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Robust anticancer efficacy of *Naja haje* venom-loaded silica nanoparticles against triple-negative breast cancer xenografts in a preclinical rat model

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ABSTRACT

Background: Breast cancer, a prevalent disease affecting women globally, is particularly aggressive and has limited treatment options.

Aim: Snake venom, containing active chemicals, has shown potential in medicine.

Methods: The study investigates the anticancer effect of Egyptian cobra *Naja haje* venom alone and in combination with Nanoparticles (NP) on TNBC *in vivo*. The study involved dividing experimental animals into five groups, each with 10 rats, each treated with different doses of crude venom, G2 and G3, respectively. The study involved loading venom onto NP-based delivery systems, measuring inflammatory cytokines and tumor markers, extracting RNA, real-time qRT-PCR gene expression, and histopathological examination of breast tissue.

Results: The study involved administering *Naja haje* crude venom at higher (1/5 LD50) and lower (1/20 LD50) dose levels in groups G2 and G3, respectively.

Conclusion: The study found that venom treatment in groups G4 and G5 significantly improved inflammatory cytokine and tumor markers levels, increased expression of tumor-suppressor genes, and increased apoptosis and necrosis.

Keywords: Triple negative breast cancer, *Naja haje* venom, Anticancer, Inflammatory cytokines, NPs.

Introduction

Breast cancer (BC) is the second most common cause of cancer-related mortality among women. (Chan and Moris, 2006). As reported by (Shah *et al*., 2014) classifications for BC have been created in an effort to treat patients more successfully. Triple-negative breast cancer (TNBC), is an extremely truculent subtype of BC (Zhu *et al.,* 2023). It is more common in young women compared to other BC subtypes, and it is linked to higher cancer risk and death (Dent *et al*., 2007).

On the surface of TNBC, there is no expression of the progesterone, estrogen, or HER2 receptors (Borri and Granaglia, 2021). Considering this fact, TNBC represents a unique and challenging entity within BC due to its aggressive behavior, limited treatment options, and poorer prognosis compared to other subtypes (Wein and Loi, 2017). Although chemotherapy is the main form of treatment for patients with TNBC, the effectiveness of chemotherapy for TNBC is still limited (Yi *et al*., 2021).

Animal poisons and venoms from a variety of species, including snakes, scorpions, cone snails, bees, and wasps, have been extensively researched over time due to their potential as a significant source of bioactive chemicals (Harvey, 2014). A wealth of active pharmacological proteins and peptides have been reported to be found in animal venoms (Mohamed *et al*., 2019). For many years numerous studies have demonstrated the significant effect of cobra venom in providing relief from various types of pain, including that associated with cancer, neuralgia, and joint-related disorders (Liang *et al*., 2015). Predominately snake venom comprises various polypeptide toxins and enzymes manifesting diverse pharmacological and biological activities contra virus, bacteria, and tumor (Salama *et al*., 2018; Roy and Bharadvaja, 2021). Venom enzymes extracted from different snake species have recently been shown in a number of studies to have potential therapeutic benefits, and this has led to a growing interest in their application in the biomedical area (Cedro *et al*., 2018).

Furthermore, snake venom has been utilized in varied communities based on its ability to decrease the cultivating of cancer cells, stifle cancer alternation and metastasis (Gomes *et al*., 2010). Its anticancer mechanisms rely on the immunosuppressive, cytotoxic, and antiproliferative strength of its proteins, which have the capability to promote cancer cells apoptosis (Mahmoud *et al*., 2019). The potential of snake venom in the treatment of cancer has been demonstrated

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by several studies. Cancer cells are cytotoxically affected by phospholipase A2, L-amino-acid oxidase, metalloprotease, disintegrin, and other peptides found in snake venom (Shanbhag, 2015).

The majority of phospholipases A2 are found in elapids, especially *Naja sp*., and they have a variety of functional pharmacological effects, including immunological modulation, hemolysis, edoema, neurotoxicity, myotoxicity, cytotoxicity, anticoagulation, and anticancer activities **(**Kang *et al*., 2011). To increase the effectiveness of treatment, NPs may be engineered to specifically target cancer cells and deliver therapeutic substances like medications or contrast agents. This can be achieved by combining biological and engineering techniques (Sun *et al*., 2020).

Several types of NPs such as solid, hollow, mesoporous, mesoporous core-shell, or hybrid forms have been widely applied as drug delivery agents for cancer diagnosis and treatment (Tiburcius *et al*., 2021). Chemical therapeutics-loaded nanoparticles (NP) have showed a lot of promise in the treatment of cancer. NPs can successfully raise medication concentrations in cancer tissues and work at the cellular level to improve antitumor efficacy when loaded with anticancer drugs (Barratt, 2003).

