Tropical Journal of Pharmaceutical Research July 2021; 20 (7): 1365-1372
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i7.7

Original Research Article

Costus speciosus J Koenig (Costaceae) exerts anti-proliferative effect on breast cancer cells via induction of cell cycle arrest and inhibition of activity of metalloproteinase-2

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Sent for review: 6 March 2021 *Revised accepted: 18 June 2021*

Abstract

Purpose: *To Investigate the antiproliferative effect of n-hexane-diethyl ether fraction of Costus speciosus (NP) on triple negative breast cancer (MDA-MB-231) cells, and the mechanism involved.*

Methods: *Maceration with methanol (CH_3OH) was used for extraction of Costus speciosus rhizomes. Chromatographic separation was used to obtain the non-polar fraction (NP) via elution with n-hexane:$(C_2H_5)_2O$ at a volume ratio of 9:1. The cytotoxic effect of NP was evaluated against two breast cancer cell lines i.e., triple negative (MDA-MB-231) and positive ER (MCF-7) employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT) assay, and the IC50 values were estimated. Cell cycle was determined with flow cytometry, while the likely mechanism involved in the cytotoxic effect was investigated using comet assay, immunofluorescence, clonogenic and scratch assays, zymography and detection of the antioxidant markers.*

Results: *NP produced potent cytotoxicity against MDA-MB-231, with IC_{50} value of 4 ± 0.03 µg/mL, whereas its IC_{50} for MCF-7 was 27 ± 1.3 µg/mL. It induced apoptosis via cell cycle arrest at G1 phase. Moreover, NP markedly decreased levels of superoxide dismutase (SOD), reduced glutathione (GSH), and matrix metalloprotease-2 (MMP-2), in MDA-MB-231 cells. Moreover, it inhibited cancer cell migration and colony formation.*

Conclusion: *Non-polar fraction of Costus speciosus (NP) exerted cytotoxic effect on triple negative breast cancer cells (MDA-MB-231) and positive ER (MCF-7). It inhibited cancer cell migration and colony formation. Interestingly, NP arrested the breast cancer cell cycles at sub-G1 phase, inhibited SOD and MMP-2, and decreased GSH levels. It induced apoptosis via DNA damage, downregulation of mutant p53, and over-expressions of the cell cycle inhibitors p21 and p27.*

Keywords: *Medicinal plants, Costus speciosus, anti-proliferation, MMP-**2***

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Cancer refers to uncontrollable growth of cells, and it affects various organs and tissues in humans. different organs of the human body. The World Health Organization (WHO) has stated that cancer is a leading cause of human mortality all over the world [1]. Indeed, cancer is the second leading cause of death, after heart diseases. Cancer was estimated as the second leading cause of death after heart diseases. In 2018, around 10 million people died from cancers [2]. Colorectal, prostate, lung, liver, and stomach cancer are the highest recorded cancers in men, while breast cancer ranks top in women, followed by colorectal, lung, cervical, and thyroid cancers. *Costus speciosus* (*C. speciosus*) is grown in Southern Asia, particularly India, Sri Lanka, Indonesia and Malaysia. It produces several therapeutic effects against a wide variety of diseases, due to its content of alkaloids, flavonoids, glycosides, phenols, saponins, sterols and sesquiterpenes [2,3].

The present study was aimed at investigating the potential anti-proliferative effect of non-polar fraction of *C. speciosus* against two breast cancer cells, MCF-7 (ER-positive) and MDA-MB-231 (triple negative). Moreover, the anti-proliferative effect of NP was evaluated against normal cells (Dental Bulb Stem cells, DBSCs) to ascertain its safety on normal cells and selective toxicity in cancer cells. The results showed that NP arrested the cell cycle of MDA-MB-231 (triple negative) at G1 phase. In addition, NP produced potent inhibitory activities against SOD and MMP-2, and also decreased levels of GSH. It induced apoptosis via DNA damage, downregulation of mutant p53, and over-expression of cell cycle inhibitors (p21 and p27).

EXPERIMENTAL

Plant material

The plant material was kindly supplied by Prof. Ayman Elkady (Department of Biological sciences, Faculty of Science, King Abdulaziz University). A voucher specimen (SCs 018-11) was deposited at Faculty of Science herbarium, KAU.

