Original Article

Acetyl Glycyrrhetinic Acid Methyl Ester as a Promising Glycyrrhizin Derivative against the Breast Cancer Cells (MCF-7)

Abstract

Background: Breast cancer remains the most potent threat to women's life worldwide. So far, no ideal drug for treatment of breast cancer, all available drugs exhibit severe side effects, poor therapeutic index, and high cost. **Objective:** Therefore, this study aimed to investigate the potential use of the natural pentacyclic triterpenoids such as *Boswellic, Betulinic (**BA**), Urosolic, Oleanolic acids, Glycyrrhizin* and their derivatives for treatment of breast cancer. **Materials and methods:** The cell viability was firstly determined after treatment with 50 μM of each compound. The effect of the treatment on cell cycle, apoptosis, cell migration and colony formation was evaluated. The ability of the new glycyrrhizin derivative to activate p53 was investigated by flow cytometry. **Results:** The cytotoxicity assay revealed that *glycyrrhizin* derivative **AM-GA** (*3-acetyl-18β-glycyrrhetinic-30-methyl ester*) and **BA** were the most cytotoxic against breast cancer cell line MCF-7 with IC50 values 4.5±0.1 and 4±0.1 μM, respectively. Both **AM-GA** and **BA** were selective towards breast cancer cells rather than the normal lung fibroblast cell line WI-38. Both **AM-GA** and **BA** were able to inhibit the cancer cell migration in the wound healing assay and inhibited colony formation. Studying the mechanism of action revealed that **AM-GA** inhibited the growth of the breast cancer cells via cell cycle arrest at sub-G1 phase, induction of apoptosis and activation of the tumor suppressor protein p53. **Conclusion:** This work highlights the unique role of **AM-GA** against breast cancer via different mechanisms and will be the gate for new potent analogues and fights different cancer types.

Keywords: *Apoptosis, breast cancer, cell cycle arrest, colony formation, cytotoxicity, glycyrrhetinic acid, selectivity index, wound healing*

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Introduction

Breast cancer is considered as the cancer with the highest incidence among women worldwide.[1] There is an apparent increase in breast cancer among women, in which 1.7 million cases are diagnosed yearly.[2] In 2018, approximately 266,000 new cases of invasive breast cancer and 64,000 new cases of noninvasive breast cancer are diagnosed among women in the US.[1] In Egypt, the estimated cases represent 38% of all newly diagnosed cancer cases.[3] Aging, physical inactivity, overweight, and smoking are considered the main causes for breast cancer.[4] The genetic factors represent the main causes of breast cancer than nongenetic or environmental factors.[4] Approximately 5%–10% of the cases are due to mutation in breast cancer gene 1 (*BRCA1*) or *BRCA2*. Approximately, *BRCA1* mutation represents 55%–65% of developing breast, whereas *BRCA2* mutation is 45%. On average, a woman diagnosed with a *BRCA1* or *BRCA2* mutation increases the exposure of getting breast cancer at older age.[5] Taking hormone replacement therapy as estrogen (>15 years) after menopause and oral contraceptive increase the risk of developing breast cancer.[5] In addition to adjuvant chemotherapy, surgery and radiotherapy and hormone therapy are mainly used at early stage of breast cancer.[6] The offered drugs for treatment have side effects such as cardiovascular and bone disorders.[7] For example, trastuzumab (Herceptin) is used in patients with HER2/neu–positive breast cancer and has showed great results; however, it leads to severe cardiac dysfunction. Because of the appearance of side effects due to the lack of selectivity, there is urgent need for new leads in treating HER-2-positive breast cancer.

More than 200 variable skeletons of triterpenoids are available in nature. They are isolated from fungi, ferns, seaweeds, angiosperms, and animals.[8] They are used as herbal medicine in China since 3000 years. Boswellic, betulinic acid (BA), ursolic acid, oleanolic acids, and their derivatives are pentacyclic triterpenoids

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and have anticancer activity against tumors cells via their cytotoxic effects, inhibition of angiogenesis, and induction of apoptosis.[8] Glycyrrhizinic acid (glycyrrhizin, GN) is a pentacyclic triterpenoid and is extracted from the root and rhizome of licorice (*Glycyrrhiza glabra*).[9] It showed diverse pharmacological effects as anticancer, anti-allergic, antiviral, anti-inflammatory, antibacterial, and antioxidant. It is composed of triterpenoid aglycone glycyrrhetinic acid (GA) linked to a diglucuronic acid. GN is hydrolyzed to GA by bacterial β-glucuronidases. GA is considered as the active form of GN with more biological activities.[10] It was found that, GA induces apoptosis in MCF-7 breast cancer cells via cell cycle arrest at sub-G1 phase via the activation and modulation of mammalian target of rapamycin (mTOR)/phosphatidylinositol phosphate (PI3K)/protein kinase B (AKT) pathways.[11] BA that has been isolated from the bark of *Betula platyphylla* induced apoptosis against breast cancer cells through activation of (Akt) pathway, p53 protein.[12] BA derivatives have shown anticancer, antimalarial, anti-inflammatory, and anti-human immunodeficiency virus activity.[13]

Over the last 25 years, our research team with multidisciplinary international scientists was able to develop more selective and effective anticancer agents.[14-24] However, there is still an increasing need to develop safe and efficacious agents for cancer therapy. The aim of this work was to investigate the potential use of pentacyclic triterpenoids as boswellic acid, BA, GN, and GN derivatives for the treatment of breast cancer. Furthermore, the effect of the most active GN derivative on the induction of apoptosis, tumor suppressor p53 protein, cell cycle, colony formation, and wound healing was evaluated in an attempt to uncover the possible mechanism of action of such compounds.

