Enhancing Anticancer Efficacy of Withania somnifera through Nanosuspension: A Study on Human Cancer Cell Lines

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Abstract

Cancer remains a leading cause of death worldwide, prompting increased research into novel anticancer agents from natural sources, particularly plants. Notably, 60-75% of FDA-approved anticancer drugs are derived from natural sources, underscoring the importance of these compounds in drug discovery. Withania somnifera known as Ashwagandha, has been utilized in traditional medicine for over a years and exhibits various therapeutic properties, including anticancer effects against multiple human cancers. Active constituents such as withanolides and alkaloids have demonstrated potential in inhibiting cancer cell proliferation and metastasis. Emerging nanotechnology, particularly nanosuspensions, offers a promising strategy to enhance the bioavailability and solubility of lipophilic drugs, addressing common challenges in drug delivery. This study aims to synthesize a nanosuspension (NSWS) from Withania somnifera root extract and evaluate its in-vitro anticancer activity against human cancer cell lines, including Prostate (DU-145), Oral Squamous (AW13516), Breast (MDA-MB-231), and Hepatoma (Hep-G2) by SRB assay. Notably, NSWS exhibited a dose-dependent anticancer effect in DU-145 and Hep-G2 cell lines, outperforming EEWS, while its impact on AW13516 and MDA-MB-231 was significant to smaller extent. These findings suggest the potential of Withania somnifera nanosuspensions (NSWS) in cancer treatment and their applications in nanomedicine.

Keywords: *Ashwagandha, nanosuspension, anticancer, SRB, human cancer cell.*

1. Introduction

According to WHO reports, the global cancer burden is expected to rise, with projections of around 37 million new cases by 2040 and approximately 20 million new cases by the end of 2025. Cancer remains a significant health challenge in both developing and developed countries, ranking among the top five leading causes of death for both men and women, and is the leading cause of death globally. High mortality rates associated with chemotherapy and radiation have led to increased research into new anticancer agents from natural sources, particularly plants. Conventional chemicals often have side effects and toxicities. Notably, 60-75% of FDA-approved anticancer drugs are derived from natural sources, demonstrating that nature is a vital source for drug discovery and development. Approximately 60% of all available anticancer drugs are natural products, and the unique chemical diversity of these sources is expected to yield new drug leads targeting various therapeutic pathways, including semisynthetic and synthetic derivatives[1, 2].

Withania somnifera (L.) Dunal, commonly known as winter cherry or Indian ginseng (Ashwagandha in Sanskrit), has been used in traditional medicine for over 3,000 years. This small, woody shrub from the *Solanaceae* family possesses various therapeutic properties, such as anti-inflammatory, sedative, hypnotic, tonic, diuretic, aphrodisiac, alterative, and deobstruent effects. The active constituents of *Withania somnifera* include alkaloids, withanolides, saponins, flavonoids, and tannins. Ashwagandha shows anticancer properties against several human cancers, including prostate, colon, lung, breast, leukemia, pancreatic, renal, and head and neck cancers[3-5].

In-vitro studies suggest that *Withania somnifera* may serve as a long-term alternative therapy to prevent breast cancer metastasis, as its root extract inhibited metastasis with minimal side effects[6]. Literature indicates its common use in herbal formulations to enhance cell-mediated immune responses due to active compounds. Additionally, research shows that *Withania somnifera*, disrupts mitosis by inhibiting survivin, a protein essential for cancer cell survival and proliferation. *Withania somnifera* demonstrate anti-tumorigenic potential against prostate tumors and its ZnONPs have also been reported to have significant tumoricidal activity through the generation of reactive oxygen species (ROS)[7]. Recent *in-vitro* studies indicate that inhibits the growth of breast and colon cancer cell lines more effectively than doxorubicin, suggesting that *Withania somnifera* extracts could serve as potent anticancer agents, potentially improving survival in cancer patients[8].

Nanotechnology, particularly nanosuspensions, has emerged as a promising approach in medicine to enhance the bioavailability and solubility of lipophilic pharmaceuticals. Many new drugs face challenges due to poor water solubility, and nanosuspensions provide a solution by creating disintegrable formulations with particle sizes ranging from 1 to 1000 nm. This technology improves medication safety and effectiveness by addressing solubility issues and modifying pharmacokinetics. Nanosuspensions enhance drug safety and efficacy by addressing poor solubility and bioavailability while altering pharmacokinetics. As a drug delivery platform, they offer significant advantages over traditional dosage forms, including improved bioavailability, reduced systemic toxicity, enhanced stability, increased pharmacodynamic action, and targeted effects[9-12]. Cell lines from humans are an essential biological resource.