Extraction and column chromatography of *C. speciosus*

The dried rhizome (150 g) was exhaustively extracted with methanol (3 times, using 250 mL for each extraction) for 24 h at 22°C. The viscous yellow extracts were combined and dried under vacuum, resulting in a yield of 18.6 g. The methanol-free extract was directly subjected to column chromatography using silica gel. The fraction eluted with 100 % *n*-hexane (fatty matter) was discarded. The fraction eluted with *n*-hexane:diethyl ether (9:1 v: v) was dried, resulting in a 2.8 g dry weight of a golden-yellow and oily material.

Cancer cells and reagents

The breast cancers [MCF-7 (ER-positive) and MDA-MB-231 (triple negative)] cell lines were obtained from Nawah Scientific, Egypt. Dental Bulb Stem Cells were obtained from the Nile Center for Experimental Research in Mansoura City, Egypt. All cells were cultured in DMEM (12-604F, Lonza Verviers SPRL, Belgium) supplemented with 10 % FBS (S-001B-BR, Life Science Group L, UK) and penicillin and streptomycin (17-602E, Lonza Verviers SPRL, Belgium). The anticancer drug cisplatin was used as positive control.

Determination of antiproliferative effect of NP

The cells were seeded in 96-well plates at a density of 5000 cells/well, and incubated overnight at 37 °C in a 5 % CO_2 atmosphere. Thereafter, the cells were treated with serial dilution of NP (50, 25, 12.5, 6.25, 3.125, or 1.56 µg/mL) for 48 h. Then, cell viability was determined using MTT (5 mg/mL) which measures the activity of mitochondrial succinate dehydrogenase in the viable cells [5]. The cells were incubated for another 4 h, and the formazan crystals formed were solubilized in 10 % SDS/PBS/0.01N HCl. The absorbance of the formazan solution was measured after 14 h at λ_{570} nm and λ_{630} nm in a BioTek plate reader (ELx808, BioTek Instruments, Inc., Winooski, VT, USA). The experiment was performed three times, and the standard deviation (SD) was calculated. Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = A_{treated}/A_{DMSO} \times 100$$

where $A_{treated}$ is absorbance of treated cells, and A_{DMSO} is absorbance of DMSO blank. The IC_{50} was calculated using GraphPad Prism 8 software.

Evaluation of the effect of NP on cell cycle

The cell cycle was evaluated by staining the cells with propidium iodide. Flow cytometry was employed for counting, examining, and sorting of microscopic particles suspended in a stream of fluid. Thus, the heterogeneous mixture of

biological cells (dead and living cells) was sorted using fluorescence method. Finally, flow cytometry was used for quantitative analysis of the cancer cell populations in the different stages of the cell cycle. The MDA-MB-231 cells were treated with NP at a dose equivalent to IC_{50} or ½ IC_{50}, with DMSO as negative control (0.5%). After 48 h of incubation, the cells were washed twice with PBS (17-516F, Lonza Verviers SPRL, Belgium) and treated with trypsin (17-161E, Trypsin-Versene (EDTA), Lonza Verviers SPRL, Belgium). The detached cells were centrifuged at 1000 rpm for 10 min. Thereafter, the cells were fixed with 70 % ice-cold ethanol for 2 h at -20°C. The fixed cells were washed with PBS and centrifuged at 5000 rpm for 15 min. The effect of the treatment on the cell cycle was evaluated by staining the cells with propidium iodide (P1304MP, Invitrogen™) for 15 min in the dark, followed by measurement of fluorescence using Accuri™ C6 Plus flow cytometer.

Determination of apoptosis with annexin V-PE

This assay is used to determine either apoptotic or necrotic effects of treatments on cancer cells. Phycoerythrin Annexin V staining was used to detect loss of membrane integrity which usually accompanies the late stages of cell death. Fluorescence was measured using Accuri C6 Plus flow cytometer. The induction of apoptosis was detected using Annexin-V and Propidium Iodide DXN kit (eBioscience, 51-66121E, Thermo Fisher Scientific™, USA). The MDA-MB-231 cells were treated with either NP or DMSO for 48 h at 37 °C in a 5 % CO_2 incubator. Then, the cells were trypsinized and washed twice with pre-chilled PBS. The cells were re-suspended in a mixture of binding buffer, 5 µL Annexin-V and 5 µL of propidium iodide, and incubated in the dark for 15 min at room temperature. Thereafter, fluorescence was measured using Accuri C6 Plus flow cytometer [6].

