Evaluation of the anticancer effect of violacein, phycocyanin and phycocyanobilin on apoptotic genes expression and glycan profiles in breast cancer cells

Neveen A. Hussein, Samia A. Ebied and Marwa M. Saleh
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I take this chance to welcome your contributions to the IJCBR and have every expectation that it will soon become one of the most respected journals in both the fields of cancer and biomedical research.

Mohamed L. Salem,
Editor in Chief
Evaluation of the anticancer effect of violacein, phycocyanin and phycocyanobilin on apoptotic genes expression and glycan profiles in breast cancer cells

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ABSTRACT

Background: Cancer researchers have been concentrated on finding the best therapeutic strategies to decline cancer mortality rates. Marine natural products with pharmacological activities have potent anticancer activities. Aim: This study investigated the anticancer effect of violacein, phycocyanin, and phycocyanobilin in MCF-7 cells. Additionally, the elucidation of combined therapy efficiency (violacein-phycocyanin) on enhancing the anticancer activity of monotherapy. Materials and Methods: The cultured untreated, and treated cells by etoposide, violacein, phycocyanin, phycocyanobilin, and combined therapy were subjected to molecular studies, and glycan profiles by real time-PCR, and MALDI-TOF respectively. Results: Bax, Bcl-2, caspase-3 genes expression, and Bax/Bcl-2 ratio in all groups were significantly upregulated compared to the untreated cells except for a significant decrease in Bcl-2 by etoposide or combined therapy and an insignificant difference in Bax/Bcl-2 by PC or PCB treatment. The intensity of tetra-, tri-sialylated N-glycan, and di-sialylated O-glycan was significantly decreased in violacein, and combined-treated cells when compared to untreated or PC-treated cells. Mono-sialylated O-glycan was also decreased in combined therapy compared to the treated or untreated cells. Conclusion: Cotreatment with violacein/PC generated synergistic, antiproliferative, and pro-apoptotic effects on MCF-7 cells. Therefore, these compounds may be promising chemotherapeutic agents for breast cancer.

Keywords: Apoptotic genes; MALDI-TOF; N-/O-glycans; Real time-PCR.

INTRODUCTION

Breast cancer (BC) represents 25% of all the cancer cases and is recognized as the second mortality cause among women (15% of all tumor deaths) (Ferlay et al., 2015). However, BC prevalence is high in developed countries, its mortality rates are rising in less developed areas (Ghoncheh et al., 2015). Although the new therapies advancement in the past decades, have improved BC survival rate, the numbers of mortalities are still significant due to drug resistance by numerous mechanisms as altered proteins expression, and reduced apoptosis. Thus, it is necessary to search for new therapies with less resistance potential to allow BC treatment. Recently, marine natural products with pharmacological potentials have been revealed to have a potent anticancer effect and fewer toxic effects. Therefore, these products have valuable development and utilization (Yue et al., 2015).

Violacein, a unique violet pigment from Chromobacterium violaceum, has fascinated attention as an immunomodulator, antioxidant, antibacterial, antiviral, and antiprotozoal activities. Violacein potentially inhibits tumor initiation and induces apoptosis in various cancers (Alshatwi et al., 2016). Phycocyanin (PC) is a bio-active nutrient component extracted from various species, as Spirulina (Devanathan and Ramanathan, 2012) and has efficient hepatoprotective, anti-inflammatory, and free radicals-scavenger properties. It is even utilized in cosmetics and food coloring because it is non-toxic and non-carcinogenic (Romay and Gonzalez, 2000). PC polypeptide chain consists
of the chromophore (phycocyanobilin, PCB) and apoprotein. PCB is a linear tetrapyrrolic molecule which has bilirubin-like structure, a valuable antioxidant, and antiproliferative activities (Vitek and Schwertner, 2007).

Apoptosis is considered a favoring target for the anticancer therapy. The intrinsic/extrinsic pathways achieve apoptosis through cleavage of multiple proteins. The inhibition of apoptosis through overexpression of anti-apoptotic proteins and down-expression of pro-apoptotic proteins in cancer cells causes intrinsic resistance to the anticancer drugs (Pfeffer and Singh, 2018).