Materials and Methods

Cell lines and drugs

The cytotoxic activity of the investigated compounds was tested against the human breast cancer cells (MCF-7, $ER^{\pm}$). The cell lines were obtained from American type culture collection. The cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified eagle medium (DMEM); Cat. No. 12-604F, Lonza Verviers SPRL, Belgium supplemented with 10% fetal bovine serum (Lonza, 14-801E) and 100 IU/mL penicillin and 100 µg/mL streptomycin (Lonza, 17-602E). Cisplatin (*cis*-diammineplatinum (II) dichloride) was obtained from Sigma-Aldrich and dissolved in 0.9% saline then stored as 1666 µM stock solution at –20°C. Boswellic acid and BA were obtained from Professor Farid Badria, Faculty of Pharmacy, Mansura University, Egypt.[25,26] GN derivatives, 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) and 3-phenyl-carbamoyl-18β-glycyrrhetinic acid (PC-GA) were synthesized by Badria *et al*,[27] dissolved in Dimethyl sulfoxide (DMSO); Cat. No. 20385.02, Serva, Heidelberg, Germany and stored at –20°C. Crystal violet (0.5% w/v) was prepared in 0.25% methanol and was used to stain the viable cells. Crystal violet binds to protein and deoxyribonucleic acid (DNA) in the adherent and viable cells so the stain is an indicator for the viability of treated cells.[28]

Cytotoxicity assay

The cells (MCF-7) were seeded in 96-well plate at 5×10^4 cells/mL (100 µL/well). Serial dilutions of the tested compounds or cisplatin (50, 25, 12.5, 6.25, 3.125, or 1.56 µM) were added after overnight incubation of the cells at 37°C and 5% CO_2. DMSO was used as a control (0.5%). The cells were incubated for 48 h. Then, 10 µL of 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) (5 mg/mL Phosphate Buffered Saline (PBS)) was added to the cells and incubated for another 4 h. After that, 100 µL of acidified sodium dodecyl sulfate solution was added to solubilize formazan crystals. The 96-well plate was incubated for another 14 h at 37°C and 5% CO_2 and absorbance was measured at 570 nm by Biotech plate reader. IC50 was calculated as the concentration that causes 50% inhibition for cell growth. The growth of the cells was monitored and the images were acquired by GX microscopes (GXM-XJL17AT, GT Vision Ltd, Suffolk, UK) (10× eyepiece).

Selectivity index calculations

The selectivity of the most active compounds toward breast cancer was evaluated by incubating normal lung fibroblast cells (WI-38) with serial dilutions after 48 h from incubation, the viability of normal cells was quantified as aforementioned. The selectivity index (SI) was calculated as described earlier.[29]

Wound-healing assay

MCF-7 cells were seeded in six-well plates as 0.2×10^6 cells per mL (2 mL in each well) and incubated overnight at 37°C and 5% CO_2. In the 2nd day, a scratch was created in each well by using sterile p200 tip, then the medium was replaced with fresh medium containing either DMSO or IC50 value or ½ IC50 value for the most active compounds (AM-GA and BA). Images were taken at different time points (0, 4, 8, and 23 h) to monitor the wound closure. After that the cells were washed twice with ice-cold 1× PBS and fixed with methanol for 20 min at 4°C. The fixed cells were washed twice with 1× PBS and stained with 0.5% crystal violet for 30 min. The extra stain was washed off by distilled H_2O until no color in the washing was observed. Size of the wound was measured by using Image J 1.51 software (Preibisch, Saalfeld et al. 2009).

Colony formation assay

Depending on the plating efficiency of MCF-7 cells, 500 cells were seeded in duplicates in six-well plates. Then, AM-GA and BA were applied after 24 h at the indicated concentration and cells were cultured without change of medium until macroscopic colonies were detected in the untreated control (7 days). Colonies were counted after fixation with ice-cold methanol and staining with 0.5% crystal violet.[30]

Cell cycle analysis

The cells were seeded at 2.5×10^4 cells/mL in a six-well plate (2 mL/well) and incubated 24 h at 37°C and 5% CO_2 to allow adhering. MCF-7 cells were treated with 2.25 µM AM-GA and

incubated for another 48 h. MCF-7 cells were washed twice with 1 mL ice-cold 1× PBS and collected after adding 0.5 mL trypsin to each well. Detached cells were centrifuged at 200 x g. MCF-7 cell pellet was treated twice with 1 mL ice-cold 1× PBS and 3.5 mL 70% ethanol, vortexed, and then stored 2 h at −20°C. The fixed cells were washed with ice-cold 1× PBS. After that, the cells were re-suspended in 500 µL propidium iodide (PI)/RNase (BD Biosciences, BDB550825, NJ, USA) staining solution at room temperature for 15 min in the dark. DNA content in each phase was measured by (BD Accuri C6 Plus flow cytometer, NJ, USA).

Apoptosis assay

The level of apoptotic cells was determined by using Annexin V phycoerythrin (PE) apoptosis DXN kit (eBioscience, 51-66121E, Thermo Fisher Scientific™, USA) following the manufacturer's instructions. Briefly, the cells were treated with 2.25 µM AM-GA and incubated for another 48 h. MCF-7 cells were washed twice with 1 mL ice-cold 1× PBS and collected after adding 0.5 mL trypsin to each well. Collected cells were washed with 1 mL ice-cold 1× PBS, then resuspended in 1× binding buffer and 5 µL of PE annexin-V, and 5 µL 7-amino-actinomycin D (7-AAD) was mixed with 100 µL of cell suspension and incubated for 15 min at room temperature in the dark. After that, the stained cells were analyzed by BD Accuri C6 Plus.

Determination of pro- or antiapoptotic protein and activated P53

The cells were treated and collected as aforementioned. After that, the cells were fixed by paraformaldehyde and permeabilized by saponin. After washing steps, the cells were stained with the antibodies specific for the proapoptotic (Bax) (Abcam, ab139543, Abcam, Cambridge, UK), antiapoptotic (Bcl-2) (BD Pharmingen, Cat. no. 556537, BD Pharmingen™, NJ, USA), apoptotic activators (caspase-8 [AA430-482] or caspase-9), or with the executioner caspase-3 (BD51-68654X). The level of activated p53 was also determined by incubating the cell suspension with anti-p53 antibody (Abcam, ab139565) for 1 h. Following the incubation, it was twice washed with PBS, and the analysis was performed within 1 h by BD Accuri C6 Plus.