Colony formation assay

The wound healing assay is a simple, inexpensive and well-developed method for studying cell migration *in vitro,* and it is suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration. The technique involves creating a scratch in a single monolayer, capturing the images at the beginning and at regular intervals during cell migration, and comparing the images to quantify the degree of migration of the cells. The MDA-MB-231 cells were seeded in 6-well plate at a density of 5 $x10^5$ cells/mL, and incubated overnight at 37 ºC in a 5 % CO_2 incubator. Thereafter, the medium was replaced with fresh medium containing either DMSO or NP. A scratch was generated using a sterile P200 tip, and pictures were taken immediately at zero time, and at 24 h of incubation. Then, the cells were fixed in methanol and stained with 0.5 % crystal violet. After washing the cells with tap water, their images were obtained using Optika B-159 (OPTIKA S.R.L, Italy). The size of the wound was measured with Image J 1.51 software.

Wound healing assay

The wound healing assay is a simple, inexpensive and well-developed method for studying cell migration *in vitro.* It is suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration. The technique involves creating a scratch on a single monolayer of cells, capturing the images at the beginning and at regular intervals during cell migration, and comparing the images to quantify the degree of cell migration. The MDA-MB-231 cells were seeded in 6-well plate at a density of 5 $x10^5$ cells/mL, and incubated overnight at 37ºC in a 5% CO_2 incubator, followed by replacement of the medium with a fresh medium containing either DMSO or NP. A scratch was generated using a sterile P200 tip, and pictures were taken immediately at zero hour, and then at 24 h of incubation. The cells were fixed in methanol and stained with 0.5 % crystal violet, after which the cells were washed with water and photographed with Optika B-159 (OPTIKA S.r.l., Italy). The size of the scratch wound was measured with Image J 1.51 software.

Evaluation of MMP-2 activity after NP treatment

The MDA-MB-231 cells were seeded in 6-well plate and treated with NP in a serum-free medium for 48 h. The medium was collected and stored at -80°C prior to analysis. The activity of MMP-2 was assayed with gelatin zymography, a highly sensitive assay for gelatinolytic enzymatic activity capable of detecting pro- and active forms of MMP-2 and MMP-9. Gelatinase zymography was performed in 10 % SDS-PAGE in the presence of 0.1% gelatin under non-reducing conditions. Each culture medium (20 µL) was mixed with sample buffer (5 µL) and loaded. Samples were not boiled before electrophoresis. Following electrophoresis, the gel was washed twice in 2.5 % Triton X-100 for 30 min at room temperature to remove SDS and renature the enzyme. The enzyme was activated by incubating the gel overnight in 50 mM Tris-HCl containing 10 mM $CaCl_2$ at pH 8.0. On the following day, the undigested gelatin was

measured by staining the gel with freshly prepared 0.5 % Coomassie Blue R250 in 50 % methanol and 10 % glacial acetic acid for 30 min. In order to visualize the digested gelatin (clear white bands) [7], the gel was de-stained. Gelatinase zymograms were scanned using LaserJet Pro MFP at 300 dpi. The intensity of the bands was evaluated using ImageJ Software.

Determination of antioxidant activities

Superoxide dismutase (SOD) activity and level of reduced glutathione (GSH)

The MDA-MB-231 cells were treated as described above and the total protein concentration of each lysate was estimated using Pierce™ BCA Protein Assay Kit. Superoxide dismutase (SOD) activity and GSH level were determined. Superoxide Dismutase (SOD) was assayed using an SOD assay kit according to the manufacturer's instructions. The assay is based on inhibition of reduction of nitroblue tetrazolium salt (NBT) in presence of phenazine methosulphate (PMS) and NADH. The reaction was carried out in a final volume of 500 µL, starting with the addition of PMS. The increase in absorbance was monitored at 560 nm every 60 sec for 5 min. Inhibition (H) was calculated as in Eq 1.

$$H\ (\%) = \{(Ac - At)/Ac\}100 \quad \ldots\ldots\ldots\ldots \quad (1)$$

where Ac and At are the absorbance of control and test samples, respectively. The results were expressed as SOD units/mg of protein in cell lysate [8].