Therefore, we examined the effects of *Najahaje* venom on the growth of TNBC cell line MDA-MB-231-bearing experimental rats in the current investigation, both alone and in conjunction with silica NPs (venom + NP).

Materials and Methods

Experimental animals

This experiment relied on fifty adult female Albino Wistar rats, aged between 7 and 9 weeks. The rats varied in weight from 100 to 120 g. The rats were obtained from the faculty of veterinary medicine, Zagazig University, Egypt. To provide suitable housing conditions, the rats were kept in plastic cages with ten rats per cage under controlled temperature and provided with standard rodent chow in the animal house, F aculty of Medicine, Zagazig University, Egypt.

Tumor cells

The triple negative BC cell line (MDA-MB-231) was acquired from the American type culture collection. Cells were cultured in RPMI replenished with 10% FBS, Penicillin 100 U/ml, and 100 mg/ML of streptomycin at criterion conditions (a humidified atmosphere at 37°C and 5% CO_2). The tumor cells then were collected out for fecundation by washing with PBS twice pursued by compendious incubation in 0.25% trypsin and 0.02% EDTA (Neudert *et al*., 2003).

Tumor cell's inoculation

To prepare for the injection of tumor cells into the mammary fat pad, the rats underwent anesthesia using metofane, and a small incision measuring 5 mm was made in the skin on the lateral thorax. This allowed for the exposure of the mammary fat pad (M.F.P). Using a 27-gauge needle, a volume of 0.1 ml of cell inoculation was injected into the tissue. The growth of tumors in the mammary fat pad was monitored on a weekly basis for a duration of 6 weeks. Subsequently, the treatment process was initiated (Price *et al*., 1990).

Venom

Different ways of extracting venom were employed from *Bothrops alternatus*, *Bothrops Neuwiedi*, and *Crotalus durissus*. These methods included: a) spontaneous ejaculation during biting; b) hand massage on glands; and c) electrical stimulus to the muscle. For these animals, electrical stimulation produces superior outcomes (di Tada *et al*., 1978). In the laboratory of the Physiology Department of the Faculty of Science, Ain Shams University, Egypt, the venom of the Egyptian cobra, *Naja haje*, was extracted by spontaneous ejaculation when biting and lyophilized. Using the Meier and Theakston method, the deadly toxic dose (LD50) of the venom was found in (1986).

Combination of snake venom with silica NPs

Silica NPs and their combination with snake venom were prepared at the Nanotechnology Laboratory of Cairo University in Egypt. Cobra venom-silica nanocomposites were synthesized using sonochemical methods, wherein ultrasonic waves induced the formation and growth of micro-bubbles. These micro-bubbles generated extreme temperature and pressure both internally and externally. As the bubbles collapsed, the cobra venom molecules were exposed to these extreme conditions, leading to the nucleation of NPs. Rapid cooling then facilitated the synthesis of cobra venom loaded into mesoporous silica NPs (MSNs). In another step, 0.05 g of MSNs were dispersed in 100 ml of double-deionized water. This dispersion was combined with a solution of 0.1 g of cobra venom in 100 ml of double-deionized water, resulting in a mixture of 200 ml. The mixture was subjected to sonication for 3 hours using specific conditions, including a pulse time of 2 seconds, a rest time of 1 second, and a temperature maintained below 50°C with an amplitude of 75%.

Experimental animal design

Lethality was assessed by injecting different doses of venom in 0.5 ml saline via the intraperitoneal route, resulting in an LD50 value of 0.568 mg/kg. The experiment included five groups, each consisting of ten rats, of which five groups had tumors.

Group 1(+ve control): served as the positive control group (a MDA-MB231induced non treated group).

Group 2 (1/5 LD_{50}V), and Group 3 (1/20 LD_{50}V) represented the treated group which included MDA-MB231 -induced mammary gland tumors for 6 weeks (Price *et al*., 1990), then intramuscular injected with a dose equivalent to 1/5 of the LD50 (0.1 mg/kg) for G1, and 1/20 of the LD50 (0.02 mg/kg) for G3 of *Nh* cobra venom for 4 weeks (twice weekly), (Markland *et al*., 2002).

Group 4 (1/5 LD_{50}V + NP) & Group 5 (1/20 LD_{50} V + NP) were similar to G2 and G3, however in addition

to the intramuscular injection with *Nh* venom, the venom was combined with MSNs (Bhowmik *et al*., 2014). At the end of the experiment, the rats were euthanized under Na thiopental anesthesia to conclude the experiment.