These *in-vitro* human biology models are employed in numerous applications such as protein synthesis, toxicological investigations, drug discovery, and biomedical research. The environment that cell lines grow in cell culture media differs greatly from that of the original tumor, where they are evolved to grow. Previous research has shown that Ashwagandha has been utilized for green synthesis; however, the nanosuspension derived from its root extract has not been investigated. Therefore, this study aims to evaluate its anticancer activity against human cancer cell lines Prostate (DU-145), Oral Squamous (AW13516), Breast (MDA-MB-231), and Hepatoma (Hep-G2). Considering the importance of this plant and its potential applications in nanotechnology, this work was designed to synthesize a nanosuspension from *Withania somnifera* roots and assess its anticancer potential.

2. Materials And Methods

2.1 Collection, Extraction and Phytochemical Screening of Plant Material

Roots of *Withania somnifera* (Ashwagandha) were sourced from the local market and identified by the relevant department. A total of 1 kg of dried root powder was extracted using petroleum ether at a temperature range of 50°C to 60°C for 72 hours in a Soxhlet apparatus. The remaining marc was then dried and subjected to extraction with 95% ethanol at 60°C to 70°C for an additional 72 hours in the same apparatus. The concentrated ethanol extract was stored in a desiccator. Preliminary phytochemical screening was performed to identify secondary bioactive metabolites, employing standard methods to detect alkaloids, flavonoids, saponins, tannins, glycosides, phenols, and steroids[13].

2.2 Formulation of Nanosuspension (NSWS)

The nanosuspension (NSWS) was prepared using a modified nano-precipitation method. One gram of the plant extract was dissolved in a 3:1 mixture of acetone and ethanol and sonicated for 60 seconds. This solution was then gradually injected into 25 ml of water containing 1.5% Polyvinyl Alcohol (PVA) at a rate of 1 ml per minute while stirring at 1000 rpm. To reduce coalescence, the emulsion was diluted with 50 ml of 0.2% PVA solution and stirred at 500 rpm for 6 hours to facilitate solvent evaporation and nanoparticle formation. The nanosuspension was subsequently cooled to -18°C and lyophilized into a dry powder for further analysis using a MAC lyophilizer[14,15].

2.3 *In-vitro* Anticancer Screening

In-vitro anticancer screening was conducted at the Anti-Cancer Drug Screening Facility, Tata Memorial Centre (ACTREC) in Navi Mumbai using SRB assay protocols. Each drug was tested at four dose levels ($1x10^{-7}$ M, $1x10^{-6}$ M, $1x10^{-5}$ M, and $1x10^{-4}$ M or 10, 20, 40, 80 µg/ml) with appropriate positive controls, and each experiment was repeated three times. Results were reported as GI50 (the concentration that produces 50% cell inhibition).

SRB Assay Protocol: Cell lines were cultured in medium with 10% fetal bovine serum and 2 mM L-glutamine. For the screening, 5000 cells/well were inoculated into 96-well plates and incubated for 24 hours at 37°C with 5% CO2. Experimental drugs, prepared at 100 mg/ml and diluted to 1 mg/ml, were added to achieve final concentrations of 10, 20, 40, and 80 µg/ml.

After 48 hours of incubation, the assay was terminated by adding cold TCA, fixing the cells, and washing them. Sulforhodamine B (SRB) solution was added to each well for staining, followed by washing to remove unbound dye. The bound stain was eluted with 10 mM Trizma base, and absorbance was measured at 540 nm with a reference at 690 nm. Percent growth was calculated based on the average absorbance of test wells relative to control wells, and percentage growth inhibition was calculated as: Percentage growth Inhibition was calculated as: [Ti/C] x 100 %. [Time zero (Tz), Control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)] [16-19].

3. RESULTS

3.1 Phytochemical Analysis and Formulation of Nanosuspension (NSWS)

The roots of *Withania somnifera* were collected from a local herbal store and authenticated by the Department of Botany at Rashtrasanta Tukadoji Maharaj Nagpur University. After thorough washing, the roots were air-dried for 48 hours and then ground into a fine powder, which were extracted by ethanol. Preliminary phytochemical screening identified several secondary metabolites. The nanosuspension (NSWS), 20mg/ml was prepared from the extract of the roots, with various formulation and process parameters optimized, including surfactant concentration, solvent-to-anti-solvent ratio, and homogenization speed (Fig. 1).