Results

Glycyrrhizin derivatives were more cytotoxic than the parent compound

To test the anticancer activity of the different pentacyclic triterpenoids against breast cancer cells (MCF-7), a concentration of 50 µM of the compounds (GN, PC-GA, AM-GA, acetyl-11-keto-β-boswellic acid (AKBA), or betulinic acid) was tested against MCF-7 cancer cells and the viability was determined after 48 h of incubation with crystal violet. The IC50 of compounds that reduced the cell viability to less than 50% in initial screening was assayed by using MTT. The tested GN derivatives (PC-GA, IC50 = 9.62 ± 0.1 and AM-GA, IC50 = 4.5 ± 0.1 µM) showed strong activity superior to that of GN itself (IC50 = 30 ± 1.8 µM) [Figure 1] [Table 1]. In this study, other triterpenoids were tested against MCF-7, BA, AKBA, and β-acetyl boswellic acid possess anticancer activity (IC50 = 4 ± 0.1, 7.1 ± 0.1, and 4.625 ± 0.1 µM, respectively) [Figure 2], [Table 1], which is in accordance with previous reports.[31-33] The most potent cytotoxic compounds against MCF-7 were tested against normal lung fibroblast cells (WI-38) to demonstrate the selectivity toward the cancer cells and the selectivity index of promising compounds was calculated.[29] Both BA and AM-GA proved to be selective to the cancer cells rather than the normal cells because the selectivity index was greater than 2 [Table 2].

Structure–activity relationship

Structure–activity relationship (SAR) is a benefit tool that helps researchers to find new analogs with superior efficacy. Different derivatives of GN have been altered at positions C-3 and C-30 by several reactions, including coupling with amino acids, esterification, or amidation.[34] Previous SAR studies revealed that insertion of acetyl, amide, carbonyl, or ester group at C-3 or C-30 increases the cytotoxicity of GN derivatives.[21] Here in this study, the parent compound [Figure 3A], GN proved to

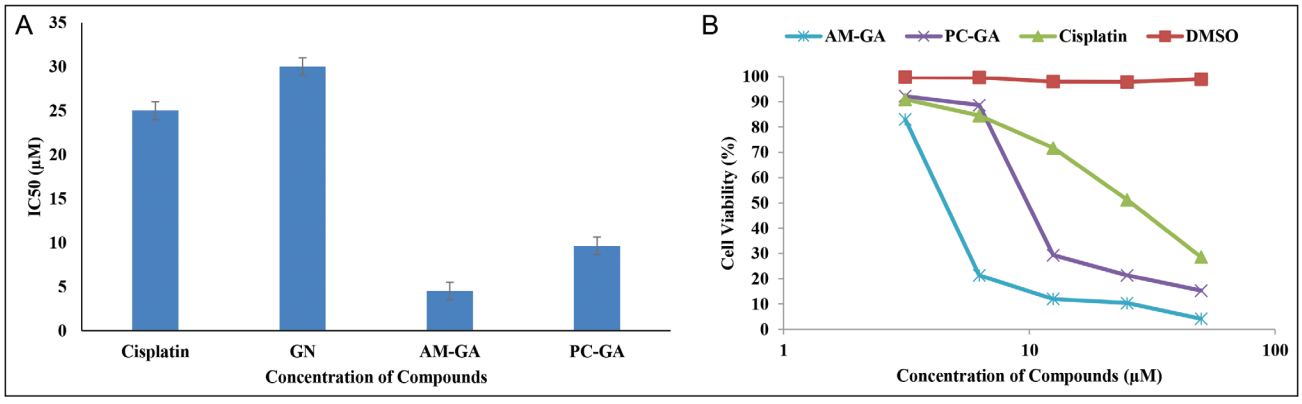

Figure 1: Cytotoxicity of the most active compounds against MCF-7 cells. The cells were incubated either with 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA), 3-phenyl-carbamoyl-18β-glycyrrhetinic acid (PC-GA), glycyrrhizin (GN), or cisplatin. (A) The derivatives AM-GA and PC-GA showed remarkable anticancer activity (IC50 = 4.5 ± 0.1 and 9.62 ± 0.1 µM, respectively) superior to the parent compound GN (30 ± 1.8 µM). (B) MCF-7 cells were treated either with AM-GA or PC-GA and DMSO was used as a negative control and cisplatin as a positive control. The cell viability was detected after 48 h of incubation by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

be less cytotoxic than the derivatives AM-GA [Figure 3B] or PC-GA [Figure 3C]. The potent cytotoxic activity of AM-GA may be attributed to the presence of acetyl group at C-3 and methyl ester group at C-30, whereas the high activity of PC-GA is attributed to the presence of phenyl carbamyl group at C-3. In other words, substitutions of the hydroxyl group at C-3 with an acyl (e.g., acetyl in AM-GA or carbamyl in PC-GA) showed superior activity, this may be explained by the presence of an ester group (COO) in these substituents. Furthermore, a small group at C-30 such as CH_3 (in AM-GA) or even no substituents (in PC-GA) may be required for the activity, along with the C-3's ester substitutions. In case of BA [Figure 3D], SAR studies revealed that C-28 carboxylic acid and three rings skeleton (A, B, and C rings) were critical for the cytotoxicity and the anticancer activity.[35]

Betulinic acid and 3-acetyl-18β-glycyrrhetinic-30-methyl ester inhibited the cell migration and colony formation

The wound-healing assay is a simple, inexpensive, and suitable method for studies on the effects of cell-matrix and cell–cell interactions in cell migration.[36] So, the effect of BA and AM-GA on cell migration was assayed either at half IC50 or the IC50 itself. BA either at 2 or 4 μM was able to inhibit the cell migration in comparison to the DMSO [Figure 4]. On the contrary, 4.5 μM of AM-GA showed inhibitory activity against the migration and wound healing in comparison to less effect by 2.25 μM [Figure 5]. Further analysis for the mechanism of anticancer activity of BA and AM-GA was confirmed by performing the clonogenic assay. A total of 4 μM of BA and 4.5 μM of AM-GA was able to reduce the number of colonies in comparison to that of DMSO-treated MCF-7 [Figure 6A and B], and the size of colonies that formed by surviving cells was smaller than that of the control cells [Figure 7].