Reduced glutathione (GSH)

The measurement of GSH content was based on its reaction with DTNB to produce a yellow substance, the absorbance of which is directly proportional to GSH concentration. The absorbance of the yellow-colored reaction product was measured at 405 nm. The concentration of GSH was expressed as mg GSH/g protein concentration in cell lysate.

Comet assay

The MDA-MB-231 cells were seeded in 6-well plate and incubated for 24 h, followed by treatment with NP or DMSO, as indicated previously. After 48 h of incubation at 37°C in a 5% CO_2 incubator, the cells were washed twice with PBS. Trypan blue (0.4% w/w) exclusion assay was used to detect viable cells. The viable cells were suspended in 0.5 % low-melting point agarose (LMPA/PBS; Invitrogen™, Cat. No. 16520050). The cells were spread on frosted microscope slides pre-coated with a layer of 1% normal melting agarose NMA/H_2O (Thermo Scientific™ R0491). After gelling, the slides were treated with lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10 % DMSO) for 2 h at 4 ºC. The slides were then placed in an electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min to allow for unwinding of DNA. Electrophoresis was carried out at 24 V and 300 mA for 30 min. The slides were then neutralized thrice with 0.4 M Tris-HCl, pH 7.5, and stained with 20 µg/mL ethidium bromide. These procedures were performed in the dark to prevent additional DNA damage. Images were obtained with Olympus BX43 connected to Olympus PEN Lite E-PL3 camera.

Immunofluorescence assay

The MDA-MB-231 cancer cells were seeded on a coverslip in 6-well plate and incubated overnight. After treatment with NP, the cells were fixed with 4 % paraformaldehyde for 15 min at room temperature, after which nuclear damage was determined by staining the cells in the dark with propidium iodide for 45 min at room temperature. The stained cells were washed twice with PBS before mounting on the glass slip, followed by image acquisition with Olympus BX43 connected to Olympus PEN Lite E-PL3 camera.

Western blot assay

The cells were seeded at a density of 2.5 x 10^5 cells/ml and treated as described above. After 48 h of incubation with NP, the cells were lysed with RIPA buffer containing protease inhibitor cocktail (THERMO SCIENTIFIC™). The protein content of the supernatant obtained after centrifugation at 4°C was determined. The proteins were separated using 15 % SDS-page at 100 V for 45 min and transferred to 0.2 µM nitrocellulose membrane. The membrane was incubated overnight with primary antibodies for P21 (CST #2947S), P27 (CST #2552), P53 (CST #2527S) OR B-ACTIN (CST #4970; loading control). This was followed by incubation at room temperature with anti-rabbit HRP-conjugated secondary antibody (CST # 7074). The blots were developed using WESTERNBRIGHT® ECL (K-12045-D20), and the signals were detected with CHEMIDOC™ imaging system (BIO-RAD Laboratories, INC).

Statistical analysis

Data are presented as mean with SD. Groups were compared with one-way ANOVA. All statistical analyses were performed using GraphPad Prism software, version 8.00 (GraphPad Software, La Jolla, CA). Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Antiproliferative activity

The potential antiproliferative effect of NP was evaluated against two breast cancer cell lines (estrogen receptor-positive MCF-7 and triple negative cells MDA-MB-231) after 48 h of incubation. Interestingly, NP was more effective against MDA-MB-231 triple negative breast cancer cells than ER MCF-7 cells, with IC_{50} values of 4± 0.03 and 27 ± 1.3 µg/mL, respectively (**Error! Reference source not found.** A and **Error! Reference source not found.**). Treatment with NP for 48 h resulted in induction of apoptosis and reduction in cell-cell attachment, as was evident in morphological changes in the treated cells (
Figure **1** B). Moreover, the IC_{50} was found at a concentration of 3.7 ± 0.1 µg/mL which indicated the effectiveness of NP in killing single cancer cells to prevent metastasis.