Glycosylation is post-translational protein modifications, where N-and O-glycans are the most common glycans attached to asparagine and serine/threonine residues respectively (Brockhausen and Stanley, 2015). Generally, glycans have been detected on the cell surface, also they participate in the interactions of cell-cell and cell-extracellular matrix. Glycans have a major role in cells growth, differentiation, pathogenesis, and innate immunity. In cancer, glycosylation modifications may control cancer development/progression, and offering novel targets for therapy (Varki, 2017). Consequently, this study examined the anticancer potential of natural products (violacein, phycocyanin, phycocyanobilin) on breast cancer cell line. Also, the elucidation of drug combination efficiency (violacein-phycocyanin).

MATERIALS AND METHODS

Cultivation of Spirulina platensis

Loop full S. platensis (Biotechnology Department, Institute of Graduate Studies and Researches, Alexandria University) was inoculated in sterile Zarrouk’s medium, growth for 15 days (30±2°C) then filtrated and washed.

Soluble proteins in Spirulina

The liquid chromatography-mass spectrometry-based proteomic (LC-MS) included: protein extraction from samples, fractionation to remove contaminants, proteins digestion into peptides by trypsin mass-spectrometer grade, post-digestion separation to get the homogeneous mixture of peptides, and analysis by LC-MS using C-18 column. (Song et al., 2015).

Preparation, characterization of PC, PCB

Spirulina platensis cells were harvested by centrifugation (10000xg, 15 min), adhesive salt was removed by double distilled water, and then biomass was dried at 50°C and homogenized. Freezing/thawing in dark were repeated 3 times. Centrifugation at10000xg for 20 min. Absorbance was evaluated at 280 and 620 nm (Lambda 25 UV/VIS spectrometer, PerkinElmer, USA). The crude extract of PC was purified by fractional precipitation with ammonium sulphate (Saran et al., 2016) and characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), fourier-transform infrared spectroscopy (FT-IR), and spectroscopy measurement (UV–Visible absorption spectra) (Patel et al., 2005). The PCB was cleaved from protein with conc. HCl (Beuhler et al., 1976), ethanol then water were added, mixture was centrifuged at 12,000xg for 5 min to extract the pigment into chloroform. The pigments were converted to their dimethyl esters and characterized by spectroscopic measurement (Schram and Kroes, 1979), mass spectrometry (MALDI-TOF) (Goetz et al., 2009) and FT-IR (Mohan, 2005).

Violacein (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and characterized by UV–Visible scanning (Ahmed et al., 2012), MALDI-TOF (Goetz et al., 2009), and FT-IR (Mohan, 2005).

Cell culture

Breast cancer-derived MCF-7 cells (RRID: CVCL_0031, Medical Research Institute, Alexandria University, Egypt) were cultured in a high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Lonza, Belgium). All MCF-7 cell culturing was held in the humidified CO2 incubator at 37°C with a permanent atmosphere (5% CO2 and 95% air). In all experiments, exponentially growing cells at 60-80% confluence were used. Cells were thawed and propagated firstly before sub-cultures and drug treatment.

Cell viability

3-(4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (MTT) was reduced to a purple formazan by mitochondrial dehydrogenases of viable MCF-7 cells.
The A260/A280 estimated RNA purity was determined at 260 nm using NanoDrop instruction. The extracted RNA concentration was determined. For untreated control, the cells were preserved in drug-free environment. After incubation for 48 h, 20 μL MTT was added, incubated 4h at 37°C, centrifuged, and 100 μL DMSO was added. Absorbance at 570 nm was generated similar impact alone. Concentration of the second drug which achieved an impact in combination. The TaqMan Assays used: Bax (ID#Hs01016548_g1), Bcl-2 (ID#Hs01048932_g1), caspase-3 (ID#Hs00234387_m1) and GAPDH “housekeeping gene” (ID#Hs99999905_m1). The relative quantification (RQ) of genes expression was estimated using comparative cycle threshold Ct ($2^{-\Delta\Delta Ct}$).

