

# Effects of gossypol on apoptosis-related gene expression in racially distinct triple-negative breast cancer cells

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**Abstract.** Apoptosis is a gene-directed mechanism that regulates cell proliferation and maintains homeostasis. Moreover, an aberrant apoptotic process can lead to several pathological conditions, such as tumorigenesis and cancer metastasis. In the present study, the apoptotic effect of the natural polyphenol compound gossypol (GOSS) was investigated in triple-negative breast cancer (TNBC) cells. The effect of GOSS was evaluated in two cell lines representative of a Caucasian-American and African-American origin, MDA-MB-231 (MM-231) and MDA-MB-468 (MM-468), respectively. A similar response to both cytotoxicity and proliferation was observed in the two cell lines. However, MM-468 cells were 2-fold more sensitive to the apoptotic effect of the compound, which was accompanied by a longer delay in colony formation. Furthermore, GOSS was found to alter the mRNA expression of many apoptosis-related genes. The compound significantly upregulated growth arrest and DNA damage-inducible 45 alpha protein (*GADD45A*), tumor necrosis factor receptor superfamily 9 (*TNFRSF9*) and BCL2 interacting protein 3 (*BNIP3*) in MM-231 cells. Similarly, *GADD45A* and *BNIP3* were upregulated in MM-468 cells. A significant finding in this study is the profound 159-fold increase in *TNF* gene expression that was observed in MM-468 cells. Moreover, the apoptosis-suppressor gene baculoviral IAP repeat containing 5 (*BIRC5*) was significantly repressed (by more than 90%) in both cell lines, as well as death-associated protein kinase 1 (*DAPK1*) in MM-231 cells and tumor protein 73 (*TP73*) in MM-468 cells. In conclusion, the data obtained in this study provide a molecular understanding of the GOSS-induced apoptosis effect and suggest the importance of this polyphenol compound targeted towards TNBC treatment, particularly in African-American women.

## Introduction

In normal cells, apoptosis is a genetically controlled process involving several pathways regulating development and maintaining homeostasis (1,2). Defects in apoptotic pathways can lead to pathological conditions such as malignancy, metastasis and chemotherapy resistance (3). Apoptosis is mediated by two basic pathways: Extrinsic (death receptor-mediated) and intrinsic (mitochondrial or Bcl-2-regulated). Initiation of the extrinsic apoptotic pathway requires the interaction of ligands such as tumor necrosis factor  $\alpha$  (*TNF- $\alpha$* ) and fatty acid synthase (FAS) with their transmembrane receptors (4,5). In particular, the intrinsic pathway maintains the balance between antiapoptotic and proapoptotic proteins (6). To resist apoptosis, cancer cells either upregulate the expression of antiapoptotic proteins such as Bcl-2 and Bcl-xL or downregulate the expression of proapoptotic proteins such as Bax and Bak, and both proteins are regulated by the p53 tumor-suppressor gene (7). Moreover, in an aggressive malignant phenotype, the overexpressed Bcl-2 protein prevents the release of cytochrome *c* from the mitochondrial membrane, which leads to interruption of the intrinsic apoptotic signaling pathway and prevents apoptotic cell death (8). Similarly, in many types of cancer, the overexpression of inhibitor of apoptosis (IAP) family members is a challenge in chemoresistance (9) and is considered a therapeutic target in apoptosis-inducing strategies (10).

Breast cancer (BC) is the most commonly diagnosed cancer and the second leading cause of death among women in the United States (11). BC is commonly classified according to the gene expression profile, and the triple-negative breast cancer (TNBC) subgroup is the most aggressive and metastatic, representing approximately 10-15% of all BC cases (12). TNBC is known to be more common among African-American (AA) patients than Caucasian American (CA) patients (2). Indeed, TNBC treatment options are limited because of the absence of the three characteristic receptors: Estrogen (ER), progesterone (PR) and human epidermal growth factor (Her2/neu) (13,14). Although TNBC has initial higher response rates to a variety of chemotherapy agents (15), approximately 30% of patients present with a poor prognosis, and treatment failure leads to a median survival of 1 year (16).

Many studies have demonstrated the medicinal importance of the polyphenol compound gossypol (GOSS), a minor

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constituent of cotton (*Gossypium hirsutum* L.) seeds (17-19). GOSS has been used in China as a male contraceptive, as well as for treating malaria and viral infections (20,21). GOSS has been suggested to be a potent anticancer agent against BC (22). Indeed, the antiproliferative and anti-metastatic effects of GOSS have been demonstrated in several human cancers, including leukemia (23), glioma (24), colon (25), prostate (26), adrenal (27) and breast cancer (28-30). The antiproliferative effect of GOSS is mediated through the induction of cellular apoptosis (31). Furthermore, the apoptotic effect of the compound was detected in different human cells, including multiple myeloma (32,33), synovial sarcoma (34) pharynx, tongue and salivary gland (35), prostate (36-38), colon (39), ovarian (40,41) gastric (42), leukemia (43,44) and pituitary (45), in addition to breast (31,46). In cancer therapy, the combination of multiple agents is key to overcoming the resistance mechanisms of the tumor (47), and GOSS has been found to induce an apoptotic effect in various human cancer cells in combination with low doses of taxanes (46), doxorubicin (34), dexamethasone (43) and valproic acid (36).

Therefore, the present study was designed to examine the effect of the natural compound GOSS on two human TNBC cell lines, MDA-MB-231 (MM-231) and MDA-MB-468 (MM-468), representing the CA and AA races, respectively (48). In the present study, we investigated the effect of GOSS on cell viability, proliferation and colony formation. We hypothesized that GOSS alters the expression of different apoptosis-related genes that mediate the antiproliferative effect of GOSS. The present study enhanced our understanding of events associated with cell death following GOSS treatment.

## Materials and methods

**Materials and reagents.** GOSS (purity  $\geq 90\%$ ), doxorubicin (purity  $\geq 99\%$ ), and cell culture flasks were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Trypsin-EDTA solution and Alamar Blue<sup>®</sup> (a solution of resazurin fluorescence dye) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), penicillin/streptomycin and Dulbecco's phosphate-buffered saline (DPBS) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated fetal bovine serum (FBS), and cell culture plates were purchased from VWR International (Radnor, PA, USA). An Annexin V-FITC Apoptosis Detection Kit Plus (cat. no. 68FT-Ann VP-S) was purchased from RayBiotech (Norcross, GA, USA). A DNA-free<sup>™</sup> kit (cat. no. AM1907) was purchased from Life Technologies, Inc. (Thermo Fisher Scientific, Inc., Waltham, MA, USA). An iScript<sup>™</sup> cDNA Synthesis kit (cat. no. 170-8890), SsoAdvanced<sup>™</sup> Universal SYBR<sup>®</sup> Green Supermix and the Human Apoptosis PCR array (SAB Target List) H96 were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

**Cell culture.** Two TNBC cell models, MM-231 and MM-468, were purchased from the American Type Culture Collection (ATCC). Both cell lines were grown in 75-ml tissue culture (TC) flasks at 37°C in a humidified 5% CO<sub>2</sub> incubator and subcultured as needed with trypsin/EDTA (0.25%). The DMEM contained 4 mM L-glutamine and was supplemented

with 10% heat-inactivated FBS (v/v) and 1% penicillin/streptomycin salt solution (100 U/ml and 0.1 mg/ml, respectively). The DMEM was supplemented with 2.5% heat-inactivated FBS.