Table 1: Cytoxoicity of NP against cancer cells

Cancer cell line	IC_{50} (µg/mL)	
	NP [a]	Cisplatin
Triple negative (MDA-MB-231)	4.0±0.03	7.3±1.00
Positive ER (MCF-7)	27.0±1.30	22.9±1.87
Normal Dental Bulb Stem Cells (DBSCs)	>50	1.7 ± 0.05

Treatment with NP (2 or 4 µg/mL) for 48 h perturbed the cell cycle and arrested cells at the G1 phase (71.8 % *vs* 61% in control cells;
Figure **2** A). Induction of cell cycle arrest usually leads to the induction of apoptosis. Indeed, NP treatment of MDA-MB-231 breast cancer cells produced increases in apoptotic cells in a dose-dependent manner (11.3% early apoptotic cells *vs* zero apoptosis in DMSO-treated cells). These results are presented in
Figure **2** B.

The cell cycle is under control of different regulators such as cell cycle inhibitors, cyclin-dependent kinases and cyclins. The cell cycle regulators are key targets in killing of cancer cells [9]. The effect of NP treatment on cell cycle inhibitors p21 and p27 was determined. The cells were treated with NP for 48 h, followed by trypsinization. Changes in the levels of p21 and p27 in the cell lysates were estimated using western blot assay. Treatment of MDA-MB-231 cells with NP induced up-regulations in the protein expressions of the two cell cycle inhibitors (p21 and p27), as shown in Figure 3.

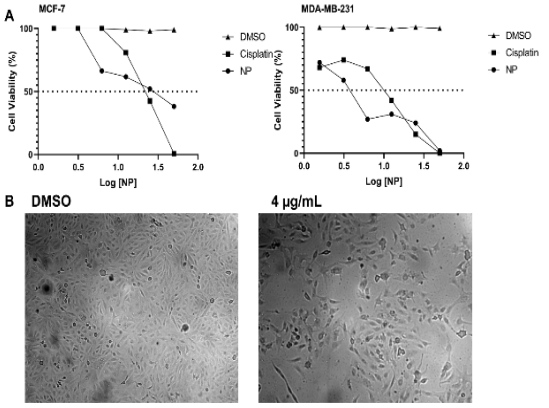

Figure 1: Antiproliferative activity of NP against breast cancer cell lines. (A) The cells were treated with serial dilutions of either DMSO, NP or cisplatin for 48 hours. After that, the viability of cells were estimated by using MTT assay. IC50 is defined as the concentration of tested compound that cause 50% reduction in cell viability. (B) the morphological alterations observed in MDA-MB-231 cells after treatment with 4 µg/mL in comparison to DMSO-treated cells.

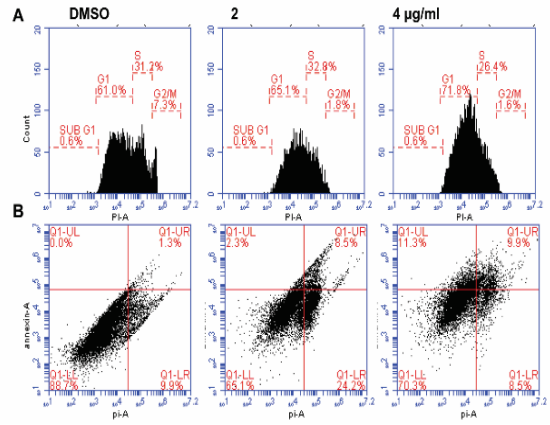

Figure 2: NP treatment led to cell cycle arrest at G1 phase in breast cancer cell line (MDA-MB-231) and induced apoptosis

It has been reported that MDA-MB-231 cells express high levels of mutant p53 (non-functional tumor suppressor) which is responsible for their aggressiveness and resistance to therapy [10]. Moreover, cells that express mutant p53 are characterized by low levels of p21 [11].

Therefore, this study determined changes in the level of mutant p53 upon treatment with NP, using western blot assay. The results revealed that mutant p53 was decreased after NP treatment; this might have contributed to the increase in p21 level (Figure 3). Increases in levels of p21 and p27 delay cell progress and cause apoptosis. Apoptosis is programmed cell death which is controlled by the activation of initiator caspases (caspase-8 and caspase-9) and executioner caspase (caspase-3) [12]. The activation of caspase-3 by proteolytic cleavage causes severe damage to cellular targets, leading to apoptosis. The NP treatment activated caspase-3, relative to control cells (Figure 3).