**Relative quantification of Bax, Bcl-2, caspase-3 expression**

**RNA extraction**

Total RNA was extracted from cultured cells (1x10^6 cells/well): untreated, and treated cells by IC_{50} of etoposide, violacein, PC, PCB and combined violacein-PC (0.63 μg/ml, 0.153 mg/ml respectively) for 48 h, using RNase-free water. The resultant pellets had been dried to yield a protein powder (Talabnin et al., 2016).
Preparation of N-glycans

The cells protein powder (1 mg) was resuspended in 25 mM ammonium bicarbonate. N-glycans were enzymatically released through adding 5mU peptide: N-glycosidase F (PNGase F) (Mechref et al., 2009) then incubated overnight (37°C).

Solid-Phase extraction of N-glycans

Glycans volume was adjusted by deionized water to 1 mL. First, the mixture had been applied to C18 Sep-Pak cartridge (Waters, Milford, MA) and circulated 5 times. O-linked glycopeptides and peptides were held on this cartridge, and the released glycan was gathered as eluents, then cartridge was washed by deionized water. Eluents that contain the released N-glycans were applied to activated charcoal microcolumns (Harvard Apparatus, Holliston, MA) that preconditioned with acetonitrile (ACN) and trifluoroacetic acid (TFA), then the microcolumn was washed with TFA. Samples were eluted with 50% ACN that contains 0.1% TFA. Lastly, purified N-glycans were evaporated until dry by using vacuum CentriVap Concentrator (Labconco Corporation, Kansas City) then permethylated (Kang et al., 2008).

Preparation of O-glycans

The cells protein powder (1 mg) was exposed to reductive β-elimination at 45°C (100 mM NaOH, that contains 1.0 M NaBH₄) for 18 h. The mixture was neutralized by 10% acetic acid and was desalted on AG50W-X8 (H⁺) column (Talabnin et al., 2016). The materials were eluted with 5% acetic acid. Boric acid was removed by evaporation with methanol. The released O-glycans had been purified by C18 Sep-Pak cartridge column and permethylated (Mechref et al., 2009).

Matrix-assisted laser desorption/ionization/time of flight mass spectrometry instrumentation

Purified N-/O-glycans (permethylated) were resuspended in methanol: water solution (50:50), containing 2.5 mM sodium acetate. Sample (0.5 mL) was spotted on the MALDI plate directly and blended with an equal quantity of 2,5 dihydroxybenzoic acid-matrix. The MALDI plate was dried under vacuum. Mass spectra were obtained utilizing MALDI TOF/TOF analyzer (Applied Biosystems, Framingham, MA) (Kang et al., 2005).

RESULTS

Characterization of PC

As shown in Figure (1), the major protein in S. platensis was PC where its efficiency by 0.1 M sodium phosphate buffer (122±4.35 mg/g) was higher than that of double-distilled water (64±5.03 mg/g). Also, PC purity ratio was improved to a considerable level and had been increased to 2.55. SDS-PAGE indicated the presence of two bands corresponded to the two subunits of PC with molecular weights nearly 15 and 24 kD. UV-Visible scanning: The purified PC spectra demonstrated a prominent peak at 620 nm, which was higher than crude extract. The peak intensity at 280 nm was low. FT-IR spectra: The protein-specific amide I band at 1655 cm⁻¹ (C=O stretching) and amide II at 1547 cm⁻¹. Shape and position of amide I band were employed for protein secondary structure analysis. The sharp amide I band for PC (at 1655 cm⁻¹) indicated α-helix as the main element of its secondary structure.

Characterization of PCB

UV-Visible scanning: The spectrum was characterized by maximum absorption at 365.5 nm. FT-IR spectra: The existence of quite strong
hydrogen bonds in the acid was specified by the broad O-H and N-H absorption bands between 3000 and 3500 cm\(^{-1}\). **Mass spectra:** The exact mass of dimethyl ester was 614 (Figure 2a).

**Characterization of violacein**

**UV-Visible scanning:** Absorption spectrum was showed maximum absorption at 585 nm. **FT-IR spectra:** It showed all the distinctive signals. The band at 1648 cm\(^{-1}\) related to carbonyl (C=O) stretching frequency. Moreover, a broad peak at 3475 cm\(^{-1}\) could be attributed to NH group on the indole nucleus. **Mass spectrum:** In the negative ion electrospray ionization-mass spectrometry (ESI-MS) spectrum, a quasi-molecular ion peak was detected at m/z 342.34 (M–H\(^+\)) (Figure 2b).