Assessment of biochemical markers

The collected blood samples were allowed to coagulate at ambient temperature for 30 minutes, followed by centrifugation at 3,000 rpm for 10 minutes. The separated serum was then analyzed for the concentrations of the tumor markers carcinoembryonic antigen (CEA) and cancer antigen 15.3 (CA15.3) by using reagent ELISA-kits obtained from Sino Gene Clon Biotech Co, China. Tumor necrosis factor alpha (TNF-α) was stately by Elisa kit with Cat. No E0082Hu and interleukin 6 (IL 6) by Bioassay Technology Laboratory ELISA kit with the cat. No E0090Hu.

Real-time quantitative PCR analysis

Total RNA was isolated from the tissue samples using the Trizol (Invitrogen; Thermo Fisher Scientific, Inc.). Specifically, 30 mg of the whole tissue was homogenized in 1 ml of Trizol reagent. Subsequently, 200 µl of chloroform was added to the homogenate, the mixture was vortexed, incubated for 3 minutes, and then centrifuged at 12,000 g for 15 minutes at 4°C. The quality of the extracted RNA was assessed by analyzing the A260/A280 ratio using a NanoDrop® ND-1000 Spectrophotometer. For complementary DNA synthesis, a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, USA) was utilized. The real-time reverse transcription-polymerase chain reaction (RT-qPCR) was performed using a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) and TOPreal™ qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics, Korea). The real-time RT-qPCR amplification was performed using the following cycling parameters: an initial denaturation step at 95°C for 12 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The gene-specific oligonucleotide primer sequences were designed and synthesized by Sangon Biotech (Beijing, China) (Table 1). The expression levels of the target genes (BAX- BCL-2, P53, Caspase-3 and BAX/BCL2 ratio) were normalized to the mRNA expression of the housekeeping gene, rat Gapdh. The results are presented as fold-changes compared to the control group, calculated using the 2-ΔΔCT method (Livak and Schmittgen, 2001).

Histopathological essay

At the conclusion of the experiment, the mammary gland tissues were harvested from the sacrificed rats. The collected tissue samples were first fixed in a 4% buffered formaldehyde solution, and then embedded in paraffin wax for sectioning. Using a manually operated rotary microtome (model CUT 4055 or 4055F, R), the paraffin-embedded tissue samples were sectioned at a thickness of 5 micrometers. These thin tissue sections were then mounted onto glass microscope slides and stained using the hematoxylin and eosin histological staining technique (Bancroft and Gamble, 2008).

Statistical analysis

Software Statistical Product and Service Solutions version 19 was used for the collection, tabulation, and statistical analysis of all the data. The information was presented using the mean ± standard deviation (SD). A one-way analysis of variance test and a least significant difference analysis were used to evaluate the difference. *p*-value: value of $p \geq 0.05$ was regarded as statistically insignificant, and a value of $p < 0.001$ as statistically significant.

Ethical approval

On June 26, 2024, the ZU-IACUC Committee and the International Animals and Use Committee accepted the updated protocol and gave it the approval number ZU-IACUC/1/F/143/2024.

Results

The expression of tumor-suppressor gene P53, Caspase-3, pro-apoptotic gene BAX, and anti-apoptotic

Table 1. Primer sequence of genes used in this trial.

Gene	Sequence
GAPDH (housekeeping gene)	F, 5—CGTCTGCCCTATCAACTTTCG—3
	R, 5—CGTTTCTCAGGCTCCCTCT—3.
BCL-2	F, 5—TGTGGATGACTGAGTACCTGAACC—3
	R,5—CAGCCAGGAGAA ATCAAACAGAG—3
BAX	F, 5—GCTGGACATTGGACTTCCTC-3
	R,5—TCAGCCCATCTTCTTCC AGA—3
P53	F, 5—CCTATCCGGTCAGTTGTTGGA—3
	R, 5—TTGCAGAGTGGAGGAAATGG—3
Caspase-3	F,5–AAGATACC GGTGGAGGCTGA–3
	R, 5–AAGGGACTGGATGAACCACG–3

Table 2. Effect of 1/5, 1/20 LD_{50} of *Nh* venom and 1/5, 1/20 LD_{50} of *Nh*venom + NP on the expression of P53, Caspase-3, BAX and BCL-2.

	G1 +VE control *n* = 10	G2 V 1/5 *n* = 10	G3 V 1/20 *n* = 10	G4 VN 1/5 *n* = 10	G5 VN 1/20 *n* = 10
P 53	0.89 ± 0.12	4.86 ± 0.36[a]	3.14 ± 0.12[ab]	10.7 ± 0.4[abc]	5.43 ± 0.28[abcd]
Caspase-3	1.2 ± 0.17	4.19 ± 0.24[a]	1.79 ± 0.12[ab]	5.91 ± 0.45[abc]	3.17 ± 0.22[abcd]
BAX	1.0 ± 0.14	5.8 0± 0.39[a]	2.19 ± 0.22[ab]	7.89 ± 0.49[abc]	4.24 ± 0.34[abcd]
BCL2	1.2 ± 0.09	0.12 ± 0.02[a]	0.37 ± 0.04[ab]	0.08 ± 0.01[ac]	0.5 ± 0.07[abcd]

[abcd]Mean ± SD in the same column with distinct superscripts differ at *p*-values of $p < 0.0001$, $p < 0.01$, and $p < 0.05$.

