Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Cytotoxic Effects and Antioxidant Mechanisms of Shilajit-Loaded Chitosan Nanoparticles on HCT116 Colorectal Cancer Cells

Asmaa E. M. Khater[1], Amira M. Elmoslemany[1], Asmaa M. EL-Gawish[1] and Amina M. G. Zedan*[2]

[1] *Department of Nutrition and Food Science, Faculty of Home Economic, Al-Azhar University, Egypt.*
[2] *Agriculture Faculty (Girls branch), Al-Azhar University, Cairo 11884, Egypt.*

Abstract

Colorectal cancer is the second leading cause of cancer deaths globally and is the most common cancer linked to obesity. This study examines Shilajit-Loaded Chitosan Nanoparticles on HCT116 Cancer Cells, organized into five groups: The first group HCT116 cells. The second group: chitosan nanoparticles-treated HCT116 cells. The third group was Shilajit extract-treated HCT116 cells. The fourth group was Shilajit-loaded chitosan nanoparticles-treated HCT116 cells. The MTT assay results demonstrated the dose-dependent cytotoxic effects of chitosan nanoparticles and shilajit-loaded chitosan nanoparticles on HCT116 cells, with IC50 values of 114.8 µg/mL and 247.5 µg/mL, respectively, compared with untreated controls. Notably, the shilajit-loaded nanoparticles exhibited minimal cytotoxicity on normal skin fibroblast cells, showing no effect up to 25 µg/mL and only a 1.64% reduction in viability at 50 µg/mL. In contrast, chitosan nanoparticles caused minimal inhibition of cell viability at various concentrations. In addition, shilajit-loaded chitosan nanoparticles dramatically decreased intracellular reactive oxygen species (ROS) and increased antioxidant enzyme compared with controls. Gene expression analysis revealed that all treatments upregulated the pro-apoptotic gene (Bax) and downregulated the anti-apoptotic gene (Bcl2) compared with controls. The extract-loaded nanochitosan exhibited the highest Bax expression, whereas shilajit resulted in lower Bcl2 expression. Interestingly, the treatments increased the expression of the oncogenes c-MYC and KRAS in colon cancer cells, with the highest expression observed in the shilajit-loaded nanochitosan group. The combination of shilajit and chitosan nanoparticles presents a promising and biocompatible delivery system for targeted cancer therapy, with minimal harm to healthy cells and potential synergy in treatment strategies.

Keywords: HCT116 cells, Chitosan Nanoparticles, Antioxidant, Shilajit-loaded chitosan nanoparticles, Colon cancer.

Introduction

Cancer is a disorder defined by the uncontrolled proliferation of particular cells in the body, which can spread to other places [1]. Cancer ranks as the second-most common cause of mortality worldwide, with 9.6 million fatalities documented in 2018, necessitating the development of novel anticancer drugs [2].

Colon cancer (CC) develops in the colon or rectum as a result of the uncontrolled growth of glandular epithelial cells. It can be caused by hereditary factors, colitis-related issues, sporadic occurrences, genetic predispositions, and environmental influences [3]. CC ranks as the second foremost cause of cancer-related mortality globally and is the most prevalent cancer linked to obesity in the United States. This cancer is influenced by various variables, including mutations, dysregulation of oncogenic drivers, altered epigenetics, and inflammation [4].

Approximately 50% of contemporary anticancer pharmaceuticals are derived from natural ingredients. Consequently, the use of natural products for the development of novel pharmaceuticals has significantly captivated researchers. Traditional Chinese Medicine employs natural items as adjunctive therapy with chemotherapy and radiotherapy to mitigate the harmful effects of these

*Corresponding authors: Amina Zedan, E-mail: AminaZedan1948.el@Azhar.edu.eg, Tel.: 0201092053103
(Received 25 March 2025, accepted 02 July 2025)
DOI: 10.21608/ejvs.2025.370936.2733

treatments and enhance overall efficacy in cancer treatment [5].

Shilajit, a thick resinous substance, originates from rock strata in high-altitude mountainous areas, particularly the Himalayas. It forms over centuries through the compression of organic and plant materials by geological layers and the gradual breakdown of vegetation. The transformation into Shilajit occurs under extreme heat and pressure, followed by exposure to sunlight on mountain slopes. This process leads to the material exuding from fissures in granite rocks, making it collectible, as described by Pandey [6] Shilajit shows considerable variations in its chemical composition depending on its location. Research indicates that more than 80% of its weight comprises humic compounds, while over 20% includes magnesium, calcium, and potassium. It also includes proteins, amino acids (especially glycine), fatty acids, bioactive chemicals like gallic acid and caffeic acid, and heavy metals such as chromium, cobalt, and selenium [7].

Shilajit has been demonstrated to function as a chemotherapeutic drug for bladder cancer and to cause apoptosis in human breast cancer cells of human [8].

The natural amino polysaccharide chitosan is made up of many functional reactive groups. It is a cationic polymer that is non-toxic, biocompatible, and made from biodegradable materials [9]. Chitosan is the bioactive deacetylated derivative of chitin and has been consistently and effectively used across various domains on human health, nutrition, medicines, and environmental applications [10]. The bioactivity, biodegradability, and usefulness of chitosan have facilitated its use across various domains, particularly in conjunction with other bioactive compounds such as nanomaterials, antibiotics, growth promoters, plant extracts, and nutraceuticals [11].

The conversion of chitosan into Nano forms enhances its functions and capacity to transport or conjugate molecules, as consistently demonstrated with numerous phytoconstituents, nanomaterials, antibiotics, enzymes, hormones, proteins, and vitamins [12]. Nano-chitosan is a natural compound exhibiting exceptional physicochemical qualities and is non-toxic to humans [13]. Chitosan nanoparticles were chosen as a delivery system due to their biocompatibility, biodegradability, and ability to effectively encapsulate bioactive compounds, enhancing targeted delivery and therapeutic efficacy in cancer treatment.

The combination of Shilajit and nano-chitosan may enhance the therapeutic efficacy against colon cancer cells (HCT-116) by promoting apoptosis and inhibiting cell proliferation more effectively than chitosan nanoparticle used alone. This synergistic effect is expected to be attributed to the unique bioactive components of Shilajit and the improved delivery and bioactivity of nano-chitosan, potentially leading to a novel natural-based anticancer therapy.

The existing literature lacks comprehensive studies investigating the synergistic effects of combining Shilajit and nano-chitosan in enhancing therapeutic efficacy against colon cancer cells, particularly in terms of promoting apoptosis and inhibiting cell proliferation, which could lead to novel natural-based anticancer therapies. Overall, this hypothesis aims to investigate whether the integration of these two natural products can result in a more effective therapeutic strategy against colon cancer, thereby contributing to the development of innovative treatments in oncology. The aim of this study is to investigate the effects of Shilajit-loaded chitosan nanoparticles on HCT116 colorectal cancer cells.