**Cell viability**

All concentrations of studied compounds were capable to inhibit the proliferation of cancer cells significantly (P<0.001) except cells treated with 0.3125 μg/ml violacein which showed insignificant difference (P=0.643) (Figure 3, A-E). The IC\(_{50}\) values were 34.22 μg/ml (58 μM), 0.8 μg/ml (2.30 μM), 1.56 mg/ml (37 μM), and 96.0 μg/ml (163 μM) for etoposide, violacein, PC, PCB respectively. The violacein IC\(_{50}\) was the lowest among all studied compounds. The violacein-PC combination had a synergistic effect at concentrations less than 1.25 μg/ml and 0.3 mg/ml of violacein and PC respectively.

**Morphological results**

**Inverted light microscope:** The treated cells were shifted from the spindle and elongated to a rounded shape. The monolayer cells turned into a rounded up, losing contact with neighboring cells, and were mostly detaching from the culture plate and floating in the medium. Cells showed shrinkage and size reduction when compared to the untreated cells that were well-spread with a flattened morphology. Also, the number of treated cells was decreased.

**Scanning electron microscope:** Untreated cells were firmly adherent. The cell surface demonstrated several links between the plasma membrane of neighboring cells and extended in all directions. Cells treated with violacein and/or PC showed shrinkage, and cell membrane abnormalities with membrane blebbing.

**TEM:** Untreated cells showed a rounded shape, a normal nucleus with microvilli like processes over the cells surface. The appearance of cell membrane abnormalities, cytoplasmic vacuoles, apoptotic bodies, and disappearance of microvilli was observed in combined-treated cells (Figure 4).

**Molecular parameters**

As revealed in Table 1, and Figures 5,6, Bax, Bcl-2, caspase-3 expression, and Bax/Bcl-2 ratio in all groups were significantly higher than untreated group (P<0.001, 0.002, 0.001 and 0.022) except a significant decrease in Bcl-2 expression by treatment with etoposide (P=0.001) or combined therapy (P<0.001) and an insignificant difference in Bax/Bcl-2 by PC or PCB treatment (P=1.000).

Comparing with etoposide-treated cells, Bax expression in the violacein-treated group was significantly upregulated (P=0.001). While in PC and PCB-treated groups, it was significantly downregulated (P=0.002 and <0.001) and in the combined-treated group, it showed insignificant difference (P=0.854). Bcl-2 expression was significantly upregulated in violacein, PC, PCB-treated cells (P<0.001). While combined-treated cells showed insignificant difference (P=0.999). Bax/Bcl-2 ratio was significantly downregulated in violacein, PC, PCB-treated cells (P<0.001). While combined-treated cells showed insignificant difference (P=1.000). Caspase-3 expression was significantly downregulated in PC, PCB, and combined-treated cells (P<0.001). While violacein-treated cells showed insignificant difference (P=0.999).

All studied parameters in PC, PCB, and combined-treated cells were significantly lower than the corresponding levels in violacein-treated cells (P<0.001, 0.002, 0.025, 0.010) except Bax/Bcl-2 ratio which was significantly high in combined-treated cells (P<0.001).

A significant decrease in all genes in PCB-treated cells when compared to PC-treated cells (P=0.001, <0.001, 0.004) except Bax/Bcl-2 which showed insignificant difference (P=1.000). In combined-treated cells, Bax and Bax/Bcl-2 were
significantly higher (P=0.049, <0.001), while Bcl-2 expression was significantly lower (P=<0.001) as compared to PC, and PCB-treated cells. Also, caspase-3 was significantly lower than PC-treated cells only (P=0.009). A positive significant correlation between all studied parameters (P=0.030, 0.001, 0.008, <0.001, 0.046) except a negative significant correlation between Bcl-2 and Bax/Bcl-2 (r=−0.650, P=<0.001) was observed.