**Cell viability assay.** In this study, cells were plated at a density of  $5 \times 10^4$  cells/well in 96-well plates and incubated overnight at 37°C. GOSS was solubilized in dimethyl sulfoxide (DMSO), and both types of cells were treated for 24 h with the compound (concentration ranges of 0-100  $\mu$ M in MM-231 cells and 0-50  $\mu$ M in MM-468 cells). Control wells were treated with DMSO at the highest concentration used ( $<0.1\%$ ), and wells treated in the same manner but without cells were used as blanks. In this study, Alamar Blue<sup>®</sup> was used to determine the cell viability as previously described (49). The fluorescent fuchsia-reduced resazurin dye was measured at an excitation/emission wavelength of 530/590 nm using a Synergy HTX Multi-Mode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Cell proliferation and clonogenic assays.** The inhibition of cell proliferation by GOSS was determined for MM-231 and MM-468 TNBC cells based on the dose-response viability study concentrations (50) using Alamar Blue<sup>®</sup>. Briefly, cells were plated at an initial density of  $1 \times 10^4$  cells/well in 96-well plates and incubated overnight at 37°C. The cells were treated for 96 h with GOSS at concentrations ranging from 0 to 100  $\mu$ M in MM-231 cells and from 0 to 50  $\mu$ M in MM-468 cells in a final volume of 200  $\mu$ l/well. Control cells were exposed to DMSO at a concentration of  $<0.1\%$ , and equivalent wells without cells were used as a blank. Doxorubicin was used as a positive control at concentrations ranging from 0 to 10  $\mu$ M in both cell lines. Proliferation was measured at different intervals up to 96 h for GOSS-treated cells and at 72 h for doxorubicin-treated cells (51) by adding Alamar Blue<sup>®</sup> as previously described (49). The plates were read at an excitation/emission wavelength of 530/590 nm.

A clonogenic assay was performed to measure the long-term effect of GOSS on MM-231 and MM-468 TNBC cells. Both cell lines were seeded and treated similarly to the proliferation study described above. After either a 1- or a 6-h exposure period, the GOSS-containing experimental media were replaced by growth media after washing the cells with DPBS. The cells were allowed to grow for 7 days, and the colonies formed in both treated and untreated cells were then evaluated based on the reduction of Alamar Blue<sup>®</sup> as previously described in the *Cell viability assay*.

**Apoptosis assay.** The apoptotic effect of GOSS was determined in MM-231 and MM-468 cells. Briefly, each cell line was seeded at an initial concentration of  $5 \times 10^5$  cells/well in 6-well plates and incubated overnight at 37°C. To induce apoptosis, cells were treated for 24 h with GOSS at concentrations ranging from 0 to 100 and from 0 to 50  $\mu$ M in MM-231 and MM-468 cells, respectively, in a final volume of 3 ml/well of experimental media. Control cells were exposed to DMSO at a concentration of  $<0.1\%$ . After the 24-h incubation period, treated and control cells from each well were harvested, pelleted and washed in phosphate-buffered saline (PBS). According to the manufacturer's protocol, the cell pellets were resuspended in 500  $\mu$ l of the provided IX Annexin V binding buffer and labeled with 5  $\mu$ l each of Annexin V-FITC and propidium iodide (PI). The apoptotic

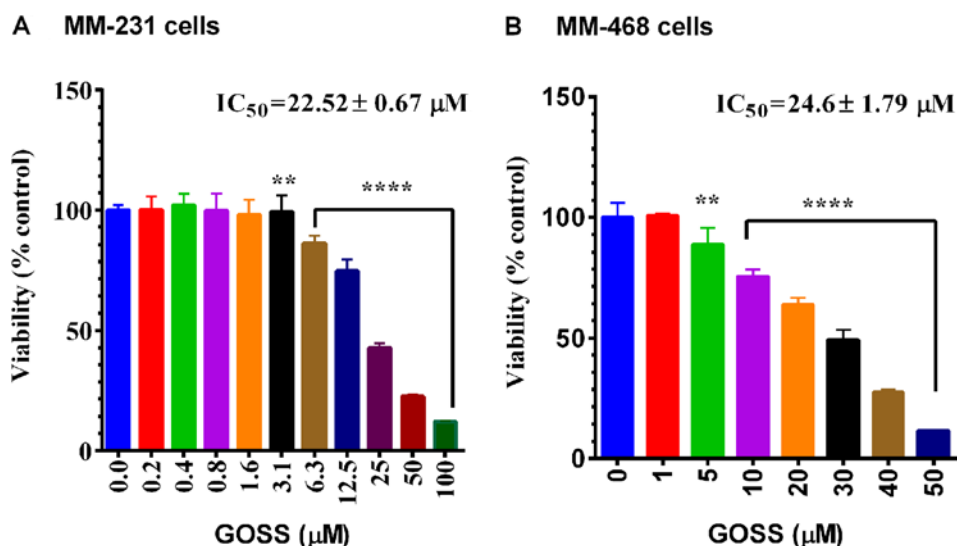


Figure 1. Effect of GOSS on the viability of MM-231 and MM-468 TNBC cell lines. Cells were treated for 24 h with GOSS at concentration ranges of 0-100  $\mu M$  (A) and 0-50  $\mu M$  (B) in MM-231 and MM-468 cells, respectively. The graph shows the viability data expressed as percentages of cell survival compared to the control. The data points represent the mean  $\pm$  SEM of three independent studies, n=4 each. The significance of the difference between the control and treated groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. The difference was considered significant at \*\* $P < 0.01$  and \*\*\*\* $P < 0.0001$ . GOSS, gossypol; MM-231, MDA-MB-231; MM-468, MDA-MB-468; TNBC, triple-negative breast cancer;  $IC_{50}$ , half maximal inhibitory concentration.

effect was quantified within 5-10 min on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For each sample,  $1 \times 10^4$  cells were examined and CellQuest software (BD Biosciences) was used for acquisition and data analysis.

**Reverse transcription-polymerase chain reaction (RT-PCR) apoptosis array.** This experiment was performed based on the data of the apoptosis assay using the concentrations that did not cause a considerable necrosis effect in either cell line: 25  $\mu M$  in MM-231 cells and 20  $\mu M$  in MM-468 cells. Briefly, two T-75 flasks of  $10 \times 10^6$  cells (control and treated for each cell line) were incubated overnight at 37°C and for an additional 24-h in the absence or presence of the previously indicated concentrations of the test compound. The cells from each sample were pelleted and mixed with 1 ml of TRIzol reagent to isolate total RNA. Subsequently, 0.2 ml of chloroform was added to each sample, vortexed, incubated at room temperature (RT) for 2-3 min and centrifuged for 15 min at 10,000  $\times g$  and at 2-8°C. The aqueous phase was collected and mixed with 0.5 ml of isopropyl alcohol to precipitate the RNAs. The RNA pellets were then dissolved in ~30-50  $\mu l$  of Nuclease-free water to measure RNA quantity and quality in each sample using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). Finally, cDNA representing the control or treated cells was synthesized using the iScript™ cDNA Synthesis kit according to the manufacturer's protocol. The obtained cDNA was reconstituted in Nuclease-free water, and the 96-well apoptosis array was loaded with 10  $\mu l$  each of the reconstituted cDNA (2.3 ng) and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) and then placed for 5 min in a shaker and centrifuged at 1,000  $\times g$  for 1 min. The PCR run was established with 39 cycles of denaturation as follows: 30-sec activation at 95°C, 10-sec denaturation at 95°C; 20-sec annealing at 60°C; and 31-sec extension at 65°C using a Bio-Rad CFX96

Real-Time System (Bio-Rad Laboratories). All real-time PCRs were performed in triplicate for each cell line. Gene expression was analyzed using the CFX 3.1 Manager software (Bio-Rad Laboratories) and verified with Student's t-test.