Table 1: IC50 of the most active pentacyclic triterpenoids and their derivatives

Compound	IC50 (μM)
Betulinic acid	4 ± 0.1
Glycyrrhizin derivatives	
GN	30 ± 1.8
PC-GA	9.62 ± 0.1
AM-GA	4.5 ± 0.1
Boswellic acid	
Boswellia resin	>50
AKBA	7.1 ± 0.1
β-acetyl boswellic acid	4.625 ± 0.1
Cisplatin	25 ± 1.4

GN = glycyrrhizin, PC-GA = 3-phenyl-carbamoyl-18β-glycyrrhetinic acid, AM-GA = 3-acetyl-18β-glycyrrhetinic-30-methyl ester, AKBA = acetyl-11-keto-β-boswellic acid
Values after ± represents the standard deviation from three independent experiments

3-Acetyl-18β-glycyrrhetinic-30-methyl ester arrested the breast cancer cells at sub-G1 phase and induced apoptosis

AM-GA is a new GN derivative and its anticancer activity was not reported[27] earlier; therefore, its mechanism of action against MCF-7 cells was partially investigated in this report. MCF-7 cells were incubated with 2.25 μM of AM-GA and it was observed that, AM-GA arrested the cells at sub-G1 phase due to high DNA content, around 65.3% of cells, indicating the induction of apoptosis [Figure 8A]. To confirm the induction of apoptosis, MCF-7 cells were treated with 2.25 μM AM-GA and the cell population undergoing either earlier events or late event of apoptosis were measured by flow cytometry. The analysis revealed that AM-GA induced the apoptosis (around 17.3% compared to control 9.4%) and around 27.5% of treated cells were undergoing early stages of apoptosis [Figure 8B].

Table 2: IC50 (μM) of the most active compounds against MCF-7 and WI-38 cells

Compound	MCF-7	WI-38	Selectivity index
Betulinic acid	4 ± 0.1	23.04 ± 1.1	5.76 ± 0.1
AM-GA	4.5 ± 0.1	>50	>11.11

AM-GA = 3-acetyl-18β-glycyrrhetinic-30-methyl ester

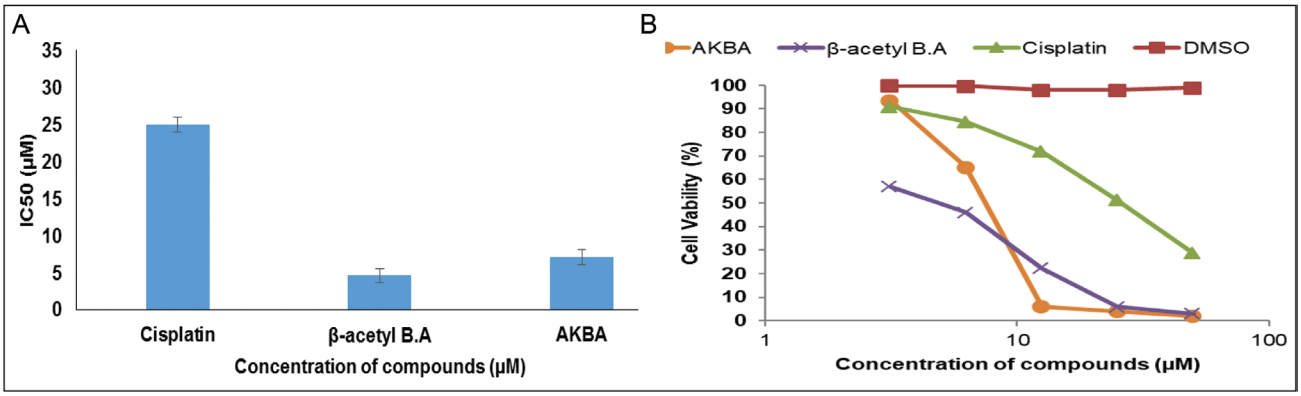

Figure 2: Cytotoxicity of boswellic acid and its derivatives against MCF-7 cells. (A) The cells were incubated either with β-acetyl B.A, AKBA (Acetyl-11-keto-β-boswellic acid), or cisplatin. The derivatives β-acetyl B.A and AKBA showed remarkable anticancer activity (IC50 = 4.625 ± 0.1 and 7.1 ± 0.1 μM, respectively). (B) MCF-7 cells were treated either with β-acetyl B.A or AKBA and DMSO was used as a negative control and cisplatin as a positive control. The cell viability was detected after 48 h of incubation by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