**Glycan mass profiles**

The exact glycans compositions of hexose (Hex), deoxyhexose (dHex), N-acetylhexose (HexNAc), and N-acetylmuramic acid (sialic acids, NeuAc) were recognized based on their accurate masses (Figure 7; Table 2). The intensity of tetra-, tri-sialylated N-glycan and di-sialylated O-glycan (structure 1, 2) was significantly decreased in violacein and combined-treated cells when compared to either untreated or PC-treated cells (P=0.001, 0.002). Furthermore, mono-sialylated O-glycan (structure 3) was significantly decreased in combined-treated cells when compared to untreated, violacein or PC-treated cells (P=0.003, 0.045, 0.004).

In PC-treated cells, all glycans structures showed insignificant difference compared to untreated or violacein-treated cells. Except for structures 1, 2 of both glycans, they were significantly higher than the corresponding intensity in violacein-treated cells (P=<0.001, 0.002). However, a significant decrease of N-glycan structure 2 when compared to untreated cells (P=0.007) and N-glycan structure 5, 7 when compared to violacein-treated cells (P=<0.001).

Treatment with violacein and/or PC did not affect N-glycan structures 4, 6, 8. Conversely, structures 5, 7 in violacein-treated cells were significantly higher than untreated cells and in combined-treated cells than untreated or PC-treated cells (P=<0.001).

**DISCUSSION**

Most of the natural products have a role in fighting malignancies and initiating identical cellular responses associated with synthetic drugs. Accordingly, these products may be used as potentially chemopreventive/chemotherapeutic agents (Demain and Vaishnav, 2012). All studied compounds reduced viability of viable MCF-7 cells in a dose-dependent manner. Etoposide had antiproliferative activity on MCF-7 viability. Etoposide bonded to tubulin and interacted with topoisomerase II, causing chromosomal disruption. It stabilized covalent enzyme-cleaved DNA compounds, producing permanent DNA breaks as well as apoptosis (Dwarakanath et al., 2004). Also, violacein markedly reduced the viability of the cells. The divergences in its IC50 value from other studies suggested the presence of various cell-type-specific mechanisms that operate in the existence of violacein. (Alshatwi et al., 2016, Santhosh and Muthusamy, 2017). The violacein antiproliferative effect achieved by reactive oxygen species (ROS) production in cancer, causing apoptosis promoting via enhances of intracellular calcium and release of cytochrome c (De Carvalho et al., 2006).

The current results proved PC had antiproliferative effects on cells. These results were in line with a previous study (Jiang et al., 2019). The discrepancy in PC IC50 value from other studies might be attributed to the effectiveness of PC that was greatly dependent on the origin of the organism, methods of extraction, purification, and storage. Also, as a protein, PC had poor stability, easy degradation, and temperature sensitivity (Gantar et al., 2012). Jiang et al. (2018) indicated that PC might efficiently prevent cell proliferation, induce tumor G0/G1 cell cycle arrest and trigger cell death in a dose-dependent manner.

Additionally, PCB had antiproliferative activity on MCF-7. The PC's pharmacological actions were exerted by its chromophore PCB since after PC administration in vivo, PC should be proteolytically degraded to PCB or PCB-linked peptides. The PCB antiproliferative effect on cancer cells was attributed to their effective antioxidant activity, and inhibition of ROS production (Konicková et al., 2014).

Interestingly, the combination of violacein and PC created a synergistic anticancer effect on MCF-7. The combined therapy improved the efficacy when compared to monotherapy since they targeted crucial pathways in the synergy or an additive manner.
Table 1. Statistical analyses of Bax, Bcl-2, caspase-3 expression and Bax/Bcl-2 ratio in MCF-7 cell line in different studied groups (n = 9)

<table>
<thead>
<tr>
<th></th>
<th>Untreated cells</th>
<th>Etoposide-treated cells</th>
<th>Violacein-treated cells</th>
<th>PC-treated cells</th>
<th>PCB-treated cells</th>
<th>Combined-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>1.04±0.3</td>
<td>4.51±0.3^a</td>
<td>8.47±1.2^ab</td>
<td>3.30±0.4^abc</td>
<td>2.0±0.16^bcd</td>
<td>4.17±0.7^acde</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>1.06±0.4</td>
<td>0.56±0.1^a</td>
<td>3.24±0.20^ab</td>
<td>2.78±1.3^abc</td>
<td>1.90±0.2^bcd</td>
<td>0.52±0.1^acde</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>1.11±0.5</td>
<td>5.52±0.6^a</td>
<td>5.45±0.7^a</td>
<td>2.98±0.5^abc</td>
<td>2.07±0.2^bcd</td>
<td>2.14±0.4^abcde</td>
</tr>
<tr>
<td>Bax/Bcl-2</td>
<td>1.06±0.4</td>
<td>8.24±1.0^a</td>
<td>2.61±0.3^ab</td>
<td>1.20±0.2^bc</td>
<td>1.06±0.2^bc</td>
<td>8.17±1.6^acde</td>
</tr>
</tbody>
</table>