**Statistical analysis.** Data for this study were analyzed using GraphPad Prism 6.2 software (GraphPad Software, Inc., San Diego, CA, USA). All data points were obtained from the average of at least two independent studies and are expressed as the mean  $\pm$  SEM. For the viability assay,  $IC_{50}$  values were determined by non-linear regression with the  $R^2$  best fit and the lowest 95% confidence interval. In both the viability and apoptosis assays, the significance of the difference between each control and its related treatment group was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. For both the proliferation and clonogenic assays, the statistical analysis was performed with two-way analysis of variance (ANOVA) between each exposure period and other(s) followed by Bonferroni's multiple comparison test. Overall, a difference was considered significant at \* $P < 0.05$  and \*\*\*\* $P < 0.0001$  (as indicated in the figures and legends). Gene expression was analyzed using CFX 3.1 Manager software (Bio-Rad Laboratories) and verified with the Student's t-test.

## Results

The anticancer effects of GOSS were examined in both MM-231 and MM-468 cell lines. As indicated in Fig. 1A and B, a highly significant effect ( $P < 0.0001$ ) of GOSS on cell viability was found in MM-231 and MM-468 cells at different concentrations of the compound (6.3-100 and 10-50  $\mu M$  in MM-231 and MM-468, respectively). In both cell lines, the obtained  $IC_{50}$  did not show a significant difference in response to the compound ( $IC_{50} = 22.52 \pm 0.67$  and  $24.6 \pm 1.79 \mu M$  for MM-231 and MM-468 cells, respectively).

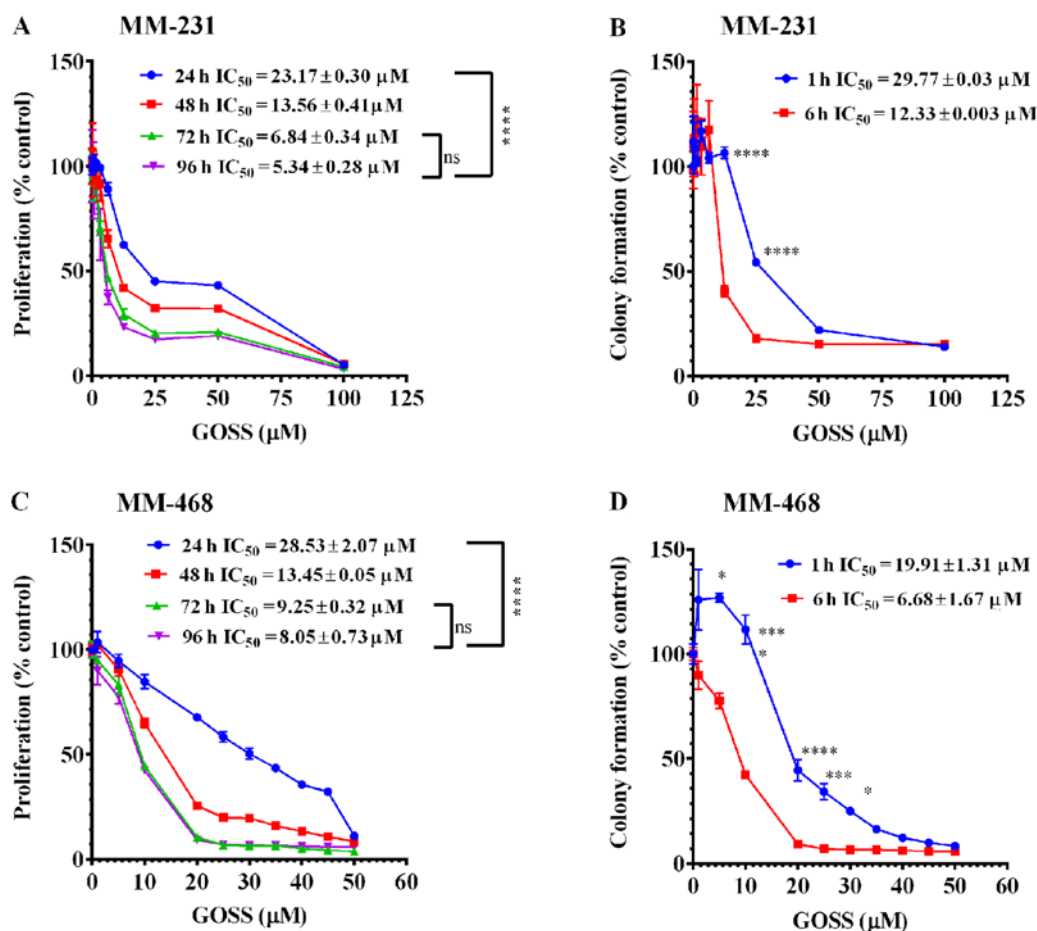


Figure 2. Effect of GOSS on proliferation and colony formation in MM-231 and MM-468 TNBC cell lines. In the cell proliferation study, cells were incubated for 96 h with GOSS at concentration ranges of 0-100  $\mu\text{M}$  (A) and of 0-50  $\mu\text{M}$  (C) in MM-231 and MM-468 cells, respectively. For the colony formation assay, MM-231 (B) and MM-468 (D) cells were exposed to similar concentrations of GOSS for either 1 or 6 h and then washed and allowed to grow in regular cell culture media for 7 days. In both assays, each data point represents the mean  $\pm$  SEM of three independent experiments,  $n=4$  each. The significance of the difference between the different exposure periods was calculated using two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test, and \*\*\*\* $P<0.0001$  indicates a statistically significant difference between 24-h vs. other exposure periods, while a non-significant difference was found between the 72- vs. 96-h exposure periods. Similarly, \*\*\*\* $P<0.0001$ , \*\*\* $P<0.001$  and \* $P<0.05$  indicate a statistically significant difference in the colony formation inhibition between the 1- vs. 6-h exposure periods at different concentrations. GOSS, gossypol; TNBC, triple-negative breast cancer; MM-231, MDA-MB-231; MM-468, MDA-MB-468; IC<sub>50</sub>, half maximal inhibitory concentration; ns, not significant.

To measure the growth inhibitory potency of GOSS in MM-231 and MM-468 cell lines, both antiproliferative and clonogenicity assays were performed. In both cell lines, GOSS inhibited cell proliferation and colony formation in a dose- and time-dependent manner compared to the control cells (Fig. 2). The inhibition of cell proliferation, as indicated by the reduction of resazurin and verified by the decrease in the IC<sub>50</sub> values at the different periods of exposure, is shown in Fig. 2A and C (MM-231 and MM-468 cells, respectively). Indeed, the IC<sub>50</sub> values were reduced significantly ( $P<0.0001$ ), from 23.17 to 5.34  $\mu\text{M}$  in MM-231 cells and from 28.53 to 8.05  $\mu\text{M}$  in MM-468 cells at the 24- vs. 96-h exposure periods. Furthermore, in both cell lines, GOSS significantly inhibited cell proliferation ( $P<0.0001$ ) at the 24-h treatment period vs. other periods, but there was no significant difference in cell proliferation inhibition between the 72- vs. 96-h treatment period.