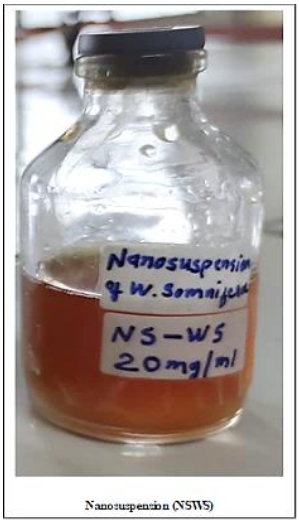

Figure 1: Formulated Nanosuspension (NSWS)

3.2 *In-vitro* Anticancer screening

Table 1: Percentage control growth of human prostate cancer cell line DU-145

1.0	**DU-145**			
	% Control Growth			
	Average Values			
	10 µg/ml	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**
EEWS	92.9	98.4	99.7	97.3
NSWS	95	95.5	90.2	82.4
ADR	-40.3	-36.9	-40.2	-46.6

EEWS= Ethanolic extract of *Withania somnifera*
NSWS= Nanosuspension from ethanolic extract of *Withania somnifera*
ADR = Adriamycin, Positive control compound

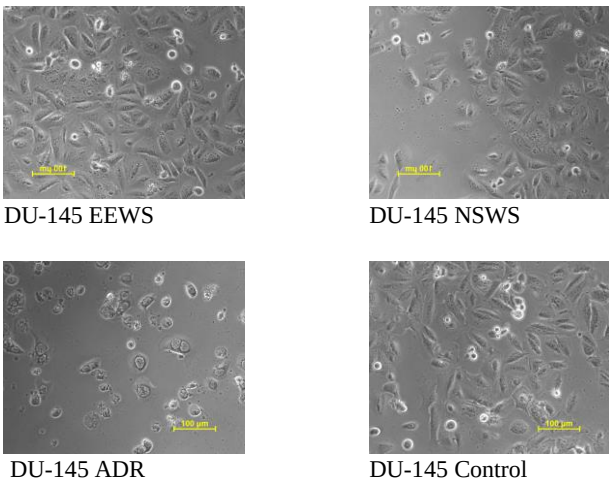

Figure 2: Efficacy of EEWS, NSWS, Adriamycin and control on prostate cancer cell line DU-145

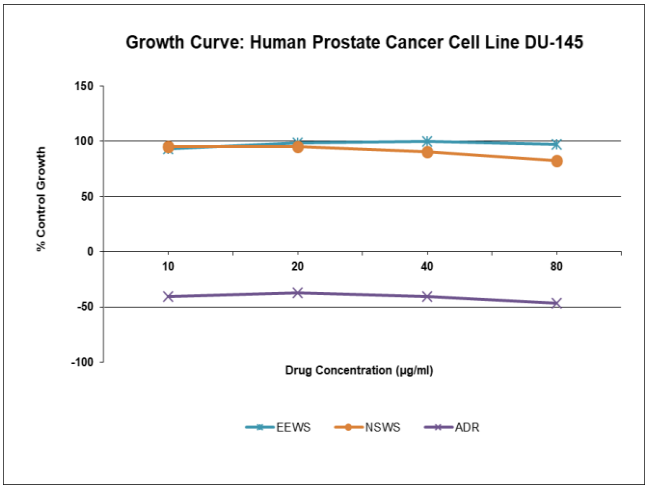

Figure 3: Effect of different concentration of EEWS, NSWS and Adriamycin on human prostate cancer cell line DU-145

Table 2. Percentage control growth of human oral squamous cell carcinoma AW13516

2.0	**AW13516**			
	% Control Growth			
	Average Values			
	10 µg/ml	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**
EEWS	87.5	92.5	94.2	91.2
NSWS	93.4	92.5	96.3	99.5
ADR	-60.4	-70.8	-77.0	-69.1