Table 3. Effect of 1/5, 1/20 LD_{50} of *Nh* venom and 1/5, 1/20 LD_{50} of *Nh*V +NP on the level of Inflammatory cytokines (TNF-α & IL-6) and on CEA and CA15.3 in all studied groups.

	G1 +VE control *n* = 10	G2 V 1/5 *n* = 10	G3 V 1/20 *n* = 10	G4 VN 1/5 *n* = 10	G5 VN 1/20 *n* = 10
IL-6	99.53 ± 9.18	52.33 ± 6.44[a]	58.67 ± 8.25[ab]	43.77 ± 3.95[abc]	47 ± 4.98[abcd]
TNF-α	50.28 ± 8.71	28.72 ± 3.23[a]	34.77 ± 2.34[ab]	22.56 ± 1.99[abc]	26.66 ± 2.58[abcd]
CEA	12.75 ± 3.52	5.52 ± 1.66[a]	8.78 ± 2.11[ab]	4.25 ± 0.81[abc]	6.99 ± 1.41[acd]
CA-15.3	52.29 ± 10.58	26.78 ± 5.99[a]	36.65 ± 7.16.11[ab]	19.99 ± 5.46.63[abc]	29.98 ± 6.56[abcd]

[abcd]Mean ± SD in the same column with distinct superscripts differ at *p*-values of $p < 0.0001$, $p < 0.01$, and $p < 0.05$.

gene BCL-2 was observed in the cancerous group (G1) and all the provided treatment groups (G2,3,4&5). For P53, Caspase-3, and BAX genes there was a significant upregulation ($p < 0.001$) in all treated groups compared to the cancerous group (+ve control) which exhibited a significant downregulation in the level of previously mentioned genes. The best result of the elevation in the level of expression of (P53, Caspase-3, and BAX) between all groups was observed in the group that was cured with 1/5 LD50 of *Nh* venom loaded on NPs (G4), and by comparing between G2 and G3 that received the crude venom with different LD50 doses, the one that treated with higher dose (1/5 LD_{50}) (G2) showed a significant increasing ($p < 0.001$) in (P53, Caspase-3, and BAX) genes than G3 that cured with lower concentrated dose (1/20 LD50) The anti-apoptotic gene BCL-2 levels were significantly elevated after inoculating of cancer cells in the mammary fat tissue in G1. When comparing the +VE control group (G1) with all treated groups, there was a significant ($p < 0.001$) lowering in the BCL-2 levels, reaching the best result in both groups that were treated with 1/5 LD_{50}*Nh* venom +NP (G4), and group (2) that received 1/5 LD_{50}*Nh* venom, where the previously mentioned groups showed non-significant ($p > 0.5$) difference in BCL-2 level of expression.

Regarding the Inflammatory cytokines (TNF-α and IL-6), In the cancerous group (G1) both TNF-α and IL-6 were significantly increased, and the administration of Nh venom in both (G2 and G3) resulted in substantial reduction ($p < 0.001$) compared to (G1) considering the higher venom dose (1/5) in G2 that exhibiting an even more pronounced effect leading to a significant reduction ($p < 0.001$) in both TNF-α and IL-6 when compared with G3. The combined treatment of (1/20) dose of venom with NPs in G5 resulted in a more effective and significant reduction ($p < 0.001$) in both TNF-α and IL-6 compared to G3 & G2 which received the same dose of crude venom. The higher venom dose (1/5) combined with NPs in G5 exhibited the best substantial reduction ($p < 0.001$) of TNF-α and IL-6 among all treatment groups. Concerning the levels of the cancer biomarkers CEA and CA15.3, both were elevated in the cancerous group (G1). The exposure to Nh venom in both G2&G3 with doses (1/5) and (1/20), respectively, exhibited a significant ($p < 0.001$) reduction in the previously mentioned parameters. Furthermore, the higher venom dose (1/5) in G2 gave significant downregulation ($p < 0.001$) in both levels of CEA & CA15.3 when compared with the lower venom dose (1/20) in G3, Also G2 showed significant downregulation ($p < 0.001$) in the level of CA15.3 when compared with G5 (1/20 VN), but for CEA levels, there was a nonsignificant difference ($p > 0.05$) between G2 and G5. The combination of the higher venom dose (1/5) with NPs in group G4 showed the most authentic and significant suppression ($p < 0.001$) of CEA and CA15.3 levels among all groups. On the other hand,