The data also showed that GOSS significantly inhibited colony formation in both MM-231 and MM-468 cells (Fig. 2B and D). In MM-231 cells, a significant delay in colony formation ( $P<0.0001$ ) was observed between the 1- vs. the 6-h

exposure periods following treatment with 12.5 and 25.0  $\mu\text{M}$  GOSS (Fig. 2B). A significant difference was also observed in its counterpart cell line, MM-468 ( $P<0.05$ - $P<0.0001$ ), throughout the concentration range of 5-30  $\mu\text{M}$ , as shown in Fig. 2D.

Nonetheless, the data indicate that GOSS has similar growth inhibitory potency on both racially distinct cell lines. The colony formation assessment indicated that after a 6-h treatment period, GOSS was ~2-fold more effective in MM-468 cells (IC<sub>50</sub>=6.68 $\pm$ 1.67  $\mu\text{M}$ ) than in MM-231 cells (IC<sub>50</sub>=12.33 $\pm$ 0.003  $\mu\text{M}$ ) (Fig. 2B and D).

On the other hand, the IC<sub>50</sub> value of cells after 72 h of exposure to doxorubicin was 1.69 $\pm$ 0.11 and 0.23 $\pm$ 0.003 in MM-231 and MM-468 cells, respectively, with  $P<0.05$ - $P<0.0001$  (Fig. 3). The apoptotic effect of GOSS on both MM-231 and MM-468 TNBC cells was examined to confirm apoptosis mediation of the antiproliferative effect and colony formation inhibition of GOSS. After 24 h of exposure, a gradual but significant increase in the number of apoptotic cells ( $P<0.0001$ ) was observed in a dose-dependent manner (Fig. 4A and B). Additionally, the results indicated that MM-468 cells were

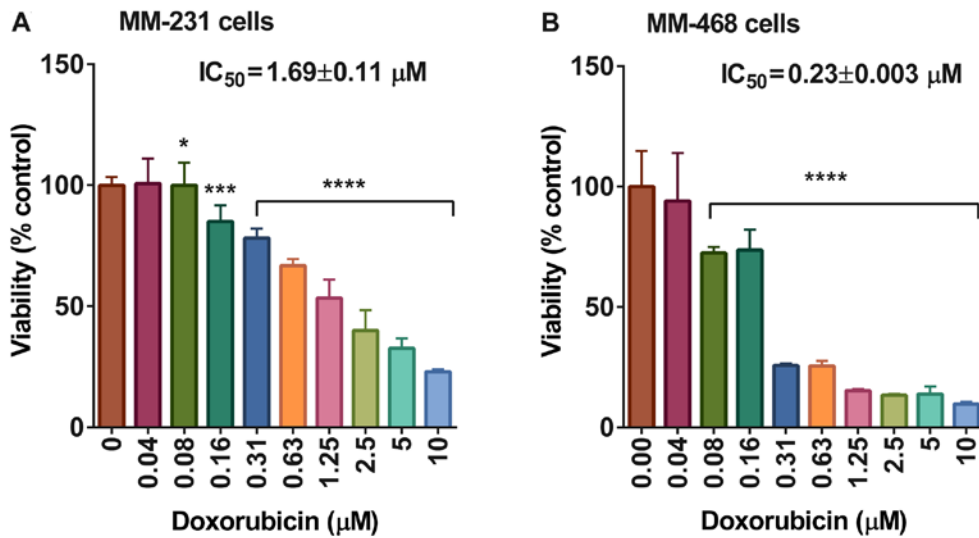


Figure 3. Effect of doxorubicin on proliferation in (A) MM-231 and (B) MM-468 TNBC cell lines. Both cell lines were incubated for 72 h with doxorubicin at concentration ranges of 0-10  $\mu\text{M}$ . Each point represents the mean  $\pm$  SEM of two independent experiments,  $n=4$  each. The graph shows the proliferation rate expressed as percentages compared to the control. The significance of the difference between the control and treated groups was determined using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. The difference was considered significant at \* $P<0.05$ , \*\*\* $P<0.001$  and \*\*\*\* $P<0.0001$ . TNBC, triple-negative breast cancer; MM-231, MDA-MB-231; MM-468, MDA-MB-468;  $\text{IC}_{50}$ , half maximal inhibitory concentration.