EEWS= Ethanolic extract of *Withania somnifera*

NSWS= Nanosuspension from ethanolic extract of *Withania somnifera*

ADR = Adriamycin, Positive control compound

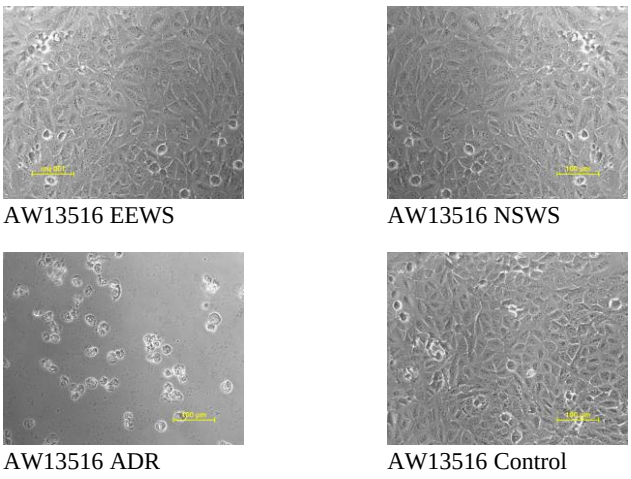

Figure 4: Efficacy of EEWS, NSWS, Adriamycin and Control on human oral squamous cell carcinoma AW13516

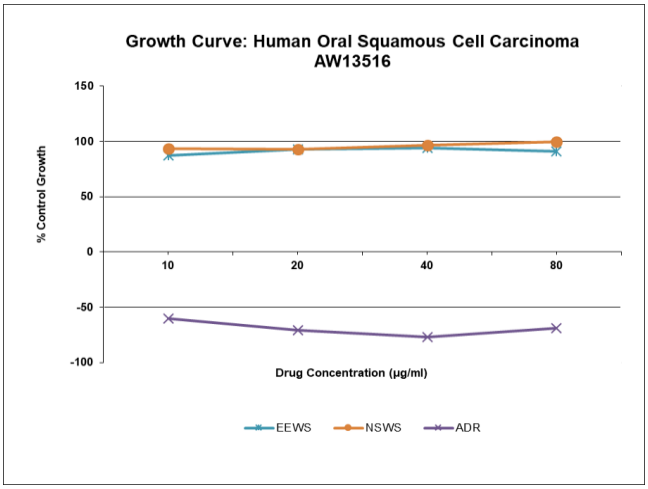

Figure 5: Effect of different concentration of EEWS, NSWS, and Adriamycin on human oral squamous cell carcinoma AW13516

Table 3. Percentage control growth of human breast cancer cell line MDA-MB-231

3.0	**MDA-MB-231**			
	% Control Growth			
	Average Values			
	10 µg/ml	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**
EEWS	89.4	83.9	86.0	90.2
NSWS	91.7	89.5	83.7	97.9
ADR	-61.0	-65.6	-60.3	-54.1

EEWS= Ethanolic extract of *Withania somnifera*
NSWS= Nanosuspension from ethanolic extract of *Withania somnifera*
ADR = Adriamycin, Positive control compound

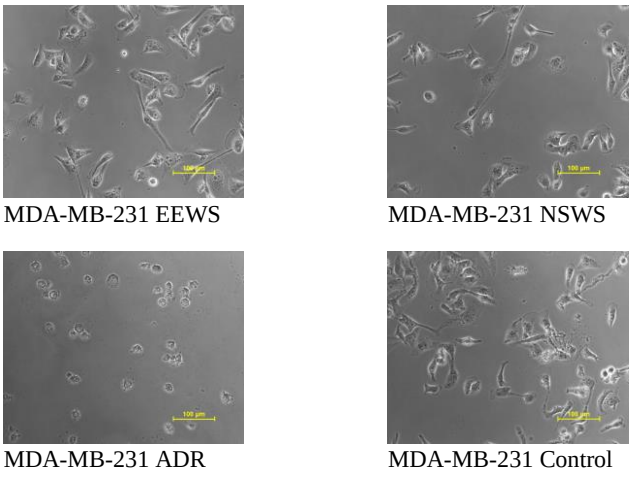

Figure 6: Efficacy of EEWS, NSWS, Adriamycin and Control on human breast cancer cell line MDA-MB-231

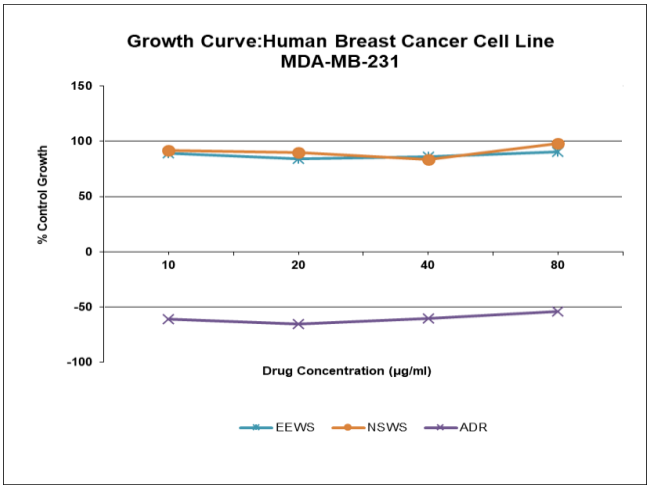

Figure 7: Effect of different concentration of EEWS, NSWS and Adriamycin on human breast cancer cell line MDA-MB-231