The results were expressed as mean± S.D, n: number of samples in each group, ANOVA test, pairwise comparison between every 2 groups was performed utilizing Post Hoc Test (Tukey), ^a: comparing with untreated cells, ^b: comparing violacein, PC, PCB, combined-treated cells with etoposide-treated cells, ^c: comparing PC, PCB, combined-treated cells with violacein-treated cells, ^d: comparing PCB, combined-treated cells with PC-treated cells, ^e: comparing combined-treated cells with PCB-treated cells, ^abcd: Significant at P <0.05.

Table 2. Major N- and O-glycan structures in MCF-7 cell line.

<table>
<thead>
<tr>
<th>Glycans</th>
<th>m/z</th>
<th>Glycan structure</th>
<th>Glycan Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-glycans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4587.59</td>
<td>![Image of glycan structure]</td>
<td>HexNAc6Hex7 dHex1NeuAc4</td>
</tr>
<tr>
<td>2</td>
<td>4226.54</td>
<td>![Image of glycan structure]</td>
<td>HexNAc6Hex7dHex1NeuAc3</td>
</tr>
<tr>
<td>3</td>
<td>2966.36</td>
<td>![Image of glycan structure]</td>
<td>HexNAc4Hex5dHex1NeuAc2</td>
</tr>
<tr>
<td>4</td>
<td>2395.27</td>
<td>![Image of glycan structure]</td>
<td>HexNAc2Hex9</td>
</tr>
<tr>
<td>5</td>
<td>2191.32</td>
<td>![Image of glycan structure]</td>
<td>HexNAc2Hex8</td>
</tr>
<tr>
<td>6</td>
<td>1987.53</td>
<td>![Image of glycan structure]</td>
<td>HexNAc2Hex7</td>
</tr>
<tr>
<td>7</td>
<td>1783.62</td>
<td>![Image of glycan structure]</td>
<td>HexNAc2Hex6</td>
</tr>
<tr>
<td>8</td>
<td>1579.36</td>
<td>![Image of glycan structure]</td>
<td>HexNAc2Hex5</td>
</tr>
<tr>
<td>O-glycans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1706.23</td>
<td>![Image of glycan structure]</td>
<td>HexNAc2Hex2 NeuAc2</td>
</tr>
<tr>
<td>2</td>
<td>1256.34</td>
<td>![Image of glycan structure]</td>
<td>HexNAc1Hex1 NeuAc2</td>
</tr>
<tr>
<td>3</td>
<td>895.25</td>
<td>![Image of glycan structure]</td>
<td>HexNAc1Hex1NeuAc1</td>
</tr>
</tbody>
</table>

This approach possibly reduced drug resistance and concomitantly provided therapeutic anticancer advantages as decreasing tumor progression, metastasis, cancer stem cell, and inducing apoptosis (Mokhtari et al., 2017).

In the current study, by using etoposide treatment, all parameters were upregulated except Bcl-2 which was downregulated. Etoposide may have cytotoxic functions through ROS induction, Akt/mTOR signaling stimulation, and caspase-3 activation (Zhang and Huang., 2013). Moreover, etoposide may cause double-strand breaks accumulation in the cell’s nuclei. Cells can identify these injure and sequentially eliminate the damaged cells by apoptosis (Jiang et al., 2015).

Bcl-2 family proteins have a significant role in promoting or inhibiting cells apoptosis, particularly mitochondrial pathway. Bcl-2 controls this pathway via blocking cytochrome c release and caspases activation. This mechanism confers Bcl-2 exert the protective effect versus etoposide-induce apoptosis. While Bax, an agonist of apoptosis by enabling cytochrome c release and activating caspases. A balance between anti-and pro-apoptotic members performs a crucial responsibility in deciding cell survival or death (Jiang et al., 2015).
Accordingly, the high ratio of Bax/Bcl-2 detected may be due to high Bax and low Bcl-2 expression in the same cells. These alterations in genes expression by etoposide may be contributed to its increased cytotoxicity.