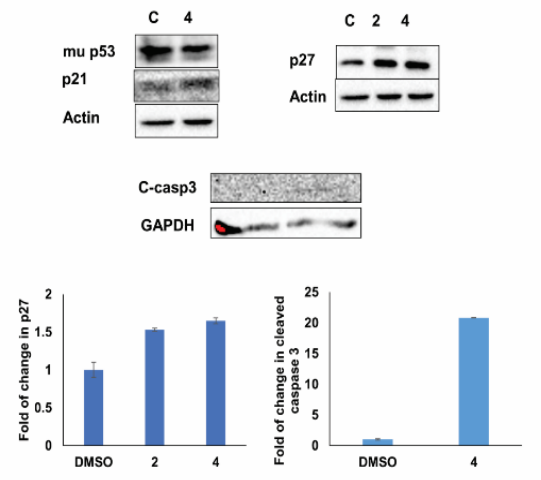

Figure 3: NP treatment increased the level of cell cycle inhibitors p21 and p27 via modulation of mutant p53 level

NP induced apoptosis and DNA damage through decreases in SOD and GSH

Treatment of MDA-MB-231 cells with NP at two different concentrations led to decreases in SOD levels (Figure 4). Decreases in antioxidant levels lead to accumulation of ROS. High level of ROS damage DNA, and damaged DNA can be detected with single-cell gel electrophoresis assay (comet assay). Thus, the level of DNA damage was evaluated after treating MDA-MB-231 cells with NP. Figure 5 A shows that NP caused DNA damage and induced migration of DNA breaks towards the anode, thereby forming comet-like tail. In addition, NP treatment led to nuclear fragmentation which eventually led to apoptotic effect (Figure 5 B). Thus, NP may be a natural product that can effectively induce apoptosis in triple-negative breast cancer cells.

Inhibition of cell migration and colony formation *via* inhibition of regulation of MMP-2

At a dose of 4 µg/mL, NP inhibited wound healing after 24 h of incubation, when compared to DMSO-treated cells (Figure 6 A). Furthermore, the ability of NP to inhibit metastasis was investigated with clonogenic assay. The single cells of MDA-MB-231 did not form colonies efficiently, when compared with the control (Figure 6 B). Based on that, the effect of NP treatment on MMP-2 activity was determined with zymography assay. As shown in Figure 4 C, treatment of MDA-MB-231 cells with NP led to decreased gelatinase activity of MMP-2, as depicted in white arrow.

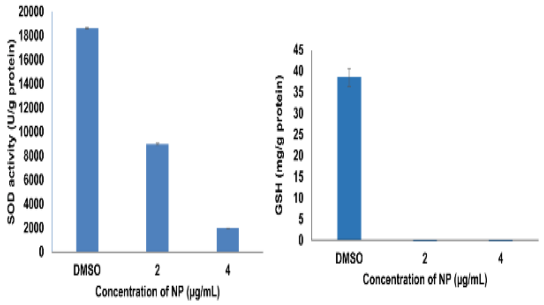

Figure 4: NP treatment decreased the level of antioxidant markers including superoxide dismutase (SOD) and reduced glutathione (GSH).

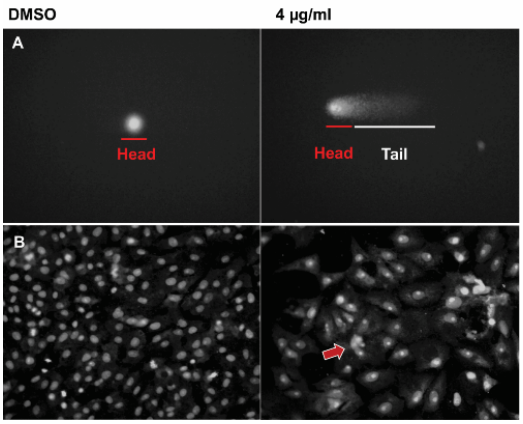

Figure 5: NP treatment caused (A) DNA damage and (B) nuclear fragmentation

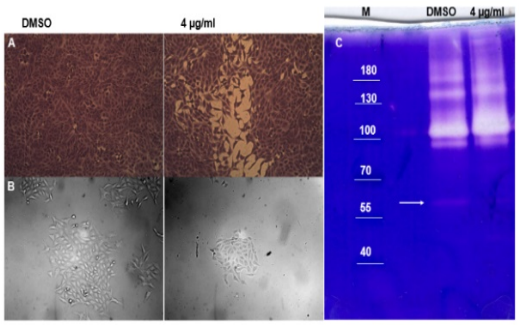

Figure 6: NP inhibited wound healing, colony formation and decreased the activity of MMP2.