Material and Methods

Materials

Shilajit was acquired from Natural Spirit Trading in Egypt.

Chitosan, with molecular weight that is medium (CAS Number 9012-76-4), was obtained from Sigma-Aldrich (USA). It has a 75%–85% deacetylation degree and a viscosity between 200 and 800 cP when dissolved in a 1% solution of acetic acid.

Acetic acid and tripolyphosphate (TPP) were obtained from Sigma-Aldrich (USA).

The human colon cancer cell line (HCT-116) and normal human fibroblasts were procured from the American Type Culture Collection (ATCC, Minnesota, USA). The cells were maintained at the National Cancer Institute in Cairo, Egypt, through serial sub-culturing. HCT-116 cells were cultured in medium of McCoy's 5A (Sigma-Aldrich, USA). The medium was provided with 10% antibiotic-free Fetal Bovine Serum (FBS, Sigma, USA), penicillin (100 U/mL), and streptomycin (2 mg/mL). The cells were kept at 37°C in a 5% CO_2 atmosphere, a 95% humidified containing.

Methods

Preparation of the Shilajit extract

Shilajit was meticulously rinsed with tap water and subsequently sectioned into minute fragments. For the preparation of the aqueous extract, 70 g of the sample was immersed in 1000 ml of distilled water on a shaker for ten hours at ambient temperature, subsequently filtered using Whatman paper (No: 41), and finally subjected to freeze-drying. The produced extract will be stored at -20°C until used.

Chitosan nanoparticles (NCT) Preparation and Conjugation

The NCT preparation was carried out following a previously established method, which involves dissolving chitosan at a concentration of 0.1% (w/v) in a solution (1% aqueous acid). At a rate of 18 mL/h, the same volume of the TPP aqueous solution with a weight-to-volume ratio of 0.05% was progressively added to the chitosan solution. The mixtures were vigorously stirred at 780 g when TPP was added and for 90 min. The resulting NCT was collected through centrifugation at 5500 g for 30 min. After being centrifuged once again, the pellet was then freeze-dried after being rinsed with distilled water [14].

To conjugate the extract of shilajit (SWE) with NCT, shilajit was solubilized in deionized water at a concentration of 0.1% (w/v). An equivalent volume of this solution was mixed into the chitosan solution while slowly adding the TPP solution. The combination was stirred at 650 g for 125 minutes. The subsequent stages were carried out as previously described.

Characterization of Nanoparticles by a Zeta-sizer

The synthesized nanoparticle formulations were characterized using a Zeta-sizer (Brookhaven). One milligram of the nanoparticles was diluted in 10 ml of distilled water (pH 7.4) for dynamic light scattering and zeta potential assessments. The analysis used an average of five successful experiments at 25 °C [15].

Analysis of HPLC for Phenolic and Flavonoid Compounds

HPLC will examine flavonoids and phenolic components in the aqueous extract. The HPLC (Agilent 1260 Infinity HPLC Series, Agilent Technologies, Santa Clara, CA, USA) will have a Quaternary pump and a Kinetex (5 µm) EVO C18 (100 mm × 4.6 mm) column (Phenomenex, Torrance, CA, USA) operated at 30 °C. Samples will be separated using a ternary linear elution gradient, consisting of grade water for HPLC with 0.2% H3PO4 (v/v), acetonitrile, and methanol. The injected volume will be 20 µL, and the VWD detector will be adjusted to 284 nm [16].

Experimental Design

The cells were organized into five groups. The first group of HCT116 cells (G1). The second group of chitosan nanoparticle-treated HCT116 cells (G2). The third group was Shilajit extract-treated HCT116 cells (G3). The fourth group was Shilajit-loaded chitosan nanoparticle-treated HCT116 cells (G4). Treatments were applied in triplicate when the cells achieved 70–80% confluence. The samples were kept for 24 h in a CO_2 incubator at 37°C and humidity of 95%.

MTT Cytotoxicity Test

Normal skin fibroblasts and Human colon cancer HCT116 cells were sourced from VACSERA in Cairo. The MTT assay was carried out as previously detailed [17]. The cells were planted in each well at a density of 1 $\times 10^4$ cells. DMEM media containing 10% FBS, 2% L-glutamine and 1% penicillin/streptomycin was added to each well. All of these components were purchased from Gibco, which is located in Waltham, Massachusetts, in the United States. We then incubated the cells for 24 h at a temperature of 37 degrees Celsius in a humidified environment consisting of 95% air and 5% carbon dioxide until they reached 80%–90% confluence. After that, the cells were subjected to a series of dilutions, ranging from 0 to 300 µg/mL, of chitosan nanoparticles and Shilajit-loaded chitosan nanoparticles. The dilutions were carried out in a sequence to decrease the concentration of the substance. The cells were incubated for an additional 4 h after the addition of 5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, Waltham, Massachusetts, United States). This was done after the cells had been treated for a period of one day. The medium was removed and replaced with 100 µL of Dimethyl sulfoxide (Sigma Aldrich, USA). Finally, the absorbance was determined by measuring it at 570 nm.

Reactive Oxygen Species Estimation

The fluorescent probe (20,70-dichlorofluorescein diacetate (DCFDA) was utilized in order to quantify the levels of reactive oxygen species (ROS) that were present within the cells. HCT116 cells were exposed to chitosan nanoparticles and Shilajit-loaded chitosan nanoparticles at concentrations corresponding to their IC50 for 2 h, thereafter treated with 25 µM H2O2 for an additional 2 h. Following PBS washing, DCFDA (5 µM) was administered, and the cells were incubated in the dark at 37 °C for 30 min. After washing in PBS, the fluorescence intensity of DCFDA was quantified using a fluorescence microplate reader. The percentage of the control was used to quantify the intracellular ROS levels [17].

Antioxidant Enzyme Detection

The activities of SOD (superoxide dismutase), CAT (catalase), and GPx (glutathione peroxidase) in human colon cancer cells will be measured using a colorimetric method, using kits that are available for purchase, and adhering to the instructions provided by the manufacturer (Biodiagnostics, Cairo, Egypt). The presentation of the antioxidant activity will be done in the form of percentages of the control [17].

qPCR for Gene Expression Analysis

A technique that has been previously described was employed to collect RNA samples from all cells. Both 1% gel electrophoresis and Nanodrop were

implemented to evaluate the integrity and concentration of RNA, respectively. Following reverse transcription by Reverse Transcriptase (Revert Aid H Minus), the expression of numerous genes related with colon cancer was assessed using 2X SYBR Green Master Mix and specific primers. All kits utilized in qPCR were procured from Thermo Scientific, USA. A total of 2 µL of cDNA, 1 µL of each primer, and 12.5 µL of Maxima SYBR Green Master Mix were included in the qPCR mixture, which had a volume of 25 µL. The process of thermal cycling consisted of a duration of ten minutes at a temperature of 95 °C, followed by 45 cycles of 95 (degrees Celsius) for fifteen seconds, 60 (°C) for 30 seconds, and 72 °C for 30 s. The technique of $2^{-\Delta\Delta C}$ was used to quantify the relative expression of the target genes. This was done by normalizing the expression of the target genes against the housekeeping gene (*β-actin*) [18].