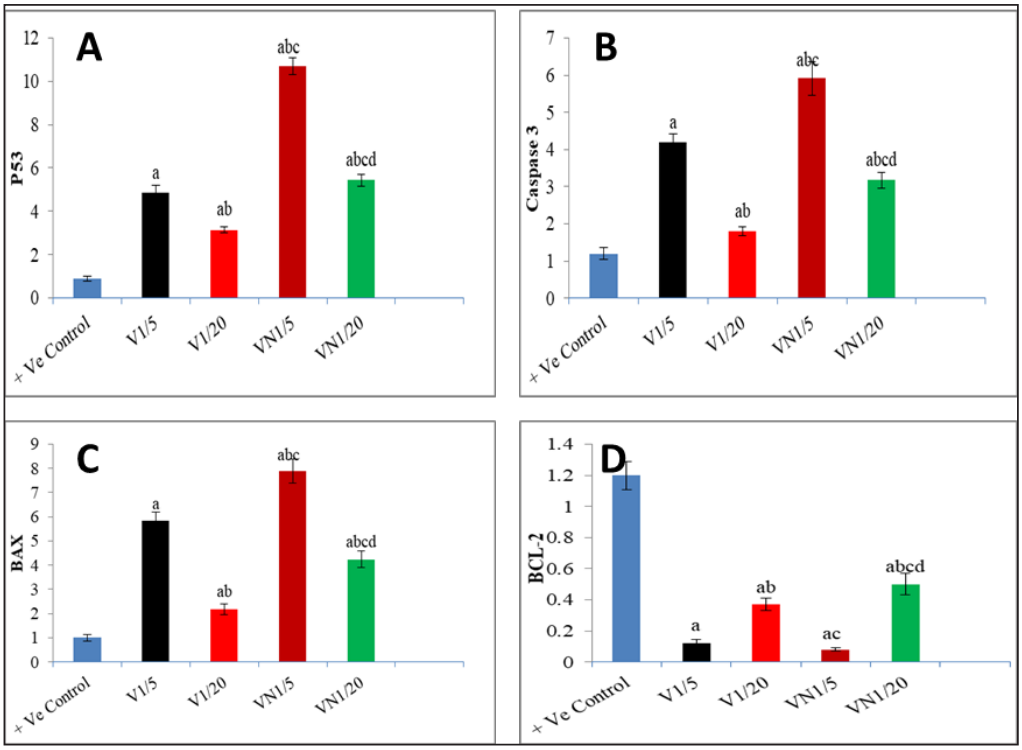

Fig. 1. Levels of genes expression (P53, Caspase3, BAX and BCL-2) in al studied groups, G1 (+Ve control), G2 (1/5 LD_{50}V), G3 (1/20LD_{50}V), G4 (1/5 LD_{50}V + NP) and G5 (1/20 LD_{50}V + NP) where (A) represents (P53), (B) represents (Caspase3), (C) (BAX) and (D) (BCL-2). [a]Significant difference with group 1, [b]Significant difference with group 2, [c]significant difference with group 3, and [d]significant difference with group 4.

G4 which represented the combination of lower venom dose (1/20) with NPs, showed a significant reduction when compared with the same dose (1/20), but for crude venom only in G2.

The levels of CEA, CA15.3, and inflammatory cytokines (IL-6 and TNF-α) were observed in all the groups under study: G1 (+Ve control), G2 (1/5 LD50V), G3 (1/20 LD50V), G4 (1/5 LD50 V + NP), and G5 (1/20 LD50 V + NP), where (A) stands for IL-6, (B) for TNF-α, (C) for CEA, and (D) for (CA15.3). Groups 1 and 2 showed a significant difference, group 3 showed a significant difference, and group 4 showed a significant difference.

Discussion

Worldwide, BC is the most frequent malignant disease that affects women. Despite the fact that BC incidence has increased substantially over the past few decades (Leong *et al*., 2010). TNBC refers to tumors that are devoid of ER, progesterone, or HER2 expression; these molecules are targets for therapeutic interventions (Malorni *et al*., 2012). Additionally, Chemotherapeutic resistance is one of the biggest hurdles to the efficient treatment of cancer. It frequently stops tumor cells from passing through phases of programmed cell death; apoptosis conducting in the survival of cancer cells and delaying the treatment process (Wilson *et al*., 2009). Consequently, focusing on the disrupted apoptotic signaling pathways has become a viable approach to tackle this persistent challenge in the treatment of cancer (Liu *et al*., 2023).

It could potentially be conceivable to steer cancer cells toward self-destruction with therapeutic drugs that have the ability to either activate pro-apoptotic proteins or inhibit anti-apoptotic proteins. This would rescind resistance mechanisms, which frequently thwart the effectiveness of traditional cytotoxic therapies (Fymat, 2017).