Table 4. Percentage control growth of human hepatoma cell line Hep-G2

4.0	**Hep-G2**			
	% Control Growth			
	Average Values			
	10 µg/ml	**20 µg/ml**	**40 µg/ml**	**80 µg/ml**
EEWS	109.7	121	124.4	120.2
NSWS	106.6	111.8	113.7	114.3
ADR	-16.5	-28.2	-55.7	-56.1

EEWS= Ethanolic extract of *Withania somnifera*
NSWS= Nanosuspension from ethanolic extract of *Withania somnifera*
ADR = Adriamycin, Positive control compound

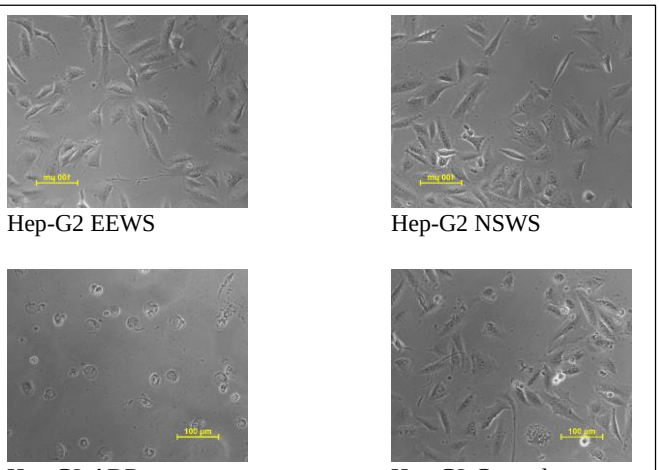

Figure 8: Efficacy of EEWS, NSWS, Adriamycin and Control on human hepatoma cell line Hep-G2

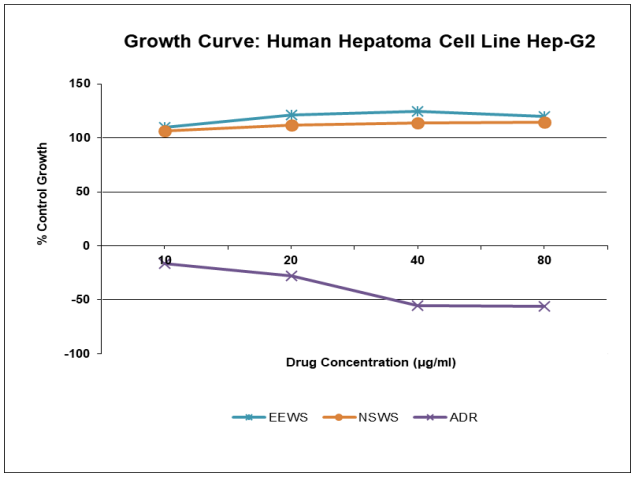

Figure 9: Effect of different concentration of EEWS, NSWS and Adriamycin on human hepatoma cell line Hep-G2

The anticancer activity of the nanosuspension (NSWS) derived from *Withania somnifera* and the ethanolic extract (EEWS) was evaluated through *in-vitro* testing using the SRB assay, which measures the effect on cell growth. Both NSWS and EEWS were compared to Adriamycin by assessing the percentage growth inhibition in human cancer cell lines using a microplate reader. Drug concentrations were determined according to National Cancer Institute (NCI) guidelines, specifically 10 µg/mL, 20 µg/mL, 40 µg/mL, and 80 µg/mL (Table 1-4). The experiment was repeated three times, and the average percentage of control growth was calculated for comparison. Different concentrations of both samples were applied to various human cancer cell lines, including Human Prostate Cancer Cell Line DU-145, Human Oral Squamous Cell Carcinoma AW13516, Human Breast Cancer Cell Line MDA-MB-231, and Human Hepatoma Cell Line Hep-G2.