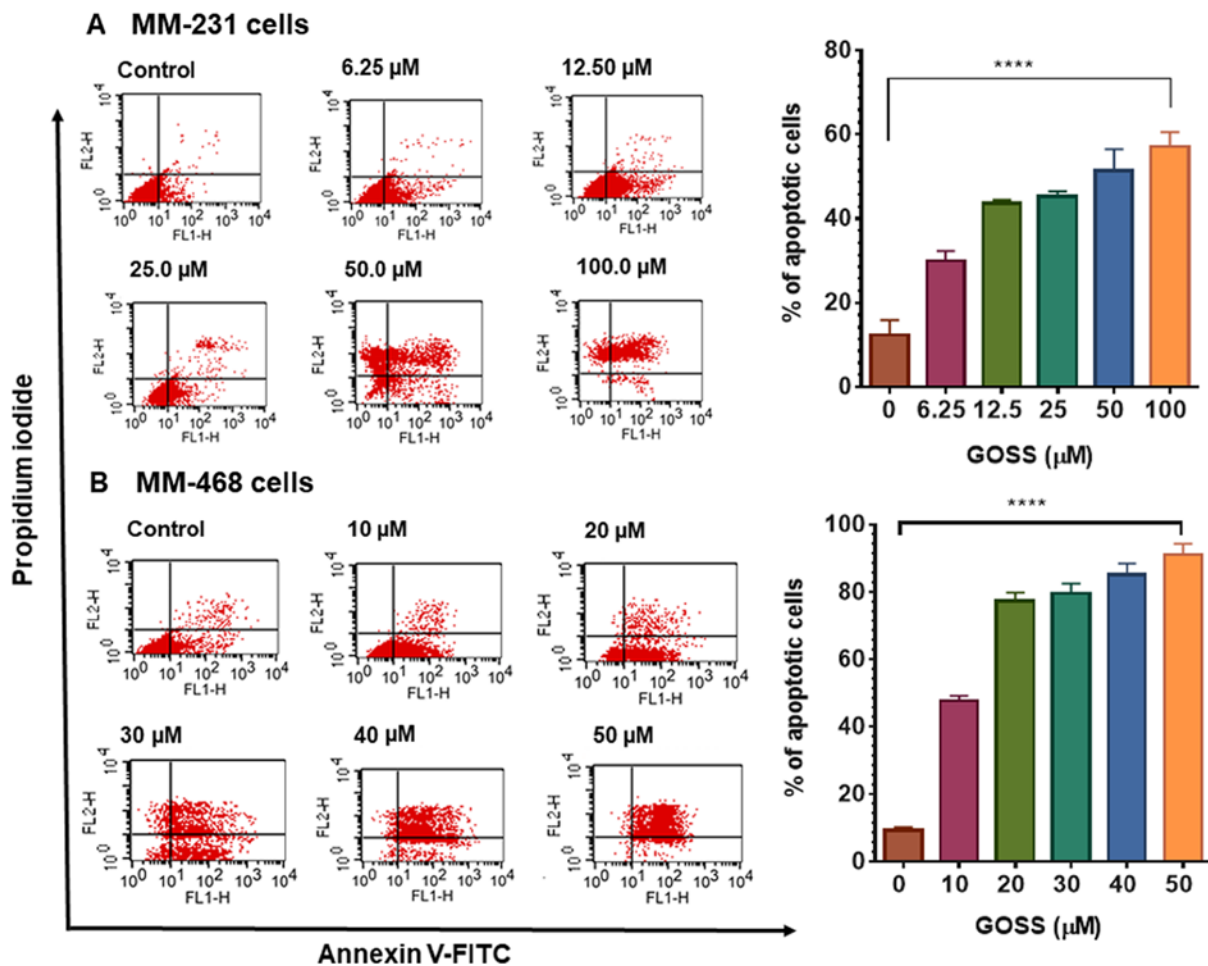


Figure 4. Apoptotic effect of GOSS on (A) MM-231 and (B) MM-468 TNBC cell lines. Cells were exposed to GOSS for 24 h at concentrations ranging from 0 to 100  $\mu\text{M}$  for MM-231 cells and 0-50  $\mu\text{M}$  for MM-468 cells. Control cells were treated with DMSO at the highest exposure dose ( $<0.1\%$ ). An Annexin V-FITC apoptosis kit was used to label treated/control cells, and a FACSCalibur flow cytometer was used to analyze the percentage of apoptotic cells compared to the DMSO-treated control cells. Each data point represents the mean  $\pm$  SEM of two independent studies,  $n=3$  each. The significance of the difference between control vs. each treatment was calculated using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. \*\*\*\* $P<0.0001$  confirmed the statistically significant difference. GOSS, gossypol; TNBC, triple-negative breast cancer; MM-231, MDA-MB-231; MM-468, MDA-MB-468; DMSO, dimethyl sulfoxide.

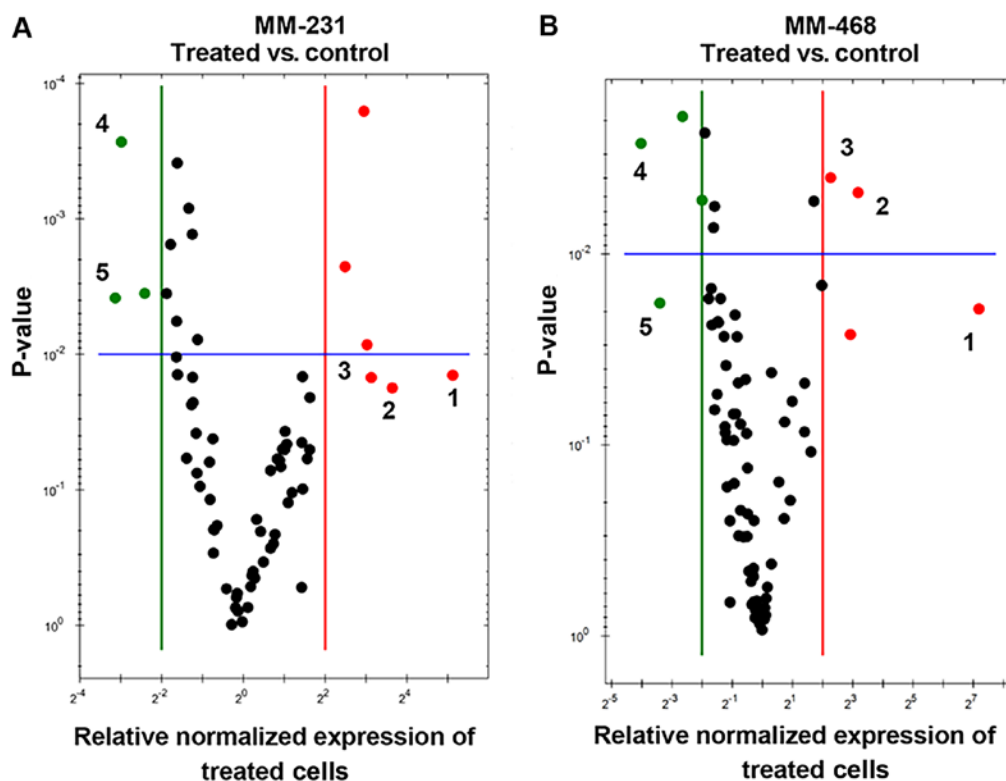


Figure 5. Scatter plots for MM-231 and MM-468 TNBC cells. Cells were exposed to GOSS for 24 h at a concentration of 25  $\mu$ M in MM-231 cells and 20  $\mu$ M in MM-468 cells and normalized the mRNA expression of target genes for the control vs. treated MM-231 (A) and MM-468 (B) cells. Based on the threshold set (green and red lines), the plot images show the following changes in apoptosis-related gene expression: Upregulation, red dots; downregulation, green dots; no change, black dots. In both cell lines and based on the fold changes, the most upregulated genes are enumerated from 1-3, while 4-5 refer to the downregulated genes. In MM-231 cells, *GADD45A*, *TNFRSF9* and *BNIP3* were upregulated, while *BIRC5* and *DAPK1* were downregulated. Similarly, in MM-468 cells, *TNF*, *GADD45A* and *BNIP3* were upregulated, while *BIRC5* and *TP73* were downregulated. GOSS, gossypol; TNBC, triple-negative breast cancer; MM-231, MDA-MB-231; MM-468, MDA-MB-468; *GADD45A*, growth arrest and DNA-damage-inducible 45 alpha protein; *TNFRSF9*, tumor necrosis factor receptor superfamily 9; *BNIP3*, BCL2 interacting protein 3; *BIRC5*, baculoviral IAP repeat containing 5; *DAPK1*, death-associated protein kinase; *TP73*, tumor protein 73.

Table I. mRNA gene expression changes in MM-231 and MM-468 TNBC cells.

Control vs. treated MM-231 cells			Control vs. treated MM-468 cells		
Target gene	Fold changes	P-value	Target gene	Fold changes	P-value
<i>GADD45A</i>	+33.42	0.028	<i>TNF</i>	+158.98	0.023
<i>TNFRSF9</i>	+12.35	0.018	<i>GADD45A</i>	+9.22	0.005
<i>BNIP3</i>	+8.75	0.015	<i>BNIP3</i>	+4.84	0.004
<i>BIRC5</i>	-7.96	0.0003	<i>BIRC5</i>	-24.32	0.002
<i>DAPK1</i>	-14.79	0.0012	<i>TP73</i>	-8.65	0.037

The left portion of the table presents the fold changes in gene expression (+/-) in MM-231 cells following treatment with 25  $\mu$ M GOSS. In contrast, the right portion presents the fold changes in gene expression (+/-) in MM-468 cells following treatment with 20  $\mu$ M GOSS. MM-231, MDA-MB231, MM-468, MDA-MB-468; TNBC, triple-negative breast cancer; GOSS, gossypol; *GADD45A*, growth arrest and DNA-damage-inducible 45 alpha protein; *TNFRSF9*, tumor necrosis factor receptor superfamily member 9; *BNIP3*, BCL2 interacting protein 3; *BIRC5*, baculoviral IAP repeat containing 5; *DAPK1*, death-associated protein kinase; *TNF*, tumor necrosis factor; *TP73*, tumor protein 73. P<0.05 was considered as statistically significant.

more sensitive (almost 2-fold more sensitive) to GOSS than MM-231 cells, and 90% of the MM-468 cells analyzed were in the apoptotic phase following treatment with 50  $\mu$ M GOSS, as shown in Fig. 4B, whereas <60% of MM-231 cells analyzed exhibited apoptotic effects at 100  $\mu$ M (Fig. 4A). The obtained

data show the strong apoptotic effect of GOSS on both TNBC cell lines.