Statistical Analysis

All results are presented as mean ± standard error (SE) based on repetitions of independent experiments. GraphPad Prism version 7.0 will be utilized to accomplish the task of evaluating the differences between the groups through the utilization of one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference test technique. A p-value less than 0.05 is necessary for a finding to be deemed statistically significant.

Results

HPLC analysis

The High-Performance Liquid Chromatography (HPLC) study detected many phenolic and flavonoid chemicals in the sample. The identified chemicals and their concentrations (in µg/g) are as follows: Gallic acid (18.38 µg/g), Protocatechuic acid (5.65 µg/g), Chlorogenic acid (5.42 µg/g), Caffeic acid (9.95 µg/g) ferulic acid (10.62 µg/g), Sinapic acid (5.12 µg/g) rosmarinic acid (3.96 µg/g) cinnamic acid (3.75 µg/g) (Figure 1 and Table 2).

Investigation of the nanoparticles of chitosan and chitosan nanoparticles loaded with Shilajit

Analyzed using dynamic light scattering (DLS), the hydrodynamic diameter was measured to be in the nanometer range. The mean diam. of chitosan and Shilajit-loaded chitosan nanoparticles at the selected concentrations was 219.1 and 231.4 nm. The polydisperity index of chitosan and Shilajit-loaded chitosan nanoparticles was 0.324 and 0.156 (Figure 2).

MTT assay

The MTT assay results indicated cytotoxic effects that were dependent on the dose for chitosan nanoparticles and chitosan nanoparticles carrying Shilajit on HCT 116 cells, with IC50 values of 114.8 and 247.5 µg/mL, with comparison to untreated (control) cells (Figure3 A,B). Shilajit nanoparticles did not harm normal skin fibroblasts at concentrations up to 25 µg ml^{-1}, with only 1.64% cell viability inhibition at 50 µg ml^{-1}. That is mean cell viability was 98.36% at 50 µg/ml. The chitosan nanoparticles also showed minimal suppression of 1.70%, 8.78% and 12.65% at concentrations of 12.5, 50, and 100 µgmL^{-1}, respectively (Figur 3 C, D)

Intracellular ROS and the Antioxidant Status

Cells treated with chitosan nanoparticles, Shilajit, and Shilajit-loaded chitosan nanoparticles exhibited markedly reduced intracellular ROS levels and significantly elevated concentrations of SOD, CAT, and GPx compared with control cells (Figure 4).

Shilajit-loaded chitosan nanoparticles were more significant than the other treatments in reducing ROS, followed by Shilajit then chitosan nanoparticles. In antioxidant enzymes, Shilajit was more effective than the other treatments in SOD, while in CAT and GPX the effect of Shilajit and Shilajit-loaded chitosan nanoparticles was equal**.**

Chitosan nanoparticles, Shilajit, Shilajit-loaded chitosan nanoparticles modulated the expression of Bax , Bcl2, c-MYC, and KRAS genes

The expression of *Bax*, *Bcl2*, *c-MYC,* and *KRAS* genes were assessed. Compared with the control cells, treatment with each extract significantly increased *Bax* expression while decreasing Bcl2 expression (Figure 5).The extract-loaded nanochitosan showed a higher expression level of *Bax*, whereas Shilajit showed a lower expression level of Bcl2. *c-MYC* and *KRAS* genes decrease in expression in colon cell cancer, whereas the treatment increases this gene's expression. The high expression was in shilajet with nanochitosan.

Discussion

Colorectal cancer is one of the leading causes of cancer-related mortality, claiming millions of lives annually. In traditional medicine, particularly Ayurveda, shilajit has been recognized for its therapeutic properties in treating chronic illnesses and various health issues. Research has shown that shilajit can induce apoptosis in human breast cancer cells and is effective against bladder carcinoma **[**8, 19]. Overall, this hypothesis aims to investigate whether the integration of shilajit and nano-chitosan can result in a more effective therapeutic strategy against colon cancer, thereby contributing to the development of innovative treatments in oncology. Therefore, the aim of this study is to examine the combined effects of these two natural products on colon cancer cells (HCT-116) to explore their potential in enhancing therapeutic efficacy and improving treatment outcomes.

The results show a strong presence of phenolic acids, particularly gallic acid, caffeic acid, and ferulic acid. These compounds are known for their high antioxidant activity. Their presence suggests a potential role in oxidative stress protection. GA has exhibited anti-cancer activities via many biological mechanisms, including metastasis, migration, cell cycle arrest, apoptosis, oncogene expression, and angiogenesis [20]. Ferulic acid exhibits a notable therapeutic benefit against colon cancer by decreasing cellular growth and facilitating apoptosis [21].

Transforming chitosan into nanoforms increases its ability to transport conjugate compounds**[12]**. Recently it has lately been investigated as a prospective drug carrier to improve medication bioavailability and water solubility [22]. The conjugation of chitosan nanoparticles with shilajit gave a new compound with a therapeutic tool in oncology.

The results from the MTT assay are especially significant. The research demonstrates that chitosan nanoparticles possess a more effective anticancer impact than those containing shilajit. This may be ascribed to the intrinsic characteristics of chitosan, which could promote improved cellular absorption or augmented interaction with malignant cells [23]. The observation that chitosan nanoparticles conjugated with shilajit exhibited negligible cytotoxicity on normal skin fibroblasts at concentrations up to 25 μg/mL is a significant advantage. This selectivity highlights the ability of these nanoparticles to specifically target cancer cells while preserving normal tissues, thus diminishing the likelihood of side effects typically associated with traditional chemotherapeutics. The 1.64% decrease in cell viability at 50 μg/mL indicates that shilajit improves the therapeutic efficacy of chitosan nanoparticles without jeopardizing their safety at lower dosages. This is a crucial factor in developing cancer medicines that emphasize effectiveness and reduce toxicity.

