values showed varying sensitivities among the cell lines, with MCF-7 cells being the most susceptible. Cell cycle analysis revealed that the formulation arrested MCF-7 and HePG2 cells in the G2 phase, while inducing G1 phase arrest in HCT 116 cells. Several previous investigations have showcased that, in addition to their robust anti-inflammatory effects, both Acetylsalicylic acid and black seed oil possess anti-tumor properties, suggesting their potential application in cancer treatment[24, 25].

Annexin V-FITC/PI staining showed a significant increase in apoptotic cell populations in MCF-7 cells treated with the acetylsalicylic acid-black seed oil nanoemulsion. The percentage of apoptotic cells reached 84.35 ± 2.5%, and similar effects were observed in HePG2 and HCT 116 cells with slightly lower percentages. Comparable results were documented, indicating that black seed oil and acetylsalicylic acid trigger apoptosis in different cell lines[25,26].

The comprehensive evaluation of the acetylsalicylic acid-black seed oil nanoemulsion's anticancer potential underscores its promise as a multifaceted therapeutic approach. The formulation exhibited cytotoxic effects across various cancer cell lines, influenced cell cycle distribution, and induced apoptotic cell death. These findings support the formulation's potential application as an effective anticancer agent.

This study provides a foundation for further investigations into the signaling pathways involved in the formulation's effects on cell cycle regulation and apoptosis induction. Preclinical studies are necessary to assess the formulation's efficacy in animal models and its potential translation to clinical applications.

Conclusion

In this study, we successfully developed and characterized an oil-in-water nanoemulsion containing acetylsalicylic acid and black seed oil for potential anticancer applications. We conducted a comprehensive investigation encompassing physical characterization, drug content analysis, in vitro drug release profiling, stability assessment, and extensive biological evaluations. The results collectively underscore the multifaceted potential of this formulation in cancer therapy.

The physical characterization revealed excellent stability and suitable particle size, crucial for efficient drug delivery. The formulation exhibited a high drug loading capacity, ensuring effective drug delivery to target tissues. Notably, the in vitro drug release profile demonstrated enhanced drug release from the nanoemulsion, suggesting its therapeutic advantage over conventional drug forms.

Our promising in vitro cytotoxicity findings underscore the formulation's significant anticancer activity. Its varying effects across different cancer cell lines highlight its potential versatility against diverse solid tumors. Furthermore, the formulation's ability to influence cell cycle distribution and induce apoptosis underscores its multifunctional therapeutic mechanisms.

The acetylsalicylic acid-black seed oil nanoemulsion represents an innovative anticancer agent with great potential for further exploration. Future research should delve into the underlying molecular pathways affected

by the formulation, facilitating the development of targeted therapies. Preclinical studies are imperative to validate its in vivo efficacy and translational potential.

In summary, the developed nanoemulsion holds promise as a versatile platform for delivering acetylsalicylic acid in cancer treatment. Its multifaceted effects on cancer cells, stability, and enhanced drug release suggest its ability to address the limitations of conventional cancer therapies. As we advance toward personalized medicine, this formulation emerges as a valuable asset in the quest for more effective and targeted cancer treatments.

Acknowledgments

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Conflict of Interest

The authors declare no competing financial interest.