Quantitative real-time PCR (qRT-PCR) was performed in both GOSS-treated cell lines to identify the genes related to GOSS-induced apoptosis. The profiling of normalized

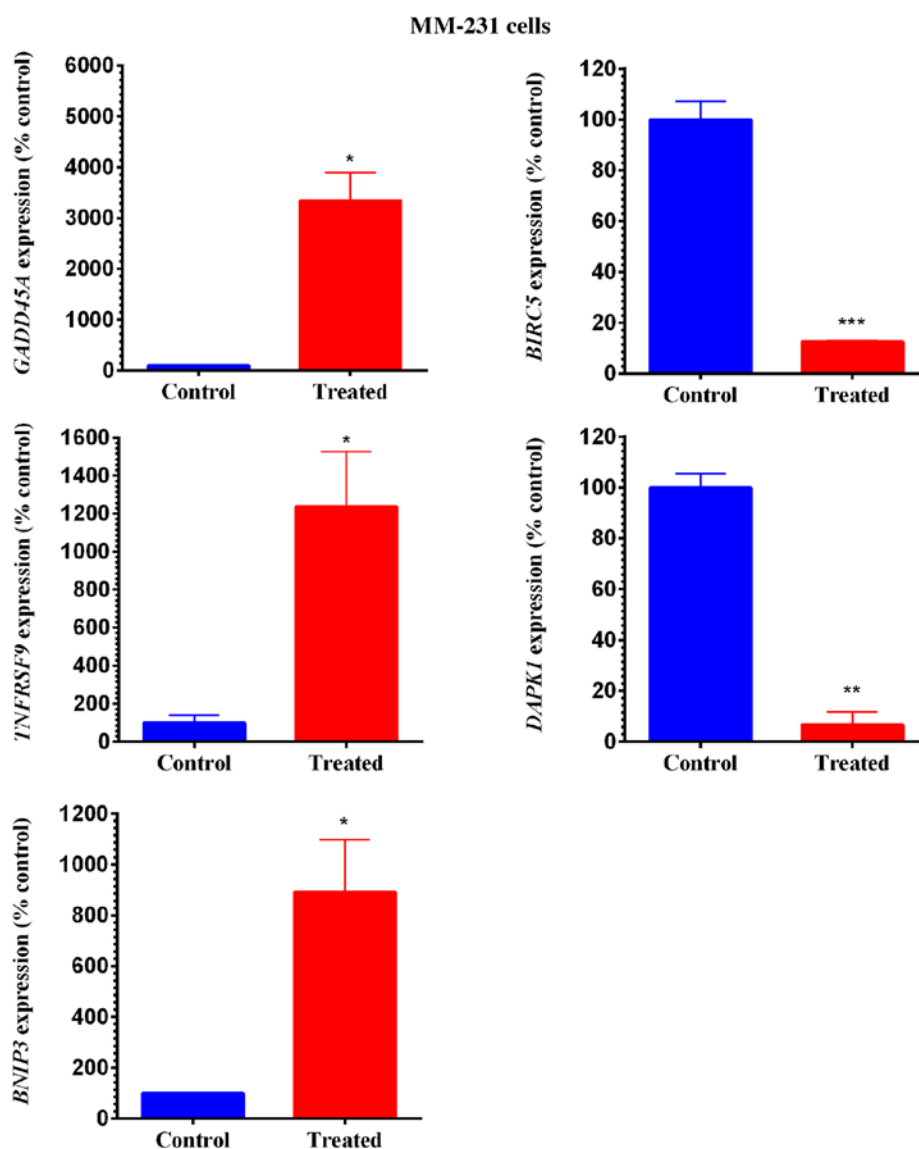


Figure 6. Gene expression quantification in MM-231 cells. Cells were exposed to GOSS for 24 h at a concentration of 25  $\mu$ M. Normalized mRNA data indicate a significant increase in three genes (*GADD45A*, *TNFRSF9* and *BNIP3*) in the GOSS-treated MM-231 cells vs. control cells, while two genes were significantly inhibited (*BIRC5* and *DAPK1*). The data points represent the mean  $\pm$  SEM of three independent studies. The significance of the difference was determined using an unpaired t-test between the resting vs. treated cells. The difference was considered significant at \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$ . GOSS, gossypol; MM-231, MDA-MB-231; *GADD45A*, growth arrest and DNA-damage-inducible 45 alpha protein; *TNFRSF9*, tumor necrosis factor receptor superfamily 9; *BNIP3*, BCL2 interacting protein 3; *BIRC5*, baculoviral IAP repeat containing 5; *DAPK1*, death-associated protein kinase.

mRNA expression in both cell lines provided insights into the influence of the compound on many apoptosis-related genes. In this study, we identified apoptosis-related genes that were significantly upregulated/downregulated by the compound. Overall, in both cell lines, as shown in Fig. 5A and B, the visually recognized red dots represent the upregulated genes, while the green dots represent those that were downregulated by GOSS.

The specifically impacted genes were quantified and are presented in Figs. 6 and 7. In MM-231 cells, GOSS significantly ( $P<0.05$ ) increased the expression of three genes (*GADD45A*, *TNFRSF9* and *BNIP3*) (Fig. 6). Similarly, in MM-468 cells, *GADD45A* and *BNIP3* were upregulated by GOSS treatment ( $P<0.05$ - $P<0.01$ ) (Fig. 7). Nevertheless, the *GADD45A* gene was significantly upregulated in both cell lines; the fold-increase in MM-231 cells was 33.42

and that in MM-468 cells was 9.22 (Table I). Moreover, the measured upregulation of *BNIP3* in MM-231 cells was approximately twice that of its counterpart cell line, MM-468 (Figs. 6 and 7 and Table I). Two members of the *TNF*/receptor family of genes were also increased. *TNF* was profoundly overexpressed by 159-fold in MM-468 cells, and an  $\sim$ 12-fold increase in the *TNF* receptor *TNFRSF9* was found in MM-231 cells. In contrast, GOSS significantly repressed the mRNA expression of many apoptosis-related genes ( $P<0.05$ - $P<0.001$ ). More than 90% inhibition ( $\geq$ 8-fold decrease) in *BIRC5* was observed in both cell lines, in addition to a 14.79- decrease and an 8.65-fold decrease in *DAPK1* and *TP73* in MM-231 and MM-468 cells, respectively (Table I). Notably, the compound GOSS did not show a significant effect on the expression of the different caspases (data not shown).