DISCUSSION

The specificity of NP for cancer cells was evaluated by testing its toxicity against isolated normal dental pulp stem cells. The NP treatment did not produce toxicity at the tested concentration of 50 µg/mL. Triple negative breast cancer is very aggressive due to lack of targeted therapies. The main chemotherapeutic option involves the use of platinum-based compounds [13]. Therefore, this study was an attempt to investigate the anticancer activity of NP against MDA-MB-231 cells in order to find a new drug lead for fighting against this aggressive type of female malignancy.

The cell cycle is under control of different regulators, among which are cell cycle inhibitors, cyclin-dependent kinases and cyclins. The cell cycle regulators are key targets, which are exploited for killing cancer cells [9]. NP treatment on cell cycle inhibitors p21 and p27 led to cell cycle arrest and induction of apoptosis. In this study, the effect of NP treatment on cell cycle inhibitors p21 and p27 led to cell cycle arrest and induction of apoptosis. It has been reported that MDA-MB-231 cells express high levels of mutant p53 (non-functional tumor suppressor) which is responsible for aggressiveness and resistance to chemotherapy [10].

Studies have shown that cells which express mutant p53 also express low levels of p21 [11]. The results of this study revealed that mutant p53 was decreased after NP treatment. This might have contributed to the increase in p21 level (Figure 3). Increases in levels of p21 and p27 delay cell progress and induce apoptosis. Apoptosis is programmed cell death which is controlled by the activation of initiator caspases (caspase-8 and caspase-9) and executioner caspase-3 [12]. The activation of caspase-3 by proteolytic cleavage causes severe damage to cellular targets, thereby leading to apoptosis. The NP treatment activated caspase-3, relative to the control cells. The ability of cancer cells to degrade the extracellular matrix so as to dissociate from each other and migrate to distant organs, is controlled in part by levels of active metalloproteases (MMPs) [14].

The activity of MMP-2 was decreased in response to NP treatment, leading to reduction in the ability of cells to migrate or form colonies (Figure **6**6C, arrow). The MMP-2 gene is a target for different cellular signaling pathways such as signal transducers and activators of transcription (STAT) pathway, wild-type p53 and nuclear factor kappa B (NFκB) [15,16].

Cancer cells are characterized by high levels of reactive oxygen species (ROS) and high level of antioxidant markers (SOD and GSH) which enable them to overcome the oxidative stress [17]. Therefore, disturbances in the antioxidant profiles are considered valuable strategies for killing cancer cells. High level of ROS causes oxidative damage to DNA, proteins and lipids resulting in apoptosis. The results obtained are in agreement with the previously reported prooxidant activity of costunolide [18].

CONCLUSION

The non-polar fraction of *Costus speciosus* (NP) exhibits cytotoxic effect on triple negative breast cancer (MDA-MB-231) and positive ER (MCF-7). Moreover, NP exerts potent inhibitory effects on SOD, GSH and MMP-2 in MDA-MB-231 cells, and inhibits cancer cell migration and colony formation. Thus, it has potentials for development as a therapeutic agent for the management of triple-negative breast cancer.

DECLARATIONS

Acknowledgement

This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah under grant number G: 766-247-1441. The authors, therefore, acknowledge with thanks DSR for the technical and financial support. The author would like to thank the Center of Excellence for Cancer Research and Genomic, Urology and Nephrology Center at Mansoura University for providing access for ChemiDoc imaging system.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Walied Alarif and Ahmed Abdel-Lateff conceived and designed the study. Fardous El-Senduny and Nahed Bawakid performed the experiments and wrote the manuscript. All authors reviewed and edited the manuscript. All authors read and approved the manuscript.

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