Shilajit extracts are rich in phenolic and flavonoid compounds and exhibit significant total antioxidant activities [24], so we hypothesized that these extracts may induce cancer cell death by reducing oxidative stress and enhancing the activities of the antioxidant enzymes. To evaluate this hypothesis, we examined the impact of these products on the intracellular ROS levels and the activities of the antioxidant enzymes. The elevation of intracellular ROS serves as a significant marker of cellular oxidative stress. As anticipated, cells treated with chitosan nanoparticles, Shilajit, and Shilajit-loaded chitosan nanoparticles exhibited markedly reduced intracellular ROS levels and significantly elevated concentrations of SOD, CAT, and GPx compared with control cells. Shilajit-loaded chitosan nanoparticles were more significant than the other treatments in reducing ROS, followed by Shilajit then chitosan nanoparticles. The noted reduction in intracellular ROS levels suggests that these substances may improve cellular defense mechanisms against oxidative stress. This is especially pertinent in cancer therapy, where oxidative stress has a dual function: it can trigger cancer cell apoptosis, while elevated levels may also facilitate tumor proliferation and metastasis [25]. By preserving equilibrium, shilajit and its derivatives may enhance the sensitivity of cancer cells to therapeutic measures while safeguarding normal cells from oxidative harm. In antioxidant enzymes, Shilajit was more effective than the other treatments in SOD, while in CAT and GPX the effect of Shilajit and Shilajit-loaded chitosan nanoparticles was equal**.**

The qPCR was utilized to evaluate the impact of treatments on the expression level of the Bax and Bcl2 genes, which are involved in cell death (apoptotic) and survival (anti-apoptotic), respectively, with c-MYC, and KRAS genes. Treatment with both extracts dramatically increased the expression level of the apoptotic gene Bax while decreasing the expression level of Bcl2 relative to the control cells. The extract-loaded chitosan nanoparticles increased Bax expression, while Shilajit decreased Bcl2. These findings indicate that the lethal effect of Shilajit and chitosan nanoparticles on HCT116 cells may be mediated by apoptosis.

c-Myc is an extensively studied proto-oncogene that promotes cellular transformation and regulates programmed cell death. Elevated *c-Myc* expression augmented *Bax* expression under hypoxic conditions and triggered cellular death [26]. *c-Myc* facilitates apoptosis by compromising mitochondrial integrity, releasing pro-apoptotic effectors such as holocytochrome c. [27].

Moreover, the levels of expression for the *c-MYC* gene exhibited a reduction at the level of transcript in ovarian cancer cases compared with the control group [28]. These may be in agreement with our results that indicated *c-MYC* genes decrease in expression in colon cell cancer whereas the treatment increase the expression of this gene. The high expression was in shilajit with nanochitosan. This study illustrates that the overexpression levels of *c-Myc* and hipper activity in colon cancer cells significantly enhances susceptibility to apoptosis produced by nanochitosan and shilajit with nanochitosan, consistent with [29].

KRAS (Kirsten rat sarcoma virus) is a small GTPase that is essential for regulating numerous cellular functions, including development, proliferation, and survival, depending on its active or inactive state. It is a crucial element in the signaling pathways of tyrosine kinase and is identified as a significant oncogene, rendering it a target for rendering it a target for tyrosine kinase inhibitors of tyrosine kinase inhibitors (TKIs) [30]. Targeting

growth factor receptors is a crucial method for developing therapeutic medicines for colorectal cancer (CRC).

This study demonstrated that treatment with chitosan nanoparticles, shilajit, and shilajit-loaded chitosan nanoparticles led to the overexpression of the *KRAS* gene. This observation contradicts the existing literature, which generally indicates the downregulation of *KRAS* following therapeutic therapies. The elevation in *KRAS* expression may suggest that these treatments do not mainly target the *KRAS* gene or its regulatory circuits, but instead affect broader cellular processes, indicating intricate connections with many signaling pathways rather than direct suppression of *KRAS*. Comprehending this gap is crucial for evaluating the ramifications of these treatments in cancer biology.

However, to translate these findings into clinical settings, in vivo studies and clinical trials are essential. Animal models should be employed to evaluate pharmacokinetics, biodistribution, systemic toxicity, immune response, and therapeutic efficacy in a complex biological environment. Additionally, dose optimization and long-term safety assessments will be necessary before human application.

From a molecular standpoint, the unexpected upregulation of the KRAS gene invites further exploration. Since KRAS is a critical oncogene often associated with resistance to therapy, understanding its regulation in response to these treatments could provide novel insights. Future studies should investigate whether the upregulation is transient, compensatory, or a result of broader cellular reprogramming—potentially using RNA sequencing or proteomic profiling to uncover the involved pathways.

In summary, the mechanisms of action for shilajit-loaded chitosan nanoparticles involve inducing apoptosis, reducing oxidative stress, enhancing antioxidant defenses, modulating gene expression, and selectively targeting cancer cells while preserving normal cells. These findings present a promising avenue for innovative treatments in colorectal cancer.

Conclusion

This study highlights the selective cytotoxic effects and antioxidant properties of shilajit-loaded chitosan nanoparticles on colon cancer cells while sparing normal fibroblast cells. The nanoparticles effectively reduced intracellular ROS levels, enhanced antioxidant enzyme concentrations, and modulated gene expression by upregulating pro-apoptotic Bax and downregulating anti-apoptotic Bcl2. Additionally, the combination of shilajit and chitosan nanoparticles presents a promising and biocompatible delivery system for targeted cancer therapy, with minimal harm to healthy cells and potential synergy in treatment strategies.

While this study highlights the significant role of shilajit-loaded chitosan nanoparticles on HCT116 colorectal cancer cells, it is important to acknowledge its limitations. First, the study was conducted in vitro, which may not fully replicate the complex interactions and microenvironment present in vivo. Therefore, further research, including animal models and clinical trials, is necessary to validate these findings and assess the therapeutic potential in a biological context.

Second, the study focused on a single cancer cell line (HCT116), which may limit the generalizability of the results to other types of colorectal cancer or different cancer types. Future studies should explore the effects of shilajit-loaded nanoparticles on various cancer cell lines to establish broader applicability.

Acknowledgments

Out of scope.

Funding statement

No funding was provided for this study.

Conflict of Interest Declaration

No conflict of interest exists, according to the authors.

TABLE 1. Primer sequences used in qPCR.

Gene name	Forward	Reverse
Bax	GGACGAACTGGACAGTAACATGGT	GCAAAGTAGAAAAGGGCGACAACA
Bcl2	TTGATGGGATCGTTGCCTTATGCC	CAGTCTACTTCCTCTGTGATGTTGT
c-MYC,	CCTGGTGCTCCATGAGGAGAC	CAGACTCTGACCTTTTGCCAGG
KRAS	CAGTAGACACAAAACAGGCTCAG	TGTCGGATCTCCCTCACCAATG
β-actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG

TABLE 2. Phenolic and flavonoid compound composition of the Shilajit extracts.