Snake venoms are increasingly considered as a potential source of biologically active compounds that hold promise for therapeutic purposes including the treatment of chronic diseases such as cancer (Li *et al*., 2018), Additionally it has been demonstrated that snake venom can induce cytotoxicity in various types of tumor cells (Bazaa *et al*., 2009).

Previous studies have reported the promising anti-cancer activity of snake venoms in vivo as well as it has demonstrated that cobra venom can inhibit the growth of inoculated hepatocellular carcinoma cells in rat models (Sun *et al*., 2003).

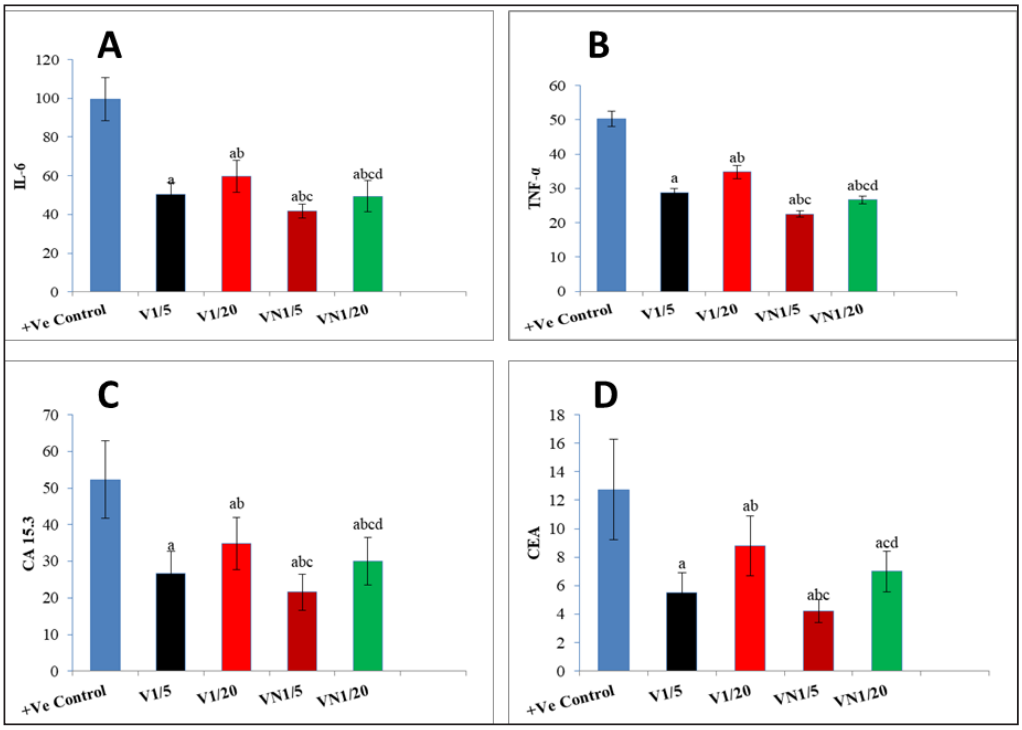

Fig. 2. The levels of CEA, CA15.3, and inflammatory cytokines (IL-6 and TNF-α) were observed in all the groups under study.

For more accuracy in the treatment process, a significant amount of cancer-related research has been conducted with the goal of creating a medication that more precisely targets tumor cells and raising drug concentration in cancer cells, for instance, NP-based drug delivery systems have featured numerous preferences in cancer treatment as rigorous targeting of cancerous cells, improve pharmacokinetic behavior of the drugs and reduction of side effects of drug resistance (Dadwal *et al*., 2018).

In this study, we investigated the potential therapeutic efficacy of the Egyptian cobra, *Naja haje* venom either alone or in combination with silica nanoparticles on BC that was developed into adult female rats. The experimental BC model was established by inoculating the rats with the MDA-MB-231 TNBC cell line.

Tumor markers are molecules that are released by both healthy and malignant cells. However, the levels of these markers are markedly elevated in the presence of cancer activity, reported by (Lohmann *et al*., 2018). Our results obtained from the tumor marker (CEA&CA15.3), following the subcutaneous inoculation of MDA-MB-231 in (+ve control group) that did not receive any treatment, revealed a significant elevation as a result of cancer development, which came in agreement with (Ebeling *et al*., 2002) and (Guo and Gau, 2022), Moreover, Wu *et al*., 2014 demonstrated that the majority of patients with BC suffering from increasing in both CA15.3 and CEA level.