References

1. Shaker, D.S., Ishak, R.A., Ghoneim, A. & Elhuoni, M.A., (2019) Nanoemulsion: A review on mechanisms for the transdermal delivery of hydrophobic and hydrophilic drugs. *Scientia Pharmaceutica*. *87*(3): 17.
2. Jaiswal, M., Dudhe, R. & Sharma, P.K., (2015) Nanoemulsion: an advanced mode of drug delivery system. *3 Biotech*. 5: 123-127.
3. Wilson, R.J., Li, Y., Yang, G. & Zhao, C.X., (2022) Nanoemulsions for drug delivery. *Particuology. 64*, pp.85-97.
4. Ahmad, A., Husain, A., Mujeeb, M., Khan, S.A., Najmi, A.K., Siddique, N.A., Damanhouri, Z.A. & Anwar, F., (2013) A review on therapeutic potential of Nigella sativa: A miracle herb. *Asian Pacific journal of tropical biomedicine. 3*(5): 337-352.
5. Ahmad, M.F., Ahmad, F.A., Ashraf, S.A., Saad, H.H., Wahab, S., Khan, M.I., Ali, M., Mohan, S., Hakeem, K.R. & Athar, M.T., (2021) An updated knowledge of Black seed (Nigella sativa Linn.): Review of phytochemical constituents and pharmacological properties. *Journal of herbal medicine.* 25: 100404.
6. Fijałkowski, Ł., Skubiszewska, M., Grześk, G., Koech, F.K. & Nowaczyk, A., (2022) Acetylsalicylic acid–primus inter pares in Pharmacology. *Molecules.* 27: 8412.
7. Rezabakhsh, A., Mahmoodpoor, A., Soleimanpour, M., Shahsavarinia, K. & Soleimanpour, H., (2021) Clinical Applications of Aspirin as a Multi-potent Drug Beyond Cardiovascular Implications: A Proof of Concept for Anesthesiologists-A Narrative Review. *Anesthesiology and Pain Medicine.* 11(5).
8. Bashir, A.U.I., Kankipati, C.S., Jones, S., Newman, R.M., Safrany, S.T., Perry, C.J. & Nicholl, I.D., (2019) A novel mechanism for the anticancer activity of aspirin and salicylates. *International journal of oncology.* 54(4): 1256-1270.
9. Almatroudi, A., (2020) The incidence rate of colorectal cancer in Saudi Arabia: An observational descriptive epidemiological analysis. *International Journal of General Medicine.* pp: 977-990.
10. Althubiti, M.A. & Eldein, M.M.N., (2018) Trends in the incidence and mortality of cancer in Saudi Arabia. *Saudi medical journal. 39*(12): 1259.
11. Abdallah, M.H., Lila, A.S.A., Unissa, R., Elsewedy, H.S., Elghamry, H.A. & Soliman, M.S., (2021) Preparation, characterization and evaluation of anti-inflammatory and anti-nociceptive effects of brucine-loaded nanoemulgel. *Colloids and Surfaces B: Biointerfaces.* 205: 111868.
12. Alotaibi, H.F., Khafagy, E.S., Abu Lila, A.S., Alotaibe, H.F., Elbehairi, S.E., Alanazi, A.S., Alfaifi, M.Y., Alamoudi, J.A., Alamrani, S.S. & Mokhtar, F.A., (2023) Anticancer potentials of metformin loaded coconut oil nanoemulsion on MCF-7, HepG2 and HCT-116 cell lines. *Artificial Cells, Nanomedicine, and Biotechnology.* 51(1): 419-427.
13. Alkhatib, M.H., Alyamani, S.A. & Abdu, F., (2020) Incorporation of methotrexate into coconut oil nanoemulsion potentiates its antiproliferation activity and attenuates its oxidative stress. *Drug delivery.* 27(1): 422-430.
14. Dacić, M., Uzunović, A., Kunić, A., Pilipović, S. & Šapčanin, A., (2020) UV-VIS determination of acetylsalicylic acid in aspirin tablets using different solvents and conditions. In *CMBEBIH 2019: Proceedings of the International Conference on Medical and Biological Engineering. 16–18 May 2019, Banja Luka, Bosnia and Herzegovina* (pp. 563-567) Springer International Publishing.
15. Sambasivarad, A., Chandra, S.R.B. & Reddy, M.H., (2016) Accelerated Stability Testing of Dosage forms as per ICH Guidelines. *World J Pharm and Med Res.* 2(3): 99-103.

16. Vichai, V. & Kirtikara, K., (2006) Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature protocols.* 1(3): 1112-1116.
17. Al Saqr, A., Wani, S.U.D., Gangadharappa, H.V., Aldawsari, M.F., Khafagy, E.S. & Lila, A.S.A., (2021) Enhanced cytotoxic activity of docetaxel-loaded silk fibroin nanoparticles against breast cancer cells. *Polymers.* 13(9): 1416.
18. Current applications of nanoemulsions in cancer therapeutics.
19. Kumar, S., Singh, J.P., Giri, D. & Mishra, S., (2021) Effect of polydispersity on the dynamics of active Brownian particles. *Physical Review E.* 104(2): 024601.
20. Asmawati, Mustapha, W.A.W., Yusop, S.M., Maskat, M.Y. & Shamsuddin, A.F., (2014), September. Characteristics of cinnamaldehyde nanoemulsion prepared using APV-high pressure homogenizer and ultra turrax. In *AIP Conference Proceedings.* 1614: 244-250.
21. American Institute of Physics.Azeem, A., Rizwan, M., Ahmad, F.J., Iqbal, Z., Khar, R.K., Aqil, M. & Talegaonkar, S., (2009) Nanoemulsion components screening and selection: a technical note. *Aaps Pharmscitech*. 10: 69-76.
22. Jaiswal, M., Dudhe, R. & Sharma, P.K., (2015) Nanoemulsion: an advanced mode of drug delivery system. *Biotech.* 5: 123-127.
23. Orellana, E.A. & Kasinski, A.L., (2016) Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. *Bio-protocol*. 6(21): e1984-e1984.
24. Ausina, P., Branco, J.R., Demaria, T.M., Esteves, A.M., Leandro, J.G.B., Ochioni, A.C., Mendonça, A.P.M., Palhano, F.L., Oliveira, M.F., Abou-Kheir, W. & Sola-Penna, M., (2020) Acetylsalicylic acid and salicylic acid present anticancer properties against melanoma by promoting nitric oxide-dependent endoplasmic reticulum stress and apoptosis. *Scientific reports.* 10(1): 19617.
25. Khan, A., Chen, H.C., Tania, M. & Zhang, D.Z., (2011) Anticancer activities of Nigella sativa (black cumin). *African Journal of Traditional, Complementary and Alternative Medicines.* 8(5S).
26. Zimmermann, K.C., Waterhouse, N.J., Goldstein, J.C., Schuler, M. & Green, D.R., (2000) Aspirin induces apoptosis through release of cytochrome c from mitochondria. *Neoplasia.* 2(6): 505-513.