Compound	Concentration (µg/g)
Gallic	18.38
Protocatechuic	5.65
Gentisic	ND
p-hydroxybenzoic	ND
Cateachin	ND
Chlorogenic	5.42
Caffeic	9.95
Syringic	ND
Vanillic	ND
Ferulic	10.62
Sinapic	5.12
p-coumaric	ND
Rutin	ND
Qurecetin	ND
Rosmarinic	3.96
Cinnamic	3.75
Apigenin-7-glucoside	ND
Kaempferol	ND
Apigenin	ND
Chrysin	ND

ND: not detected

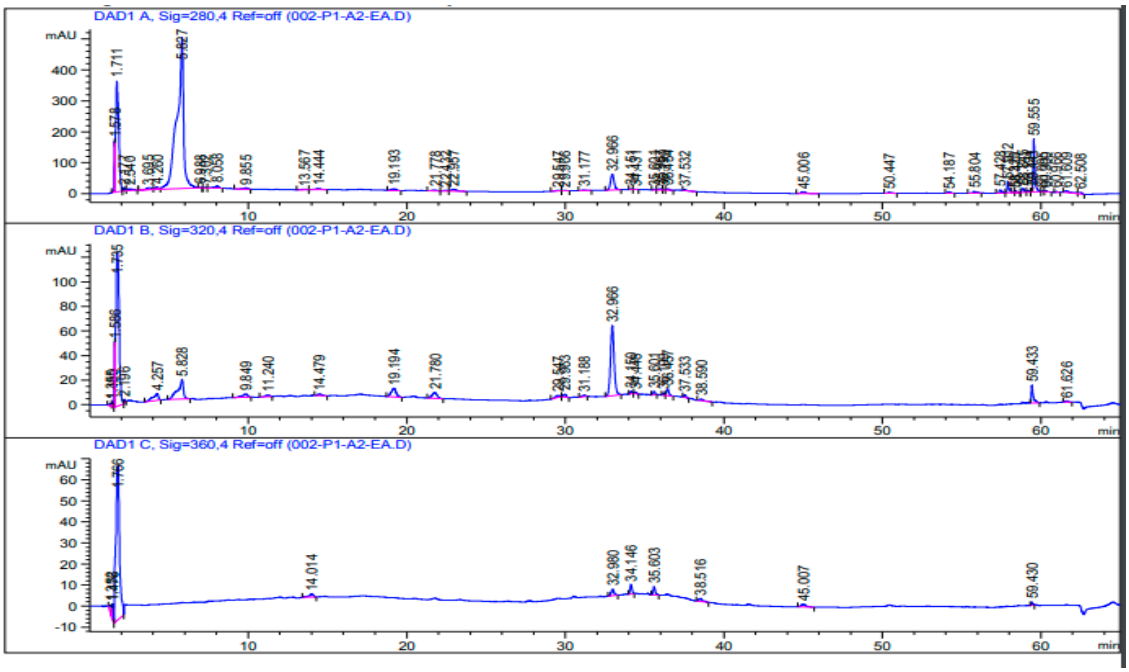

Fig. 1. HPLC chromatograms of Shilajit

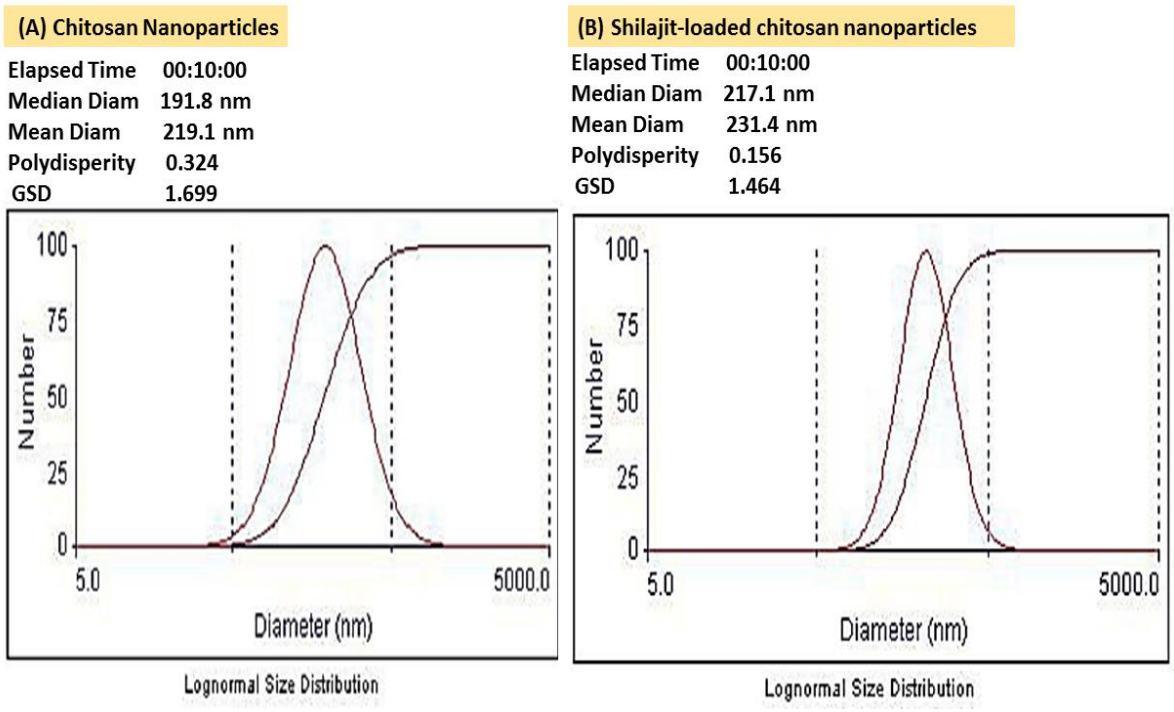

Fig. 2. Nanosizer of chitosan nanoparticles and Shilajit-loaded chitosan nanoparticles