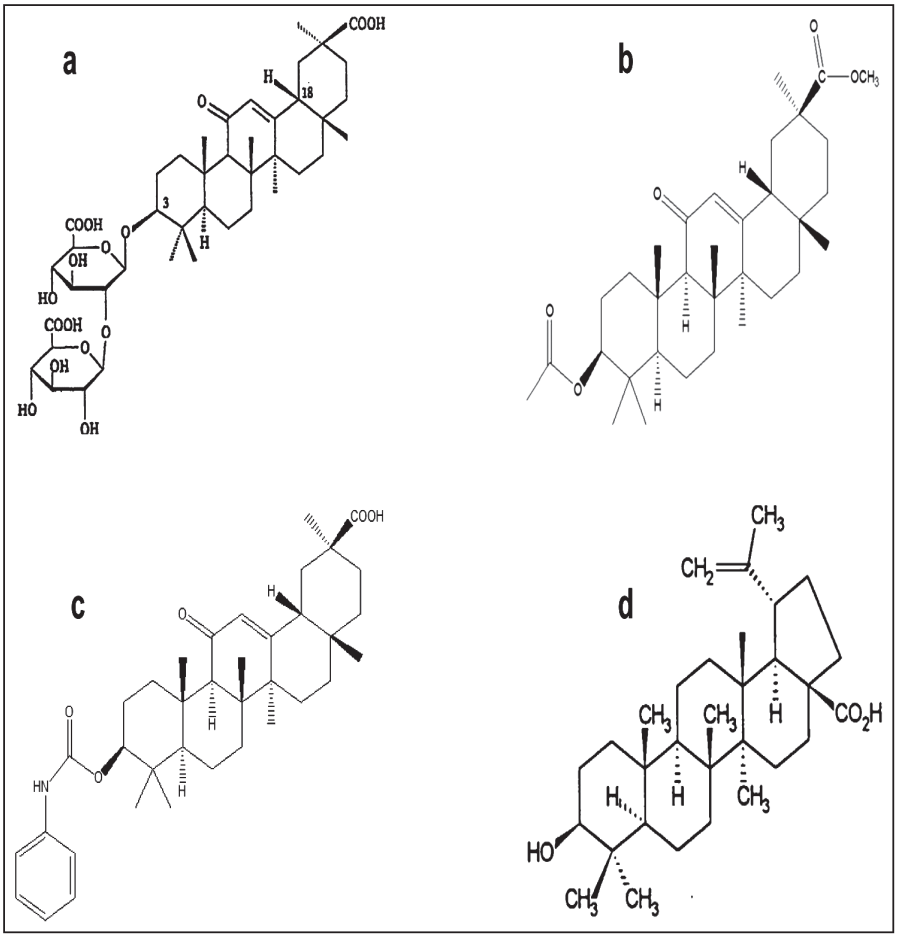

Figure 3: Structure–activity relationship study revealed the importance of acetyl or phenyl carbamyl group at C-3 and methyl ester group at C-30 in glycyrrhizin derivatives. The cytotoxicity of 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) (B) compound (IC50 = 4.5 ± 0.1 μM) is attributed to substitution of hydroxyl group at C-3 of parent glycyrrhizin compound (A) (IC50 = 30 ± 1.8 μM) with acetyl group at C-3 and methyl ester at C-30 rather than carboxylic group. 3-phenyl-carbamoyl-18β-glycyrrhetinic acid (PC-GA). (C) Compound is another derivative of compound A and has cytotoxic effect at IC50 = 9.62 ± 0.1 μM due to the presence of phenyl carbamyl group at C-3. The cytotoxicity of betulinic acid (D) is due to the presence of carboxylic group at C-28 and rings A, B, and C

AM-GA changed the level of pro- and antiapoptotic proteins through in part the activation of p53

The treatment of MCF-7 cells with 2.25 μM of AM-GA for 48 h led to the activation of the executioner caspase-3 [Figure 9A] via the activation of both caspase-9 and -8 [Figure 9B and C, respectively]. AM-GA revealed to have anticancer effect on MCF-7 cells through in part activation of p53 [Figure 10A], which led to the decrease in the level of antiapoptotic protein Bcl-2 [Figure 10B] and the overexpression of the apoptotic protein Bax [Figure 10C] in comparison to that of the untreated one.

Discussion

Triterpenoids are widely used in most Asian countries for many purposes including anti-inflammatory, analgesic, antipyretic, hepatoprotective, and cardiotonic effects.[37,38] Most of the triterpenoids revealed cytotoxicity against a variety of cancer cells without showing any side effects on normal cells.[39,40] In this work, BA and AM-GA showed potent cytotoxicity against MCF-7 breast cancer cells without affecting the viability of normal lung fibroblast cells (WI-38) with selectivity index of 5.76 and 11.11, respectively. The BA results are in accordance with recently reported work by Zeng *et al.*[33] Zeng *et al.*[33] showed that BA impaired the cell migration and invasion via the downregulation of metalloproteases and the overexpression of matrix metalloproteinases inhibitor (TIMP-2) via the inhibition of the signal transducer and activator of transcription 3 and focal adhesion kinase signaling pathways.[33] In this study, we focused on the new GN derivative AM-GA. A wide range of triterpenoids have been synthesized by structural modification of parent compounds for the generation of new analogs with high bioactivity and potent anticarcinogenic properties. It was found that addition of varied groups as esters, amides, or nitrile at C-30 position of GA; addition of double bond between C-1 and C-2; modification with electronegative groups such as CN and CF_3 at C-2 position; or conversion of hydroxyl group at C-3 to carbonyl group significantly increased cytotoxicity.[34,41] Here in this study, the cytotoxicity of GN was highly improved by the presence of acetyl group at C-3 and methyl ester group at C-30. The inhibition

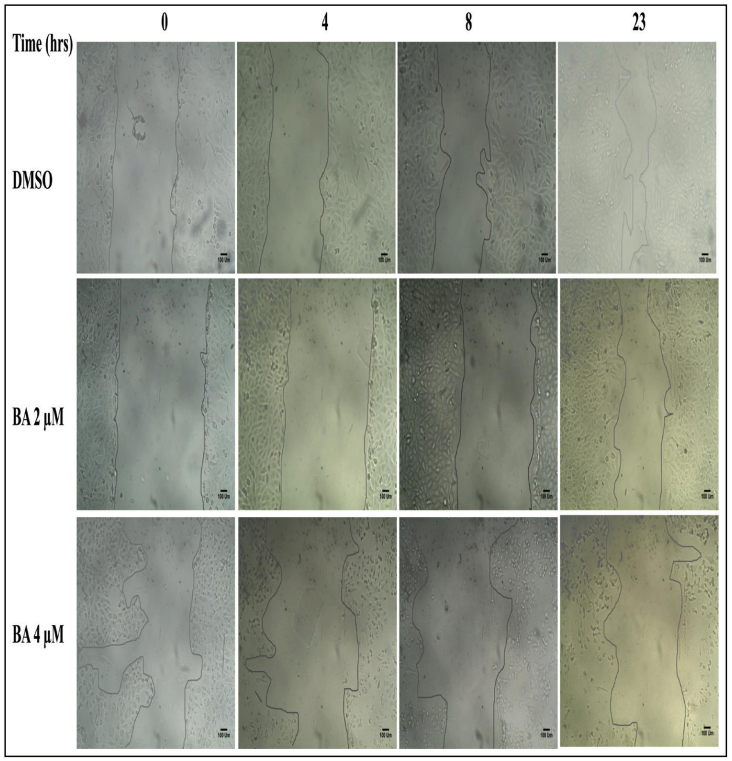

Figure 4: Effect of betulinic acid (BA) on wound healing at concentrations 2 and 4 µM. Images were taken after 0, 4, 8, and 23 h of incubation, then images were processed by using ImageJ 1.51 software to measure the size of wound of DMSO or BA-treated MCF-7 cells. Results revealed that BA was able to inhibit the cell migration either at 2 or 4 µM.