The significant increase of pro-apoptotic genes (Bax, caspase-3) in all groups and the significant decrease of anti-apoptotic gene (Bcl-2) in etoposide and combined therapy groups compared to untreated cells may indicate that these natural products can play an important role in promoting cells susceptibility to apoptosis. Consequently, this alternation leads to varying Bax/Bcl-2 ratio which significantly increased in etoposide, violacein, and combined treated-cells. The altered ratio was skewed to a greater extent to apoptosis and may have been responsible for membrane potential reduction, cytochrome c leakage and associated downstream events of apoptosis as endonucleases and caspases activation. Furthermore, caspase-3 activation led to cellular substrates cleavage and finally cell death (Wen et al., 2019).

Alternatively, Bcl-2 expression in violacein, PC, PCB-treated cells was higher than untreated or etoposide-treated cells. These results seem contradictory to its apoptotic regulatory function so the overexpression may be attributed to the molecular machinery effort of cells to survive and escape the apoptosis pathway (Sagar et al., 2014).

As compared to etoposide-treated cells, violacein revealed a significant overexpression in Bax expression, whereas an insignificant difference in caspase-3. Also, the combined therapy showed insignificance difference in Bax, Bcl-2 and their ratio. These results may indicate that etoposide, violacein and combined therapy have a comparable effect which resulted in the
Figure 2. UV-Visible scanning, FT-IR, and mass spectrum for (a) PCB, (b) violacein

Figure 3. Viability % of untreated and treated MCF-7 cells with different concentration (A) etoposide, (B) violacein, (C) PC, (D) PCB, (E) combined therapy (violacein-PC).
Figure 5. Amplification plot of (A) Bax, (B) Bcl-2, (C) caspase-3, (D) GDPH expression and (E) genes relative expression in different studied groups.
Anticancer activity of violacein, PC, PCB.

Comparing to violacein-treated cells, all parameters in PC, PCB, combined-treated cells were significantly decreased except Bax/Bcl-2 ratio in combined-treated cells which was significantly increased. These results may indicate the high cytotoxicity of violacein on tumor cells through induction of apoptosis. Interestingly, the three genes were significantly decreased in PCB-treated cells than PC-treated cells. Although PC antioxidant activity was attributable to PCB, the possible contribution of apoprotein to the total antioxidant activity of PC could not be excluded since amino acids of the protein may contribute to its antioxidant activity (Madhyastha and Vatsala, 2010).

The significant increase of Bax, and Bax/Bcl-2 ratio and the significant decrease of Bcl-2 by combined therapy than PC or PCB-treated cells may indicate the effectiveness of combined therapy. Besides, violacein-PC combination caused downregulation of Bax, Bcl-2, caspase-3 as compared to single treatment of either violacein or PC except for higher Bax expression than PC. An elevated Bax/Bcl-2 ratio by combined therapy was also observed. These results suggested the combined therapy may exert its apoptotic effect by influencing intrinsic pathway. The combined therapy did not achieve the expected results for caspase-3 and this may refer to the need of combined therapy for more incubation time to exert its effect.

The correlation between pro-, anti-apoptotic and Bax/Bcl-2 ratio was reasonable since the apoptotic pathway was activated through Bax/Bcl-2 related caspase-3 activation and preferred cells entry apoptotic pathway. The localizing Bcl-2 and Bax in the same cells might suggest the two opposing mechanisms that attempt to decide cells fate (Yang et al., 2002) since Bax may modulate Bcl-2 effect and that explain the observed positive correlation between them. Our results proved that all studied compounds induced apoptosis in MCF-7. This observation confirmed by cells morphological alternations which were typical of the apoptosis process.