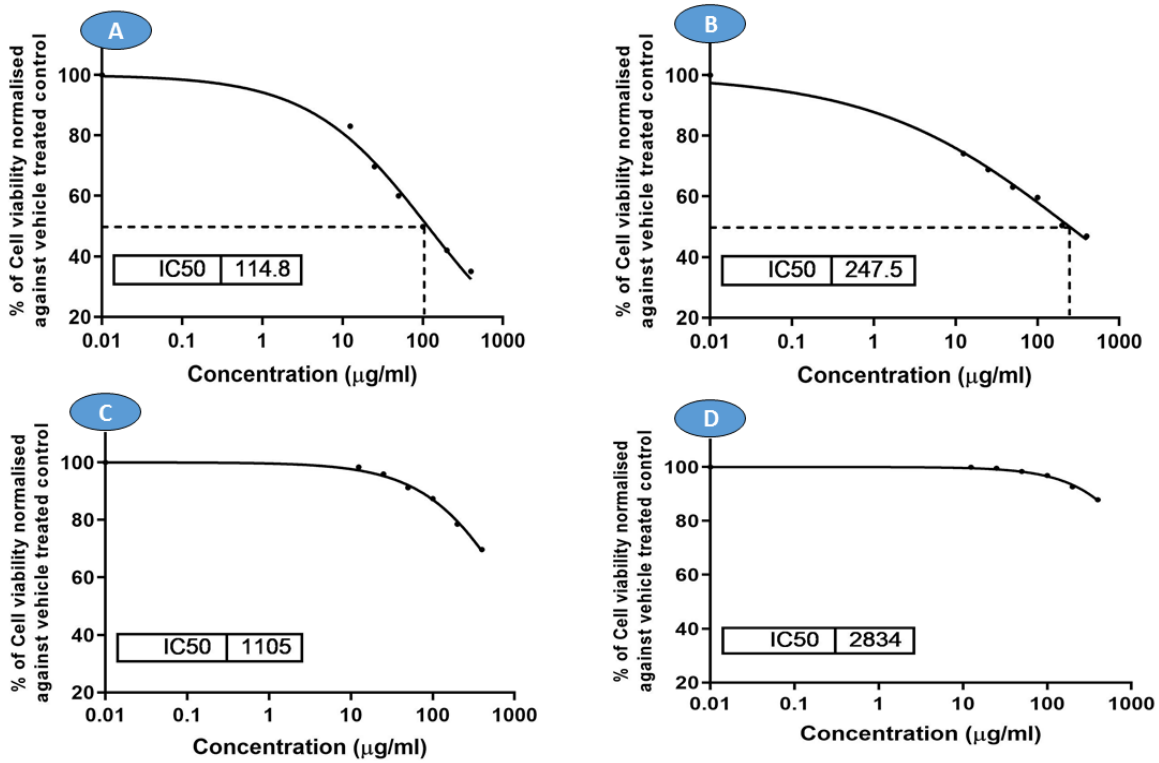

Fig. 3. Cytotoxicity analysis of the chitosan nanoparticles (A) and Shilajit-loaded chitosan nanoparticles (B) using the MTT assay on HCT 116 cells and on skin fibroblast normal cells (C) and (D), respectively. Dose–response curves show IC50 after 24 h. The mean percentage viability ± SEM was used to express the data, which were normalized to untreated control cells. There were three separate trials with a total of three replicates of the samples.

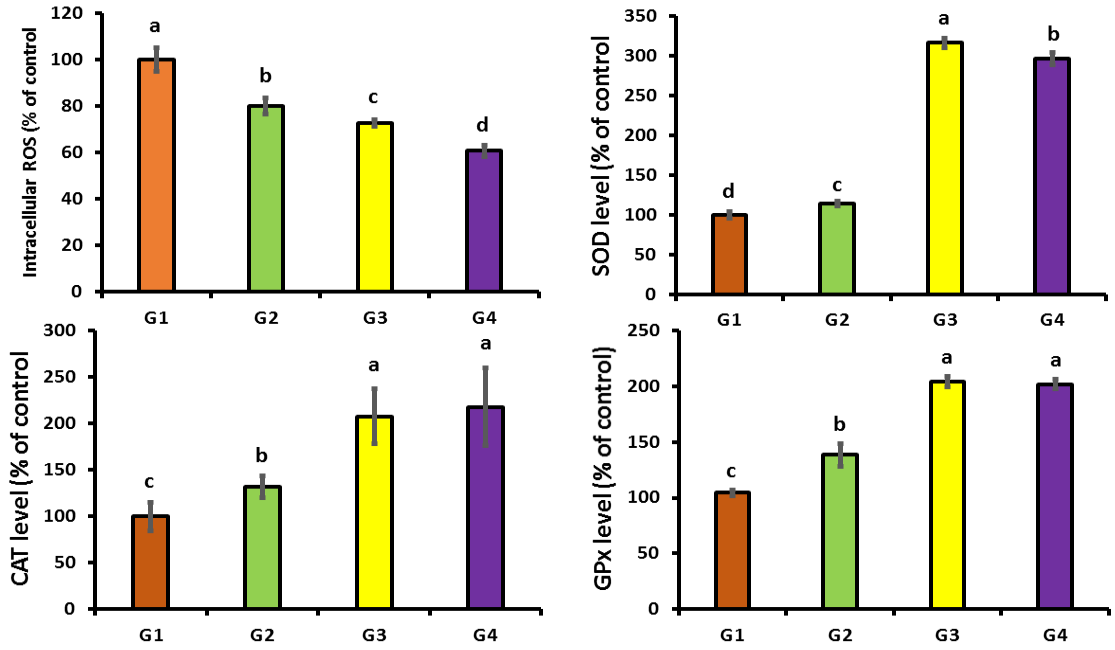

Fig. 4. Impact of chitosan nanoparticles, Shilajit, and Shilajit-loaded chitosan nanoparticles on the activity of ROS and antioxidant enzymes (SOD, CAT and GPx) in HCT 116 cells. Data are presented as a percentage of the control ± standard error of the mean (SEM). Samples were conducted in triplicate across three independent experiments, n = 5. Groups denoted by different letters exhibit significant differences at $p < 0.05$.