Lee *et al*., 2013 reported that increased tumor load has been shown to be strongly correlated with increasing serum levels of the tumor markers CA15.3 and CEA, the higher the concentrations of these biomarkers, the greater the risk of the cancer spreading and developing systemic metastases, which came in confirmation with our histopathological examination of the breast tissue in the same group where breast ducts were found to be dilated and lined by atypical epithelial cells exhibiting increased mitotic activity and prominent nucleoli. Similarly, Marangoni *et al*., 2007 depended on the inoculation of BC fragments into the fat pad of old female mice, resulting in the presence of necrotic areas in the ductal structure, inflammatory areas, and infiltrating ductal carcinoma, as well as, Angeline Kirubha *et al*., (2012), who depended on 7,12-Dimethyl benz(a)anthracene (DMBA) to evaluate mammary cancer, founded a ductal carcinoma and abnormal epithelial proliferative breast lesions situ plus central necrosis of rat mammary tissue, Along with that, Roy *et al*., (2019) who also demonstrated a atypical hyperplasia, proliferation of ductal epithelial lining in the mammary tissue of the cancerous rat group.

There is a well-established correlation between the induction of cancer development and the chronic inflammation. This connection is primarily mediated through the action of inflammatory cytokines as TNF-α and IL-6 which not only promote the inflammation but also drive the epigenetic alternation in the promoter

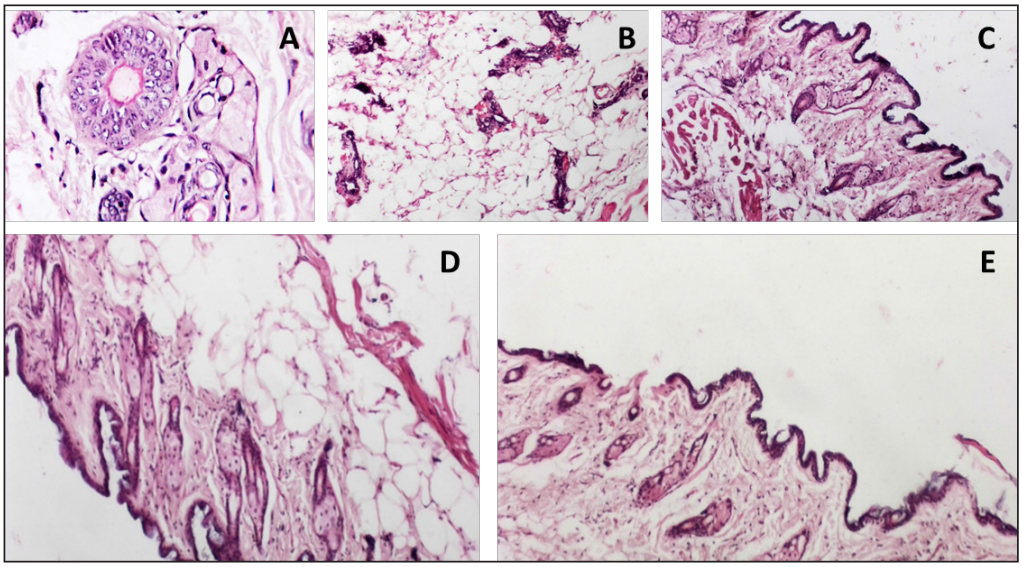

Fig. 3. Histopathological examination of mammary gland tissues in the studied groups. (A) Group (1) the cancerous group showed dilated breast duct lined by atypical epithelial cells with increased mitosis and prominent nucleoli. (B) Group (2) breast cancer group that received (1/5) LD50 of *Nh* venom showed dilated breast duct containing secretory material surrounded by moderate inflammation and fibrosis with obvious decrease of atypical cell lining. (C) Group (3) breast cancer group that received (1/20) LD50 of *Nh* venom revealed dilated breast duct containing secretory material surrounded by mild inflammation and fibrosis with obvious decrease of atypical cell lining. (D) Group (4) (1/5 LD_{50}V) and (E) Group (5) (1/20 LD_{50}V + NP) both showed near normal breast architecture with replacement of the abnormal ducts with adipose tissue.

regions of tumor suppressor genes and cell cycle regulatory genes Hodge *et al*., 2005. Our TNF-α & IL-6 findings exhibited high levels of both cytokines in the inoculated cancerous group which is similar to Al-Hassan *et al*., 2012 who also reported elevated serum IL-6 and TNF-α within newly diagnosed BC patients. Analogous to Gulbahce-Mutlu *et al*., 2021 who announced that the DMBA-induced BC group had the highest IL-6 levels.