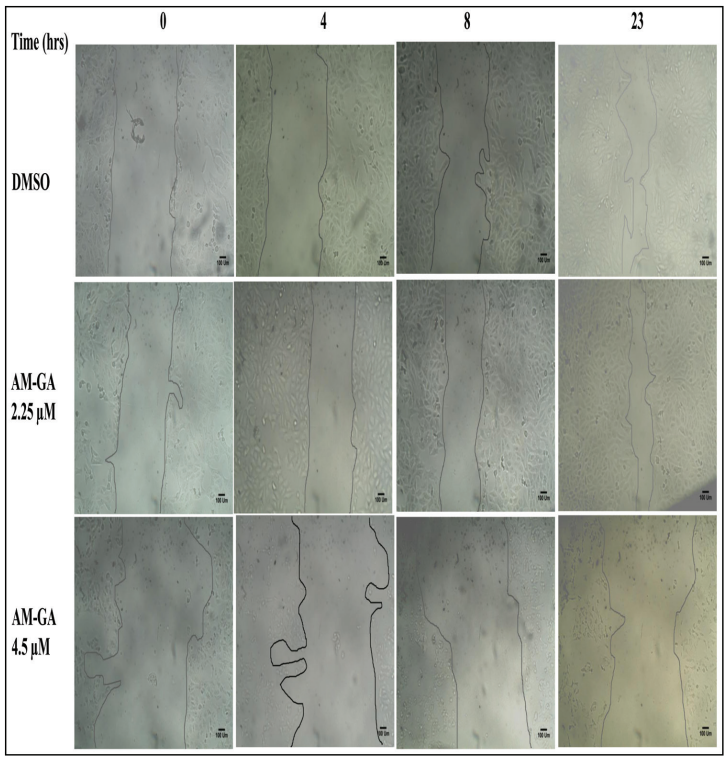

Figure 5: Effect of 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) on wound healing at concentrations 2.25 and 4.5 µM. Images were taken after 0, 4, 8, and 23 h of incubation then images were processed by using ImageJ 1.51 software to measure the size of wound of the DMSO or AM-GA-treated MCF-7 cells. Results revealed that compound AM-GA at concentration 2.25 µM has little effect on wound healing and cell migration than control (DMSO), but at concentration 4.5 µM, it inhibited cell migration and prevented cells from metastasis

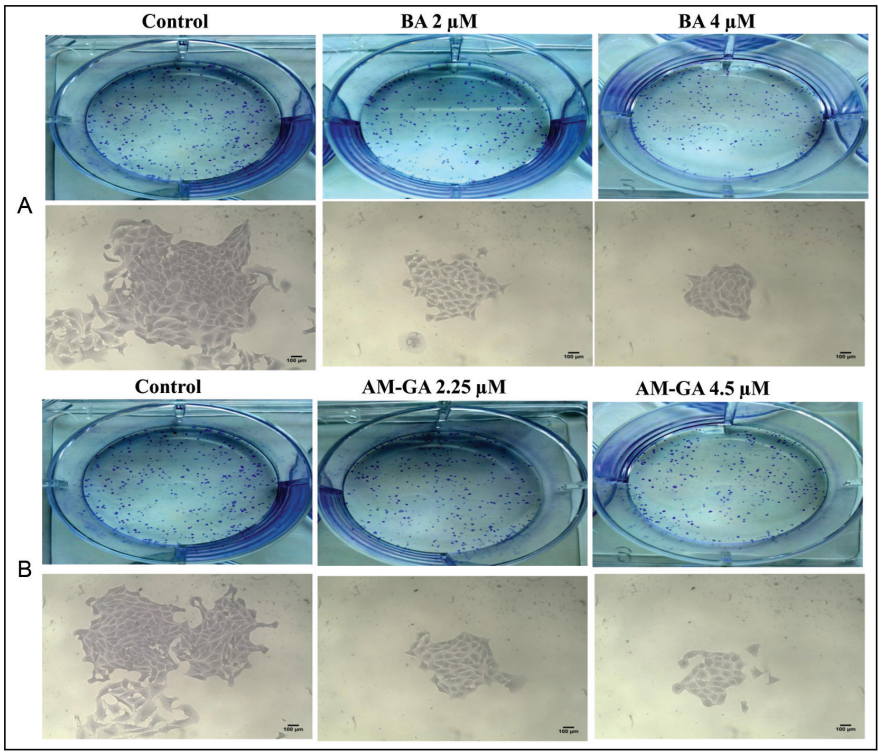

Figure 6: Effect of betulinic acid (BA) on colony formation of MCF-7 cells. (A) The images showed the ability of BA at 2 or 4 µM to inhibit colony formation in comparison to DMSO. (B) The images showed the ability of 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) at 2.25 or 4.5 µM to inhibit colony formation in comparison to that of DMSO and in reducing the size of individual colony in comparison to that of the control