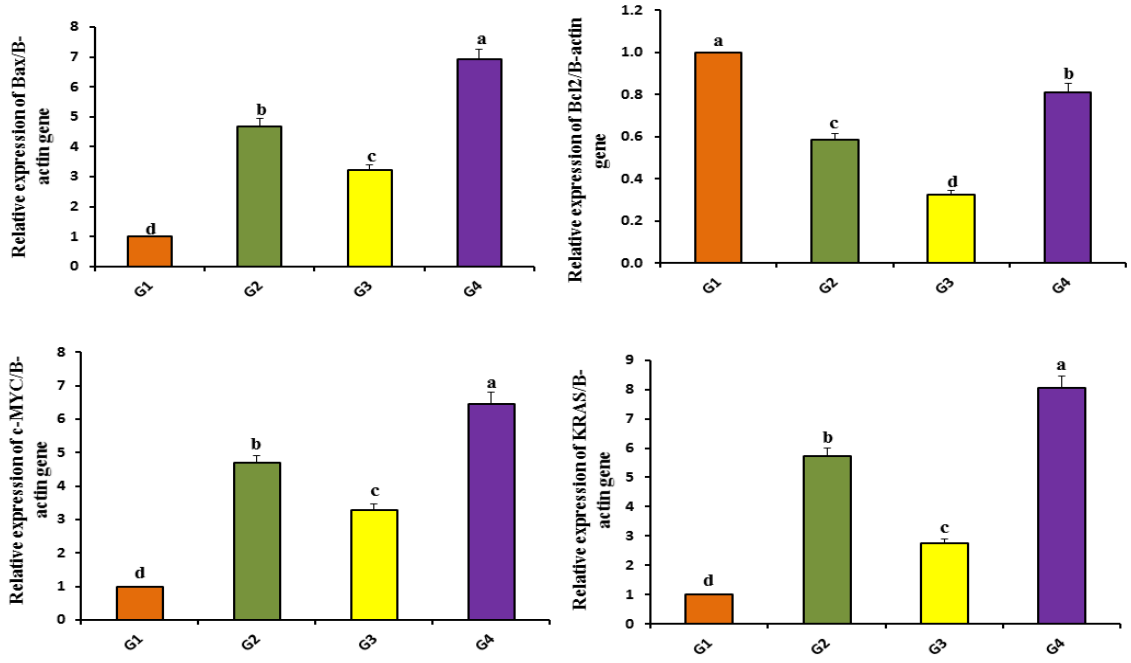

Fig.5. Visualization of a real-time quantitative PCR experiment of the expression levels of *Bax*, *Bcl2*, *c-MYC*, and *KRAS* genes.

References

1. Brown, J.S., Amend, S.R., Austin, R.H., Gatenby, R.A., Hammarlund, E.U. and Pienta, K.J. Updating the definition of cancer. *Molecular Cancer Research*, **21** (11), 1142-1147 (2023).

2. Koparir, P., Parlak, A.E., Karatepe, A. and Omar, R.A. Elucidation of potential anticancer, antioxidant and antimicrobial properties of some new triazole compounds bearing pyridine-4-yl moiety and cyclobutane ring. *Arabian Journal of Chemistry*, **15** (7), 103957 (2022).

3. Hossain, M.S., Karuniawati, H., Jairoun, A.A., Urbi ،Z., Ooi, D.J., John, A., Lim, Y.C., Kibria, K.K., Mohiuddin, A. and Ming, L.C. Colorectal cancer: a review of carcinogenesis, global epidemiology, current challenges, risk factors, preventive and treatment strategies. *Cancers*, **14** (7), 1732 (2022).

4. Ghimire, J., Collins, M.E., Snarski, P., King, A.N., Ruiz, E., Iftikhar, R., Penrose, H.M., Moroz, K., Rorison, T. and Baddoo, M. Obesity-facilitated colon cancer progression is mediated by increased diacylglycerol O-acyltransferases 1 and 2 levels. *Gastroenterology*, **168**(2),286-299 (2024).

5. Ling, C.-q., Yue, X.-q. and Ling, C. Three advantages of using traditional Chinese medicine to prevent and treat tumor. *Journal of Integrative Medicine*, **12** (4), 331-335 (2014).

6. Pandey, P.S. Shilajit-a wonder drug of ayurveda: an overview. *Int. J. Pharm. Sci. Rev. Res.*, **59** (1), 140-3 (2019).

7. Kamgar, E., Kaykhaii, M. and Zembrzuska, J. A comprehensive review on Shilajit: what we know about its chemical composition. *Critical Reviews in Analytical Chemistry*, 1-13 (2023).

8. Kloskowski, T., Szeliski, K., Krzeszowiak, K., Fekner, Z., Kazimierski, Ł., Jundziłł, A., Drewa, T. and Pokrywczyńska, M. Mumio (Shilajit) as a potential chemotherapeutic for the urinary bladder cancer treatment. *Scientific Reports*, **11** (1), 22614 (2021).

9. Desai, R., Pachpore, R., Patil, A., Jain, R. and Dandekar, P. (2021) Review of the structure of chitosan in the context of other sugar-based polymers. In Chitosan for Biomaterials III: Structure-Property Relationships, pp. 23-74, Springer.

10. Ways, T.M., Lau, W.M. and Khutoryanskiy, V.V. Chitosan and its derivatives for application in mucoadhesive drug delivery systems. *Polymers*, **10** (3), 267 (2018)

11. Sivanesan, I., Gopal, J., Muthu, M ،.Shin, J., Mari, S. and Oh, J. Green synthesized chitosan/chitosan nanoforms/nanocomposites for drug delivery applications. *Polymers*, **13** (14), 2256 (2021).

12. Zhao, D., Yu, S., Sun, B., Gao, S., Guo, S. and Zhao, K. Biomedical applications of chitosan and its derivative nanoparticles. *Polymers*, **10** (4), 462 (2018).

13. Benettayeb, A., Seihoub, F.Z., Pal, P., Ghosh, S., Usman, M., Chia, C.H., Usman, M. and Sillanpää, M. Chitosan nanoparticles as potential nano-sorbent for removal of toxic environmental pollutants. *Nanomaterials*, **13** (3), 447 (2023).

14. Zayed, M., Elkafafi, S., Zedan, A.M. and Dawoud, S.F. Effect of nano chitosan on growth, physiological and biochemical parameters of Phaseolus vulgaris under salt stress. *Journal of Plant Production*, 8(5), 577-585 (2017).

15. Abou El-Naga, H.M.H., El-Hashash, S.A., Yasen, E.M., Leporatti, S. and Hanafy, N.A. Starch-based hydrogel nanoparticles loaded with polyphenolic compounds of Moringa oleifera leaf extract have hepatoprotective activity in bisphenol A-induced animal models. *Polymers*, **14** *(*14*)*, 2846 (2022).

16. Kim, K.-H., Tsao, R., Yang, R. and Cui, S.W. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry*, **95** (3), 466-473 (2006).

17. Zedan, A.M., Sakran, M.I., Bahattab, O., Hawsawi, Y.M., Al-Amer, O., Oyouni, A.A., Nasr Eldeen, S.K. and El-Magd, M.A. Oriental Hornet (*Vespa orientalis*) larval extracts induce antiproliferative, antioxidant, anti-inflammatory, and anti-migratory effects on MCF7 Cells. *Molecules*, **26** (11), 3303 (2021).

. 18. El-Magd, M.A., Zedan, A.M., Zidan, N.S., Sakran, M.I., Bahattab, O., Oyouni, A.A.A., Al-Amer, O.M., Alalawy, A.I. and Elmoslemany, A.M. Avocado seeds-mediated alleviation of cyclosporine a-induced hepatotoxicity involves the inhibition of oxidative stress and proapoptotic endoplasmic reticulum stress. *Molecules*, 27 (22), 7859 (2022).