The cytotoxic effects were observed under an inverted microscope, revealing morphological changes such as cell shrinkage, reduced cell counts, and irregular shapes indicative of cytotoxicity. At a concentration of 80 µg/mL, the NSWS showed an average cell viability of 82.4%, 99.5%, 97.9%, and 114.3%, while the EEWS demonstrated average viabilities of 97.3%, 91.2%, 90.2%, and 120.2% (Fig. 2-9). These results suggest that the nanosuspension (NSWS) exhibits greater anticancer potential compared to the ethanolic extract (EEWS) at the same concentration.

4. Discussion

In this study, the nanosuspension (NSWS) was formulated with dersired particle size and stability, and its *in-vitro* anticancer activity was investigated. The ethanolic extract of *Withania somnifera* roots, known for its various traditional medicinal uses, has gained significant interest in nanotechnology. Nanosuspensions are unique submicron colloidal dispersions of drug particles stabilized by surfactants or polymeric stabilizers, which are essential for their effectiveness and stability. Poor water solubility and biodistribution may limit the anticancer efficacy of *Withania somnifera*, and the lack of extensive clinical studies on its anticancer potential may stem from these challenges. Nanoformulations could help overcome barriers associated with natural phytochemicals like *Withania somnifera*, which encompass a diverse array of compounds. Another significant advantage is the ability of these formulations to enhance the effects of traditional antineoplastic agents without altering their therapeutic properties. Given the rising resistance to standard chemotherapy, combination therapies have gained attention as innovative cancer treatment strategies. *Withania somnifera* has shown a chemo-sensitizing effect on various cancer types[20, 21].

To evaluate the anticancer activity of nanosuspension (NSWS) and the ethanolic extract (EEWS), different concentrations of samples were tested on various human cancer cell lines, including Prostate (DU-145), Oral Squamous (AW13516), Breast (MDA-MB-231), and Hepatoma (Hep-G2). The growth curves for all four cell lines were constructed by plotting drug concentration against percentage control growth[22]. The nanosuspension (NSWS) derived from *Withania somnifera* demonstrated anticancer potential by inhibiting cell growth, achieving average viabilities of 82.4%, 99.5%, 97.9%, and 114.3% at a concentration of 80 µg/mL. Similarly, the ethanolic extract (EEHI) also showed anticancer effects, with average viabilities of 97.3%, 91.2%, 90.2%, and 120.2% at the same concentration. However, both treatments were less effective than the positive control, Adriamycin (ADR)[23, 24]. The NSWS exhibited a dose-dependent anticancer effect in the Human Prostate Cancer Cell Line DU-145 and the Human Hepatoma Cell Line Hep-G2, outperforming the ethanolic extract (EEHI) in these contexts. Conversely, its impact on the Human Oral Squamous Cell Carcinoma AW13516 and Human Breast Cancer Cell Line MDA-MB-231 was less pronounced.

These findings suggest a correlation between particle size and cancer cell type, as the cells exhibit different anatomical and physicochemical properties. The nanosuspension (NSWS) appears to selectively adhere to specific cancer cell types. Notably, at a drug concentration above of approximately 80 μg/mL above, total growth inhibition and cytotoxicity were may observed in the corresponding cancer cells. This interaction likely triggers kinetic and thermodynamic exchanges at the particle-cell interface. Therefore, further research is needed to optimize the dosage formulation for effective cancer treatment.

5. Conclusions

This study successfully developed a nanosuspension (NSWS) from the ethanolic extract of *Withania somnifera* root, which demonstrated enhanced anticancer activity compared to traditional formulations. The nanosuspension approach serves as an innovative and adaptable strategy to improve the therapeutic efficacy of hydrophobic drugs, enabling better bioavailability and effectiveness across various routes of administration. The incorporation of phytochemicals such as *Withania somnifera* into cancer treatment regimens, particularly when combined with conventional chemotherapeutics, holds promise for increasing overall therapeutic effectiveness. This synergy could potentially overcome some of the limitations associated with standard chemotherapy, such as drug resistance and systemic toxicity. The anticancer effects observed with the NSWS may be attributed to its unique formulation properties, which facilitate improved interaction with cancer cells. The mechanism behind the observed growth inhibition warrants further investigation, but it indicates the potential of utilizing this nanosuspension as a natural, cost-effective therapeutic option in cancer treatment. Overall, the findings suggest that the nanosuspension (NSWS) could serve as a promising candidate for cancer therapy, offering a natural alternative with minimal or no adverse side effects. Future studies should focus on optimizing the formulation and exploring its full therapeutic potential, including *in-vivo* evaluations and clinical applications.

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