Despite the improvement in the understanding of cancer genome, the full characteristics of cancer glycoproteome and glycome are still under research. Glycans in cancer are involved...
in essential molecular and cellular processes. Generally sialic acid terminates the outer end of glycans (sialoglycans), and its expression on membrane glycolipids and glycoproteins as well as its secretion into microenvironment is increased in cancer cells (Varki, 2017). Alteration of sialyltransferases activity causes increasing of glycans sialylation, specific tumor associated-carbohydrate antigens, genes of sialic acids biosynthesis, and sialidases activity (Häuselmann and Borsig, 2014).

From glycan profiles, N-/O-glycans structure1,2 intensity was significantly lower in violacein than untreated cells and combined-treated cells than either untreated or PC-treated cells. Furthermore, O-glycan structure3 was significantly decreased in combined therapy compared to either untreated or treated cells.

Cell death resistance is one of the malignant cell characteristics. Down-regulation or mutation of molecules implicated in Fas receptor–Fas ligand
(FasR– FasL) pathway is an ordinary mechanism utilized by malignant cells to escape apoptosis where cells can hypersialylate FasR (Swindall and Bellis, 2011). FasR acts as a substrate for β-galactoside α 2,6 sialyltransferase (ST6Gal I). In cancer cells, silencing ST6Gal I expression enhances FasL-induced apoptosis, while ST6Gal I overexpression hinders Fas-mediated apoptosis. α2,6-sialylation of FasR impairs its internalization and inhibits the initiation of death-inducing signaling complex (DISC) through preventing Fas-associated adaptor domain (FADD) binds to FasR death domain. Naturally, internalization of FasR results in more DISC structure and serves as a positive feedback loop used for Fas-mediated apoptosis. The FasR siaylation prevents this signal amplification loop and disrupts the downstream apoptotic signaling cascades, thus enabling cancer to deactivate the main apoptotic pathway (Bull et al., 2014). Accordingly, treatment of MCF-7 cells with violacein or combined therapy may have an obvious role in reducing siaylation which favors inducing extrinsic apoptotic pathway. These observations were in line with the obtained genomic results.

The observed high intensity of di-sialylated N-glycan in combined-treated cells only may be due to the specific changes in glycosyltransferase expression that play role in MCF-7 cells glycosylation. Moreover, the raise in some high-mannose N-glycans (structure5,7) intensity proposes the early termination of glycosylation pathway and trouble in their synthesis which prohibits deletion and consequent a supplement of sugar residues. Certainly, variations in the earlier part of a biosynthesis pathway are recognized to increase the number of particular structures besides limit other structures (Varki, 2017). The increased existence of high-level mannose glycans (hybrid-type structures or vis-à-vis complex), has significances for protein functions where they may modify the protein ability to interact with substrates, stability, half-life, communication properties of proteins (De Leoz et al., 2011).

The significantly increased N-/O-glycans structure1,2 concomitant with significantly decreased of structure5,7 in PC-treated cells compared to violacein-treated cells indicate a rise in the glycosylation process which decreases some high mannose N-glycan to produce complex and hybrid oligosaccharides. Alternatively, the other glycan structures showed insignificance difference compared to untreated or violacein-treated cells. This observation may indicate that the treatment with PC is less effective than violacein.

**Conclusion:** for the first time, this study shows that cotreatment with violacein and PC has antiproliferative and pro-apoptotic effects on MCF-7 cells through the intrinsic and extrinsic pathways. Violacein-PC combination generates a synergistic effect leading to increase in their anticancer activity. Therefore, this combined therapy may be a promising chemotherapeutic applicant for BC.

Anticancer effect of violacein, PC, PCB on MCF-7 cells is mediated by apoptosis induction in a caspase-dependent manner. Cancer cells respond to these drugs takes place under genetically programmed mechanisms proves the probability that selective interference in the process which can be modified by a regulated expression of particular genes, as Bax, Bcl-2, caspase-3 may affect the course of the disease.

Finally, this study yielded some basic information about N-/O-glycans in violacein and/or PC-treated MCF-7 cells. The difference in the intensity of specific glycan structures can indicate their impact on cancer progression. Also, the terminal sialic acid decline suggests its role in cell apoptosis.

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**CONFLICT OF INTEREST**

All authors declared no conflicts of interest.

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Egyptian Association for Cancer Research (EACR)
http://eacr.tanta.edu.eg/

EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcb.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

EACR Chairman,
Prof. Mohamed Labib Salem, PhD
Professor of Immunology
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