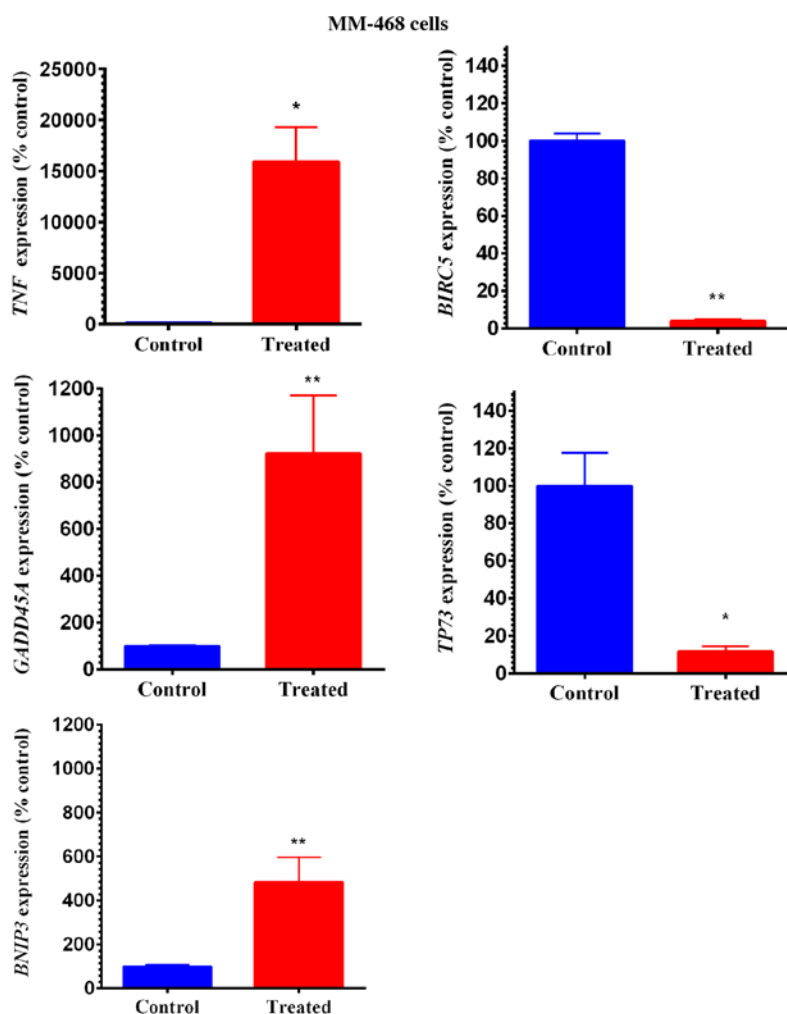


Figure 7. Gene expression quantification in MM-468 cells. Cells were exposed to GOSS for 24 h at a concentration of 20  $\mu$ M. Normalized mRNA data indicate a significant increase in three genes (*TNF*, *GADD45A* and *BNIP3*) in the GOSS-treated MM-468 cells vs. control cells, while two genes were significantly inhibited (*BIRC5* and *TP73*). The data points represent the mean  $\pm$  SEM of three independent studies. The significance of the difference was determined using an unpaired t-test between the resting vs. treated cells. The difference was considered significant at \* $P < 0.05$  and \*\* $P < 0.01$ . GOSS, gossypol; MM-468, MDA-MB-468; *TNF*, tumor necrosis factor; *GADD45A*, growth arrest and DNA-damage-inducible 45 alpha protein; *BNIP3*, BCL2 interacting protein 3; *BIRC5*, baculoviral IAP repeat containing 5; *TP73*, tumor protein 73.

## Discussion

Apoptosis is a pivotal cellular process that maintains genomic integrity (52). Genomic alterations, including the upregulation of genes involved in DNA synthesis, cell division, proliferation, and cell cycle progression, have been shown to be key mediators in BC development and progression. (53,54). In particular, the apoptosis methylation-mediated silencing of genes is the most important epigenetic mechanism for regulating normal gene expression (1).

The present study provides evidence for the anticancer effect of the natural polyphenol gossypol (GOSS) in two triple-negative breast cancer (TNBC) cell lines: MDA-MB-231 (MM-231) and MDA-MB-468 (MM-468). However, future studies are needed to determine the relative toxicity of GOSS in TNBC cells compared to non-cancer cells. This compound impacted the molecular apoptotic pathway by altering the mRNA gene expression of specific apoptosis-related genes (Figs. 5-7 and Table I). Overall, the obtained data are consistent with those from previous studies (22,29,35). This study shows similar cytotoxic (Fig. 1A and B) and antiproliferative (Fig. 2A and C) effects in

both cell lines. Notably, MM-468 cells showed a higher response during the colony formation (Fig. 2B and D), doxorubicin antiproliferation (Fig. 3) and apoptotic (Fig. 4) assays. However, doxorubicin was an extremely potent antiproliferative agent, and the obtained IC<sub>50</sub> values were compatible with those previously reported (51). However, meager previous investigations have examined GOSS effects on diet, and a previous study on women with refractory metastatic breast cancer (BC) indicated that the maximally tolerated dose of GOSS was 40 mg/day (55).

In normal cells, several molecular pathways control apoptosis and orchestrate the balance between cell proliferation and cell death to maintain homeostasis. However, in cancer cells, triggering the genes involved in these signaling pathways leads to uncontrolled cell proliferation and tumorigenesis. Therefore, understanding the mechanisms of apoptosis and targeting the expression of these apoptotic genes are crucial in the development of targeted cancer therapy (56). The profiling of apoptosis-related genes in both MM-231 and MM-468 TNBC cell lines revealed that GOSS could induce both intrinsic and extrinsic apoptotic pathways. In the present study, GOSS was found to upregulate the proapoptotic genes



*GADD45A*, *BNIP3*, *TNF* and *TNFRSF9*, the critical initiators of the extrinsic apoptotic pathway. Moreover, the compound was efficiently able to attenuate the expression of the survivin *BIRC5* gene, a well-known inhibitor of intrinsic apoptosis, as well as two additional proapoptotic genes, *DAPK1* and *TP73*. Although GOSS increased *GADD45A* expression in both cell lines, the gene was highly upregulated in MM-231 cells (33-fold increase) compared with MM-468 cells (Table I). The proapoptotic gene, growth arrest and DNA damage-induced 45 alpha (*GADD45A*) is a member of the stress sensor family *GADD45*, which normally controls many cellular functions, including DNA repair, apoptosis, cell cycle regulation and genotoxic stress (57). However, *GADD45* genes are epigenetically inactivated in different types of cancer cells (1). In TNBC, there is a strong association between a low level of the *GADD45A* gene and the lack of the three hormone receptors, ER, PR and Her2/neu (58). Moreover, in BC, the hypermethylated *GADD45A* gene is regulated by two major tumor suppressor proteins: p53 and *BRCA1* (59). Furthermore, the upregulation of *GADD45A* may be involved in apoptosis by activating the JNK and/or p38 MAPK signaling pathways (60,61).

In both MM-231 and MM-468 cell lines, GOSS upregulated *BNIP3* expression. However, the increase in gene expression in MM-231 cells was ~2-fold greater than that in its counterpart cell line, MM-468 (Figs. 6 and 7 and Table I). The death-inducing mitochondrial protein *BNIP3* is a member of the proapoptotic Bcl-2 family of proteins that promotes both apoptosis and autophagy. *BNIP3* contains a C-terminal transmembrane (TM) domain as well as a sequence resembling a BH3 domain, and both are essential for apoptosis induction (62,63). Moreover, *BNIP3* mediates novel necrosis-like mechanisms by opening the mitochondrial permeability transition (PT) pore independent of caspase activation, cytochrome *c* release, Apaf-1, or the nuclear translocation of apoptosis-inducing factor (64). Similar to our finding, in BC, the induction of *BNIP3* was found to induce apoptosis via (FAS) inhibition, which leads to the suppression of cell proliferation (63,65).