(TNF-α) and (IL-6) appear to play an essential role in diverse stages of tumor development, including formation, invasion, and metastasis. This is primarily assignable to their ability to activate a range of oncogenic transcription factors, such as the signal transducer and activator of transcription (STAT) proteins STAT1, STAT3, and STAT5, and induce epigenetic alterations in the promoter regions of tumor suppressor proteins and cell cycle regulatory genes causing inactivation of P53 a tumor suppressor gene (Su *et al*., 2019). P53 is involved in the progression of many biological processes such as cell cycle arrest and apoptosis through the transcriptional regulation of the corresponding genes as BAX and BCL-2 genes. BCL-2 is an anti-apoptotic protein that enhances survival via cytochrome c residue binding. Contrarily BAX is a pro-apoptotic protein participant in the release of cytochrome C and inducing the caspase-dependent apoptotic pathway (Um, 2016) and (Renault *et al*., 2017). At the molecular level and in consistency with our inoculated group with the tumorous cell line, the decreased activities of P53 in BC models and liver carcinoma are accompanied by downregulation of both BAX and caspase-3 levels of expression besides elevating in the BCL-2 levels as reported by (Roy *et al*., 2019) and (Changizi *et al*., 2021) which came in similarity with our findings for the previously mentioned genes.

The present study comprehensively evaluated the antineoplastic efficacy of crude *Naja haje (Nh)* venom at two dose levels, 1/5 LD50 (high dose) and 1/20 LD50 (low dose), as well as the effect of Nh venom loaded on nanoparticles delivery systems at the corresponding LD50 doses.

The results demonstrated that both the high-dose (1/5 LD50) and low-dose (1/20 LD50) of the crude *Nh* venom were effective in significantly suppressing the growth of TNBC implanted in the rat model. However, the high-dose (1/5 LD50) crude venom treatment showed a more pronounced anti-tumor activity compared to the low-dose (1/20 LD50) crude venom group. Captivatingly the 1/5 LD50 *Nh*V + NP showed the most potent inhibitory effects on TNBC tumor

development. These findings analogous to the seminal work by (Omran, 2003), which previously demonstrated a remarkable cytotoxic and anticancer properties of high doses of *Najahaje* venom against both breast and prostate cancer cell lines in contrast to the diminished effects shown at lower venom concentrations.

Abe *et al*., 2002 reported that apoptosis induction by venom have been resulted in the inhibition of the tumor cells. Badr *et al*., 2014 revealed that *Walterinnesia aegyptia* venom, both alone and in combination with nanoparticles (NPs) increased the activities of caspase-3, caspase-8, and caspase-9 in human breast and prostate cancer cells. Furthermore, Shebl *et al*., 2012 illustrated the induction of apoptosis after treatment with *Viperalebtina* snake venom by elevating the expression of the pro-apoptotic p53 and BAX genes and in the contrary downregulation of the BCL-2 level of expression. These data are in harmony with a study reporting that crud cobra snake venom helps in suppressing human breast and liver carcinoma progression via increasing the expression of BAX and decreasing the expression of BCL-2 published by El-Sharkawi *et al*., 2015. All the previously mentioned studies came in agreement with El-Ghani and Amr 2020 who observed that levels of Caspase-3 was elevated in the group treated with Egyptian Nh venom similarly El hakim *et al*., 2011, illustrated the induction of mitochondrial apoptosis pathway and over expression of caspase-3 in the groups treated with *Nh* venom. The real-time RT- PCR results in our study demonstrated that the Nh and Nh + NP treatment activated P53, increased BAX and Caspase3 levels of expression, and decreased the BCL-2 gene level of expression which indicated of initiating apoptosis cascade and initiating the healing process in a response to the venom treatment. Consistent with the induction of apoptosis at molecular levels, the levels of tumor markers CEA & CA15.3 inflammatory cytokines TNF-α and IL-6 were noteworthy downregulated in all treatment groups with the preferable outcome in G4 that received 1/5 LD50 *Nh* + NP compared to either the cancerous group or to the other treatment groups. Aslam *et al.*, 2024 found that *Naja oxiana* venom suppress inflammation by attenuating pro-inflammatory cytokines pathway, Cui *et al*., 2014 agreed to this report by demonstrated in a study depended on *Naja naja atra* venom to mend pulmonary fibrosis by downregulation of TNF-α.

Snake venoms is a complex mixture of diverse bioactive constituents, such as growth factors, poisons, enzymes, activators, and inhibitors. Furthermore, the combined between the multifarious components of *Naja haje* (*Nh*) venom probably participate to the observed anti-tumor effects and the mechanistic pathways demonstrated in the present study. This study revealed the unique biological effects of Nh and *Nh* + NP with both high dose and low dose on Triple negative BC cell line inoculated in rat model which may permit these compounds to be utilized in treatments for breast cancer.

Conflict of interest

The authors declare no competing interests.

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Authors' contributions

The study was conceptualized and designed by all authors, with the experiment being supervised by N.A.S. and A.A.S.H.A.M. carried out the study's practical components, conducted data analysis, S.M.A and M.A.A. wrote and revised the manuscript. All authors read and appropriate the manuscript.

Data availability

The raw data are available and can be provided upon reasonable request from the corresponding author.

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