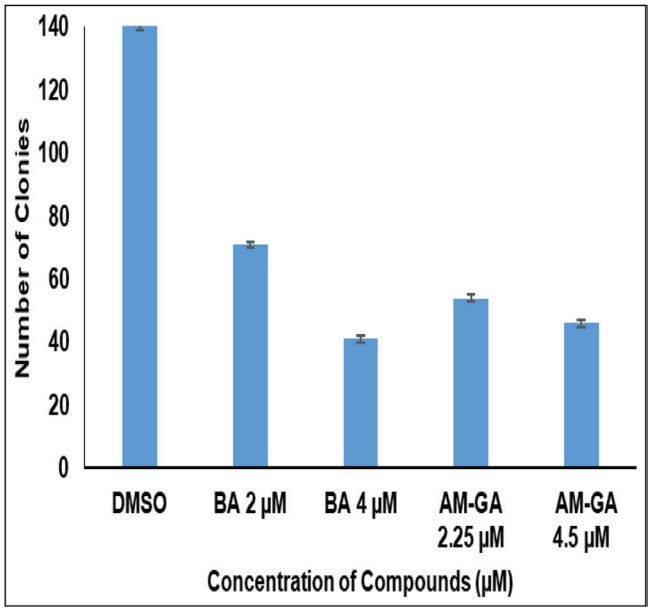

Figure 7: The inhibitory effect of betulinic acid (BA) and 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) on the number of colonies formed after treatment in comparison to DMSO. Error bars represent mean ± standard deviations (SD) from three independent experiments

of wound healing in breast cancer cells MDA-MB-468 was found to be mainly due to the inhibition of metalloproteinase-2 and -9 via the inhibition of Akt signaling pathway.[42,43] From these data, it was found that AM-GA may alter the regulatory factors and gene expression related to cell migration and wound healing and this needs to be confirmed in future studies on AM-GA.

The anticancer activities for pentacyclic triterpenoids were found to be through the regulation of different pathways that induce apoptotic pathway via the elevation of the proapoptotic Bax protein and the inhibition of the antiapoptotic Bcl-2 protein. They also inhibit different signaling pathways such as mitogen-activated protein kinase, Akt/mTOR, and epidermal growth factor receptor/Her2.[44] In this study, it was found that AM-GA induced apoptosis in MCF-7 cells through caspase activation and upregulation of Bax and downregulation of Bcl-2 proteins, which play a unique role in controlling apoptosis. The increase in Bax leads to a change in mitochondrial membrane, followed by release of cytochrome *c* in the cytoplasm and the formation of Apaf-1/cytochrome *c* complex, which induces and activates caspase-9, which in turn activates caspase-3, -7 and -6. It is known that the level of Bcl-2 in the cell determines whether the cell will undergo apoptosis or growth arrest.[45] Also, AM-GA was able to induce the apoptosis via the activation of caspase-8, which means that AM-GA may activate the death receptor complex, extrinsic apoptotic pathway.[46] The ability of p53 to induce cell cycle arrest is due to its ability to induce the expression of the cell cycle inhibitor genes such as $p21^{WAF1}$, growth arrest, and DNA damage (*GADD45*) and *14-3-3*[47,48] in case if the DNA damage can be repaired, but if the damage is excessive then p53 promotes apoptosis through the regulation of transcription of target genes such as *Bax* and *Bcl-2*.[49] Moreover, the activated p53 induces apoptosis through cytochrome *c* release and activation of caspase-9.[50] Here in this study, AM-GA was able to activate p53, which

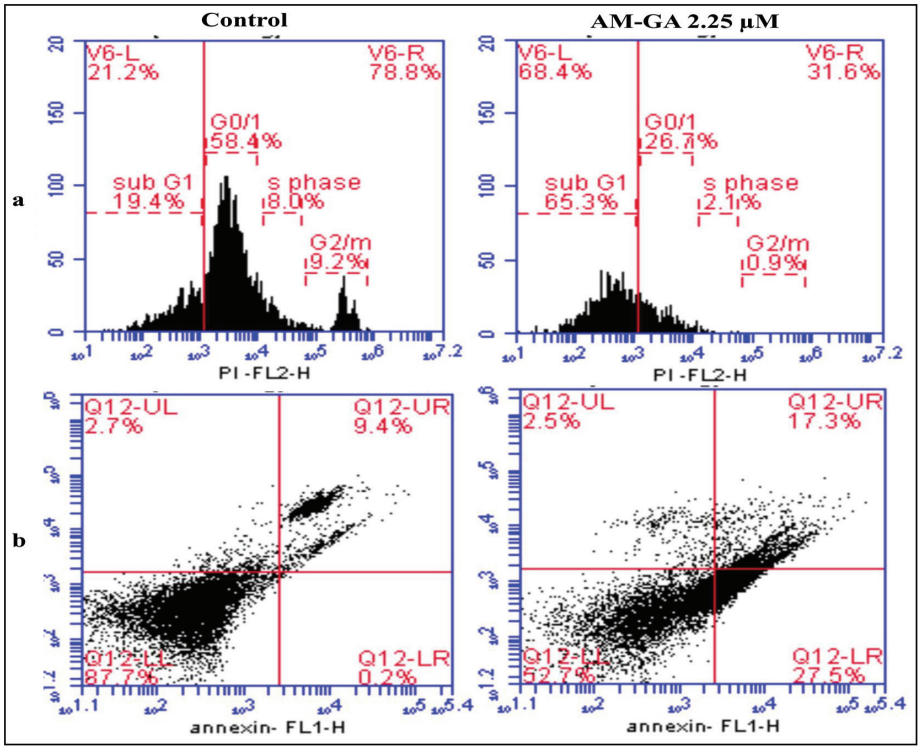

Figure 8: Treatment of breast cancer cells (MCF-7) by 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) led to an arrest of cell cycle at sub-G1 phase. (A) The DNA content was quantified by propidium iodide and analyzed by BD Accuri C6 Plus. (B) Treatment of cells with AM-GA at concentration 2.25 µM led to induction of apoptosis. Cells detached gently after treatment with trypsin and washed twice with PBS then stained with 7-amino-actinomycin D (7-AAD) and phycoerythrin (PE) annexin-V and analyzed within 1 h by BD Accuri C6 Plus flow cytometer