Various stimuli can trigger apoptosis, one of which is the signaling protein *TNF* superfamily (66). In the present study, *TNF*, also known as the *TNF- $\alpha$*  gene (67), was the most upregulated in MM-468 cells. The compound GOSS markedly increased the expression of *TNF* by 159-fold (Fig. 7 and Table I). The fact that *TNF* is the most potent inducer of apoptosis in the *TNF* superfamily (68) can explain the higher response of MM-468 cells to the apoptotic effect induced by the compound. Furthermore, multifunctional *TNF* can trigger both cell proliferation and cell death. In particular, the upregulation of NF- $\kappa$ B-related genes increases cell viability and proliferation. However, the activation of different caspases leads to apoptosis induction (68,69). Contrary to healthy cells, the multifunctional proinflammatory gene *TNF- $\alpha$*  is detected in many cancers, including ovarian, renal and breast (70-72), and has been found to increase cell survival and proliferation through NF- $\kappa$ B activation (73). Additionally, the *TNF* superfamily also plays a crucial role in regulating tissue homeostasis by activating the extrinsic apoptotic pathway (74) and triggering various signaling pathways, including the activation of caspases, impacting proliferation and inducing apoptosis (75).

In MM-231 cells, a 12-fold upregulation in *TNF* receptor superfamily 9 (*TNFRSF9* also known as CD137, 4-1BB, or ILA)

was found (Fig. 5 and Table I). *TNFRSF9* is a costimulatory receptor that is involved in apoptosis. *TNFRSF9* is expressed by activated monocytes and lymphocytes (76), while its ligand is expressed by monocytes and B cells (3). In MM-231 cells, the most important ability is for CD137 to synergize the apoptotic effect of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor with anticancer properties (77).

GOSS markedly and similarly decreased the expression of *BIRC5* in both TNBC cell lines, as shown in Figs. 5 and 6 and Table I. *BIRC5*, or the survivin gene is the smallest member of the IAP family (78). In the human genome, the gene encoding *BIRC5* is among one of the highly specific tumor genes (79) that prevent apoptosis by inhibiting the activity of different caspases (80), leading to aggressive tumor behavior and poor clinical outcomes (81). Compared to normal tissues, an increase in *BIRC5* expression has been demonstrated in different types of cancers, including pancreatic (82), lung (83), colon and ovarian (84), esophageal and skin (85), colorectal and lymphoma (86) and prostate (87). In particular, in TNBC cells, the highly expressed *BIRC5* is considered a marker in the early diagnosis of BC and was found to correlate with the resistance to radiation and chemotherapy (79,81,84). Additionally, two more apoptosis-related genes, *DAPK1* and *TP73*, were significantly downregulated in GOSS-treated MM-231 and MM-468 cells, respectively (Figs. 6 and 7 and Table I).

The proapoptotic death-associated protein kinase 1 (*DAPK1*) is characterized by its C-terminal death domain and plays an essential role in cell survival, proliferation and death (88). Compared to normal cells, lower expression of *DAPK1* mRNA has been observed in different cancer cell lines; however, in the presence of *TNF- $\alpha$* , the gene can decrease cell growth (89). The present study indicated repression of *DAPK1* gene expression in treated MM-231 cells. In addition, *TNF- $\alpha$* -stimulated apoptosis was inhibited by overexpression of *DAPK1* (89). Taken together, our results suggest that *DAPK1* downregulation may be a promising target in cancer therapy, particularly in MM-231 TNBC cells.

Surprisingly, the compound GOSS downregulated the gene *TP73* in MM-468 cells. Tumor protein p73 (*TP73*) is a member of the TP53 (p53) tumor-suppressor gene family. This family is characterized by a proapoptotic role (90) and is involved in cell cycle regulation and apoptosis (91). In TNBC MM-231 cells (92,93), upregulated *TP73* can replace (92) or activate P53 gene transcription, induce apoptosis and considerably affect tumor progression (94). On a molecular basis, gene polymorphisms can modify their specific functions (95). Certainly, there is an association between the *TP73* polymorphism (G4C14-A4T14) and cancer risk (94). Although it was not found in BC, in sympathetic neurons, the *p73* isoform lacking the transactivation domain has been found to act as a neuronal antiapoptotic protein and counteract the proapoptotic function of p53 (90).

The data obtained in this study provide evidence that the apoptotic effect of GOSS is related to either the upregulated proapoptotic genes and *TNF- $\alpha$*  or repression of the inhibitor of apoptosis genes or both. However, previous studies have shown the ability of GOSS to induce apoptotic effects by DNA fragmentation (96), DNA synthesis inhibition (30), and arresting S-phase without affecting RNA and protein synthesis (97). The molecular mechanism of GOSS-induced apoptosis was previously determined to be associated with the interaction between

the compound and the antiapoptotic proteins Bcl-2 and Bcl-xL in the mitochondria (98). In other words, the BH3 mimetic GOSS ectopically expresses Bcl-2 and Bcl-xL (25), inhibiting the Bcl-2/Mcl-1 pathway (33) and the heterodimerization of Bcl-xL/Bcl-2 by releasing proapoptotic proteins (apoptosis-inducing factor, AIF) (37). GOSS can also induce apoptosis by releasing cytochrome *c* and activating the proapoptotic protein Bak or Bax/Bak via conformational changes in overexpressed Bcl-2 (99,100). Moreover, inhibiting the phosphorylation of ERK1/2 and AKT, stimulating p38 and JNK1/2 protein phosphorylation, and inhibiting ErbB2 protein expression have been found to be mechanisms of GOSS-induced apoptosis (35).

In conclusion, the present study elucidated the genes involved in the apoptotic molecular mechanism of the polyphenol compound GOSS in two different TNBC cell models, MM-231 and MM-468. This study demonstrated that GOSS was more potent in MM-468 cells in inducing apoptosis and delaying colony formation. However, the impact of the compound on cell viability and proliferation was almost the same, and the data obtained did not show a considerable difference in cell response. In parallel, GOSS upregulated two proapoptotic genes (*GADD45A* and *BNIP3*) and attenuated the expression of *BIRC5* in both MM-231 and MM-468 cells. Additionally, GOSS increased the expression of *TNFRSF9* and repressed *DAPK1* in MM-231 cells, while an increase in *TNF* gene expression (159-fold) and repression in *TP73* were detected in MM-468 cells. The results obtained emphasize the importance of the polyphenol gossypol as a compound that can induce cancer cell apoptosis through extrinsic apoptosis-related genes. This compound may be targeted for TNBC treatment, particularly in African-American patients.

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#### Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

#### Authors' contributions

Conceptualization of the study was achieved by SSM and KFAS. The research methodology was designed by SSM, HA, CC, NOZ, PM and KFAS. Formal analysis of the data was conducted by SSM and KFAS. Funding acquisition was accomplished by KFAS. Project administration was carried out by KFAS, and study resources were obtained by SSM and KFAS. Software analysis of data and figures was conducted by SSM and KFAS, and supervision of the research was conducted by KFAS. Writing of the original draft was undertaken by SSM, and writing, review and editing of the manuscript were carried out by SSM, NOZ, PM, HA, CC and KFAS. All authors read and approved the manuscript and agree to be accountable for all aspects of the

research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors state that they have no competing interests.

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