19. Jafari, M., Forootanfar, H., Ameri, A., Foroutanfar, A., Adeli-Sardou, M., Rahimi, H.R., Najafi, A., Zangiabadi, N. and Shakibaie, M. Antioxidant, cytotoxic and hyperalgesia-suppressing activity of a native Shilajit obtained from Bahr Aseman mountains. *Pakistan Journal of Pharmaceutical Sciences*, **32** (5),2167-2173 (2019).

20. Jiang, Y., Pei, J., Zheng, Y., Miao, Y.-j., Duan, B.-z. and Huang, L.-f. Gallic acid: A potential anti-cancer agent. *Chinese journal of integrative medicine*, **28** *(*7*)*, 661-671 (2022).

21. Alazzouni, A.S., Dkhil, M.A., Gadelmawla, M.H., Gabri, M.S., Farag, A.H. and Hassan, B.N. Ferulic acid as anticarcinogenic agent against 1, 2-dimethylhydrazine induced colon cancer in rats. *Journal of King Saud University-Science*, **33** (2), 101354 (2021).

22. Bhoopathy, S., Inbakandan, D., Rajendran, T., Chandrasekaran, K., Kasilingam, R. and Gopal, D. Curcumin loaded chitosan nanoparticles fortify shrimp feed pellets with enhanced antioxidant activity. *Materials Science and Engineering: C*, **120**, 111737 (2021).

23. Aibani, N., Rai, R., Patel, P., Cuddihy, G. and Wasan, E.K. Chitosan nanoparticles at the biological interface: implications for drug delivery. *Pharmaceutics* **13** (10), 1686 (2021).

24. Al-Salman ،F., Redha, A.A. and Al-Zaimoor, Z. Inorganic analysis and antioxidant activity of Shilajit. *Int. J. Sci. Res. in Chemical Sciences*, **7**(3), 71 (2020).

25. de Sá Junior, P.L., Câmara, D.A.D., Porcacchia, A.S., Fonseca, P.M.M., Jorge, S.D., Araldi, R.P. and Ferreira, A.K. The roles of ROS in cancer heterogeneity and therapy. *Oxidative medicine and cellular longevity*, **2017** (1), 2467940 (2017).

26. Lin, X., Sun, R., Zhao, X., Zhu, D., Zhao, X., Gu, Q., Dong, X., Zhang, D., Zhang, Y. and Li, Y. C-myc overexpression drives melanoma metastasis by promoting vasculogenic mimicry via c-myc/snail/Bax signaling. *Journal of Molecular Medicine*, **95**, 53-67 (2017).

27. Juin, P., Hunt, A., Littlewood, T., Griffiths, B., Swigart, L.B., Korsmeyer, S. and Evan, G. c-Myc functionally cooperates with Bax to induce apoptosis. *Molecular and Cellular Biology* , **22** *(*17*)*, 6158-6169 (2002).

28. Olbromski, P.J., Bogacz, A., Bukowska, M., Kamiński, A., Moszyński, R., Pawlik, P., Szeliga, A., Kotrych, K. and Czerny, B. Analysis of the Polymorphisms and Expression Levels of the BCL2, BAX and c-MYC Genes in Patients with Ovarian Cancer. *International Journal of Molecular Sciences*, **24** *(*22*)*, 16309 (2023).

29. Arango, D., Mariadason, J., Wilson, A., Yang, W., Corner, G., Nicholas, C., Aranes M. and Augenlicht, L. c-Myc overexpression sensitises colon cancer cells to camptothecin-induced apoptosis. *British Journal of Cancer*, **89** (9), 1757-1765 (2003).

30. Nalli, M., Puxeddu, M., La Regina, G., Gianni, S. and Silvestri, R. Emerging therapeutic agents for colorectal cancer. *Molecules*, **26** (24), 7463 (2021).

آثار السمية الخلوية وآليات مضادات الأكسدة لجزيئات النانو من الكيتوزان المحملة بالشيلجيت على خلايا سرطان القولون

أسماء العشري خاطر[1]، اميرة المسلماني[1]، أسماء الجاويش[1] وأمينة محمد جاد زيدان[2]

[1] قسم التغذية وعلوم الاطعمة، كلية الاقتصاد المنزلي ، جامعة الازهر، مصر.
[2] كلية الزراعة (فرع بنات)، جامعة الازهر، مصر

الملخص

نتائج اختبار MTT أظهرت تأثيرات سمية خلوية تعتمد على الجرعة لجزيئات النانو من الكيتوزان وجزيئات النانو المحملة بالشيلجيت على خلاياHCT116 ، مع قيم IC50 تبلغ 114.8 ميكروغرام/مل و247.5 ميكروغرام/مل على التوالي، مقارنةً بمجموعة التحكم غير المعالجة. ومن الجدير بالذكر أن جزيئات النانو المحملة بالشيلجيت أظهرت سمية خلوية ضئيلة على خلايا الفيبروبلاست الجلدية الطبيعية، حيث لم تظهر أي تأثير حتى 25 ميكروغرام/مل وبتقليل بنسبة 1.64% في البقاء عند 50 ميكروغرام/مل. في المقابل، تسببت جزيئات النانو من الكيتوزان في تثبيط ضئيل لبقاء الخلايا عند تركيزات مختلفة. بالإضافة إلى ذلك، قامت جزيئات النانو المحملة بالشيلجيت بتقليل مستويات أنواع الأكسجين التفاعلية (ROS)داخل الخلايا بشكل كبير وزيادة تركيزات إنزيمات مضادات الأكسدةSOD) ، CAT، (GPxمقارنةً بمجموعة التحكم. كان الشيلجيت فعالاً بشكل خاص في تعزيز مستوياتSOD ، بينما أظهرت جزيئات النانو المحملة بالشيلجيت والشيلجيت تأثيرات مماثلة على CAT و.GPx أظهر تحليل التعبير الجيني أن جميع العلاجات زادت من تعبير الجين المؤيد للاستسحاب الخلوي Bax وخفضت تعبير الجين المعادي للاستسحاب الخلوي Bcl2 مقارنةً بمجموعة التحكم. كانت جزيئات النانو المحملة بالمواد المستخرجة تمتلك أعلى تعبير لـBax ، بينما أدى الشيلجيت إلى تعبير أقل لـ.Bcl2 من المثير للاهتمام أن العلاجات زادت من تعبير الجينات المسرطنة c-MYC و KRASفي خلايا سرطان القولون، مع أعلى تعبير لوحظ في مجموعة جزيئات النانو المحملة بالشيلجيت.

الكلمات الدالة: HCT116 : جزيئات نانو الكيتوزان، شيلجيت، مضادات الأكسدة.