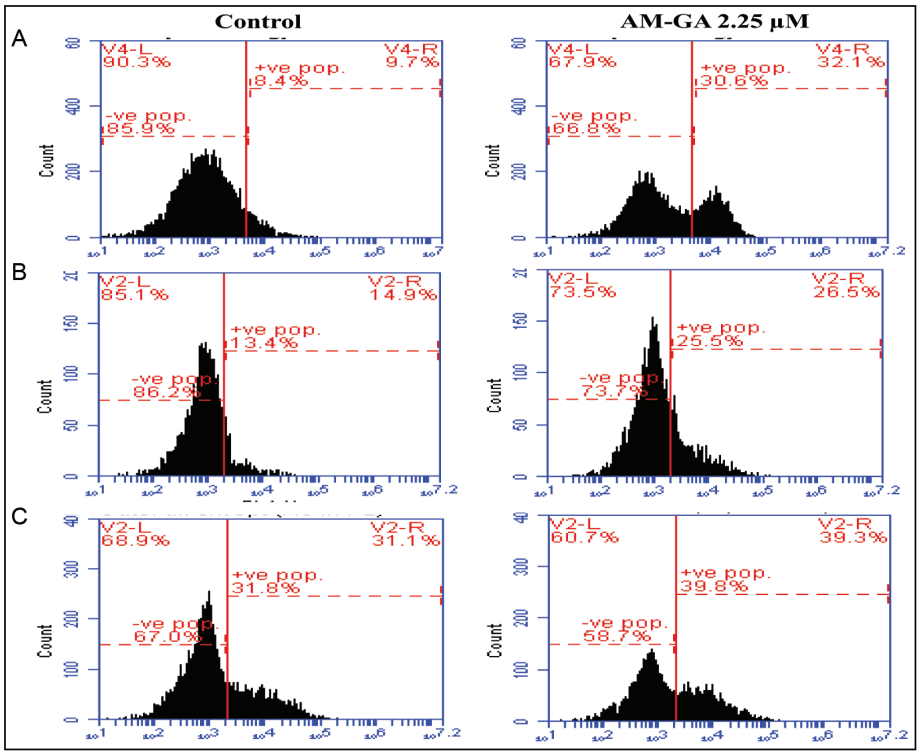

Figure 9: Effect of 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) treatment on the activation of extrinsic and intrinsic apoptotic pathways. The breast cancer cells were treated with AM-GA at concentration 2.25 µM for 48 h. Then, the cells were detached and washed twice with ice-cold PBS, fixed with 500 µL of PFA. The cells were permeabilized by 0.5% saponin for 30 min at room temperature. After that, 100 µL of suspension was treated with specific antibody. The analysis was performed by BD Accuri C6 plus flow cytometer. (A) Treatment of MCF-7 cells with 2.25 µM of AM-GA, led to activation of executioner caspase-3 via the activation of both caspase-9 (B) and caspase-8 (C)

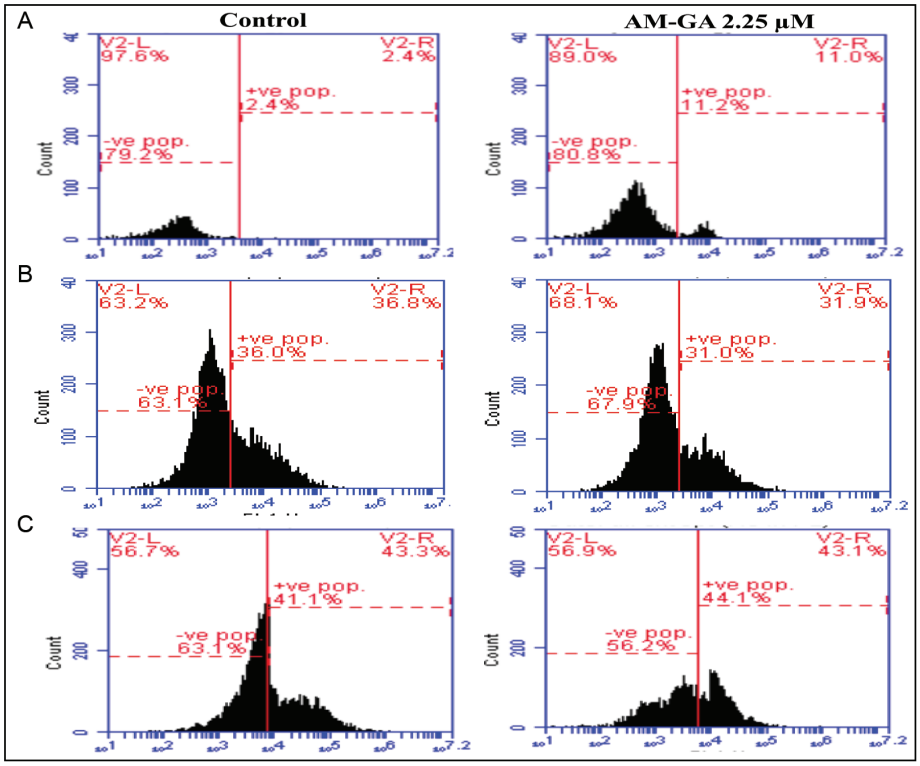

Figure 10: Effect of 3-acetyl-18β-glycyrrhetinic-30-methyl ester (AM-GA) treatment on the level of pro- and antiapoptotic proteins and p53. After the treatment of the MCF-7 cells with AM-GA at concentration 2.25 µM for 48 h, the cells were detached and washed twice with ice-cold PBS then fixed with paraformaldehyde. The fixed cells were permeabilized by 0.5% saponin for 30 min at room temperature. After that, 100 µL of suspension was treated with specific antibodies for 1 hour followed by washing twice with PBS. The analysis was carried out by BD Accuri C6 plus flow cytometer. AM-GA revealed to have anticancer effect on MCF-7 cells through in part activation of p53 (A), which led to the decrease in the level of antiapoptotic protein Bcl-2 (B) and the overexpression of the apoptotic protein Bax (C)

may contribute in part to its induction of apoptosis. The presented data need further deep investigation for discovering the other possible signaling pathways that may be affected by AM-GA treatment.

Conclusion

This work highlights the cytotoxicity of AM-GA against MCF-7 breast cancer cells. The presented results showed that AM-GA inhibited wound healing and colony formation, induced apoptosis through activation of extrinsic and intrinsic pathways, activation of proapoptotic protein (Bax), and downregulated antiapoptotic protein (Bcl-2). In summary, this new analog of GN worth further *in vivo* studies.

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Conflicts of interest

There are no conflicts of interest.

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