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Welcome to the Int J Cancer and Biomedical Research (IJCBR)**!**

It is with great pleasure that I write this editorial to welcome you to the IJCBR. This journal provides a platform for publication of original and reviews research articles, short communications, letter to editor, thesis abstract, conference report, and case studies. These types of publication are directed at the interface of the fields of cancer and biomedical research.

The IJCBR relies on a distinguished expert of the Advisory and Editorial Board Members from the top international league covering in depth the related topics. They timely review all manuscripts and maintain highest standards of quality and scientific methodology and ethical concepts. Meanwhile, we take all possible means to keep the time of the publication process as short as possible.

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

INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

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.

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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), adhere salt was removed by double distilled water, and then biomass was dried at 50ºC 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 CO_2 incubator at 37°C with a permanent atmosphere (5% CO_2 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

(Mosmann, 1983). Cells ($1x10^6$ cells/mL) were plated and after an overnight growth, cells were treated with different concentrations of etoposide (Sigma) as conventional chemotherapeutic agent, positive control, (100, 50, 25, 12.5, 6.25, 3.125 µg/ml), violacein (10, 5.0, 2.5, 1.25, 0.625, 0.3125 µg/ml), PC (2.0, 1.0, 0.5, 0.25, 0.125, 0.0625 mg/ml), PCB (100.0, 50.0, 25.0, 12.5,6.25, 3.125 µg/ml), and combined of violacein (2.5, 1.25, 0.625, 0.3125, 0.15625 µg/ml) and PC (0.6, 0.3, 0.15, 0.075, 0.0375 mg/ml). 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 assessed using a microplate reader (Bio-Rad, USA). Experiments were done in triplicate and their mean absorbance were calculated. The drug concentration equivalent to 50% inhibition of cell proliferation in drug-treated cells (IC_{50}) was determined.

Combination index (CI) of violacein and PC

CI used for analysis the synergistic, antagonistic or additive effects of two drugs. CI>1 antagonism, CI=1 additive, CI<1 synergy effect (Chou and Talalayn, 1984).

$$CI= [(D)_1/(D_x)_1) +((D)_2/(D_x)_2]$$

- $(D)_{1=}$ Concentration of the first drug which achieved an impact in combination.
- $(D_x)_1=$ Concentration of the first drug which caused similar impact alone.
- $(D)_2=$ Concentration of the second drug that achieved an impact in combination
- $(D_x)_2=$ Concentration of the second drug which generated similar impact alone.

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 RNeasy Mini Kit (Qiagen) according to the manufacture's instruction. The extracted RNA concentration was determined at 260 nm using NanoDrop Spectrophotometer (thermo Scientific, USA). The A260/A280 estimated RNA purity.

Reverse transcription reaction

QuantiTect Reverse Transcription Kit (Qiagen) was used for synthesis cDNA from total RNA using a PCR thermocycler (Applied Biosystem, Foster City, USA). The reactions contained 10 pg-1 µg template RNA, 4 µl Quantiscript RT Buffer, 2 µl gDNA Wipeout Buffer, 1 µl Quantiscript Reverse Transcriptase, 1µl RT primer mix, and RNase-free water.

Real-time PCR

The genes expression was analyzed on real-time PCR (Stratagene MX3000, USA) using 2 µl Template cDNA, 1 µl TaqMan primer/probe (Applied Biosystem), 10µl oasigTM 2x qPCR Master Mix (Qiagen) and 7µl DNase-RNase-free water. The reaction mixtures were initially activated at 95°C for 30 sec, followed by 40 cycles at 95°C (5 sec), 60°C (30 sec), and 72°C (60 sec) and then final extension at 72°C (10 min). 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 ($2^{-\Delta\Delta Ct}$).

BAX :
forward primer (FP) 5′-TGGCAGCTGACATGTTTTCTGAC-3′
reverse primer (RP) 5′-TCACCCAACCACCCTGGTCTT-3′
Bcl-2: FP 5′-TCGCCCTGTGGATGACTGA-3′
RP 5′-CAGAGACAGCCAGGAGAAATCA-3′,
Caspase-3: FP 5′-GGACAGCAGTTACAAAATGGATTA-3′
RP 5′-CGGCAGGCCTGAATGATGAAG-3′
GAPDH: FP 5′-TGA AGGTCGGAGTCAACGGATTTGGT-3′
RP 5′-CATGTGGGCCATGAGGTCCACCAC-3′

Glycan profiles

Preparation of MCF-7 protein powder

The harvested cells (100 µl); untreated, and treated with violacein (IC_{50}), PC (IC_{50}), and combined violacein-PC (0.63 µg/ml, 0.153 mg/ml respectively); were homogenized in cold methanol (50%). The homogenate has been adjusted to 4:8:3 of chloroform/methanol /water, extracted at 37°C for 2 h, then centrifuged for 15 min at 3000 rpm. 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 trifluroacetic 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 $NaBH_4$) 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).

Morphological Changes

The nuclear morphology was investigated using an inverted microscope in untreated and all treated cells. Additionally, the scanning electron microscope was done to show the impact of violacein and/or PC on cell morphology. Transmission electron microscopy (TEM**)** for untreated and violacein-PC-treated cells was done.

Statistical analysis

The data were evaluated statistically using IBM SPSS software package version 20. Kolmogorov-Smirnov test was applied to verify the normality of distribution. One-way analysis of variance (ANOVA) and then Tukey's test were utilized to compare difference among groups. A correlation was assessed applying Spearman's coefficient. $P<0.05$ was considered significant.

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-acetylneuraminic (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 (structure3) 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 IC_{50} 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 IC_{50} 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 (Konícková et al., 2014).

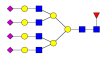
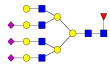
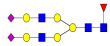
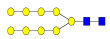
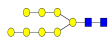
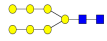
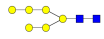
Interestingly, the combination of violacein and PC created a synergistic anticancer effect on MCF-7. The combined therapy improved the efficacy when compared to monotherapy since they targeted crucial pathways in the synergy or an additive manner.

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)

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

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, ^{abcde}: Significant at P <0.05.

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

Glycans	m/z	Glycan structure	Glycan Composition
N-glycans			
1	4587.59		HexNAc6Hex7 dHex1NeuAc4
2	4226.54		HexNAc6Hex7dHex1NeuAc3
3	2966.36		HexNAc4Hex5dHex1NeuAc2
4	2395.27		HexNAc2Hex9
5	2191.32		HexNAc2Hex8
6	1987.53		HexNAc2Hex7
7	1783.62		HexNAc2Hex6
8	1579.36		HexNAc2Hex5
O-glycans			
1	1706.23		HexNAc2Hex2 NeuAc2
2	1256.34		HexNAc1Hex1 NeuAc2
3	895.25		HexNAc1Hex1NeuAc1

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).

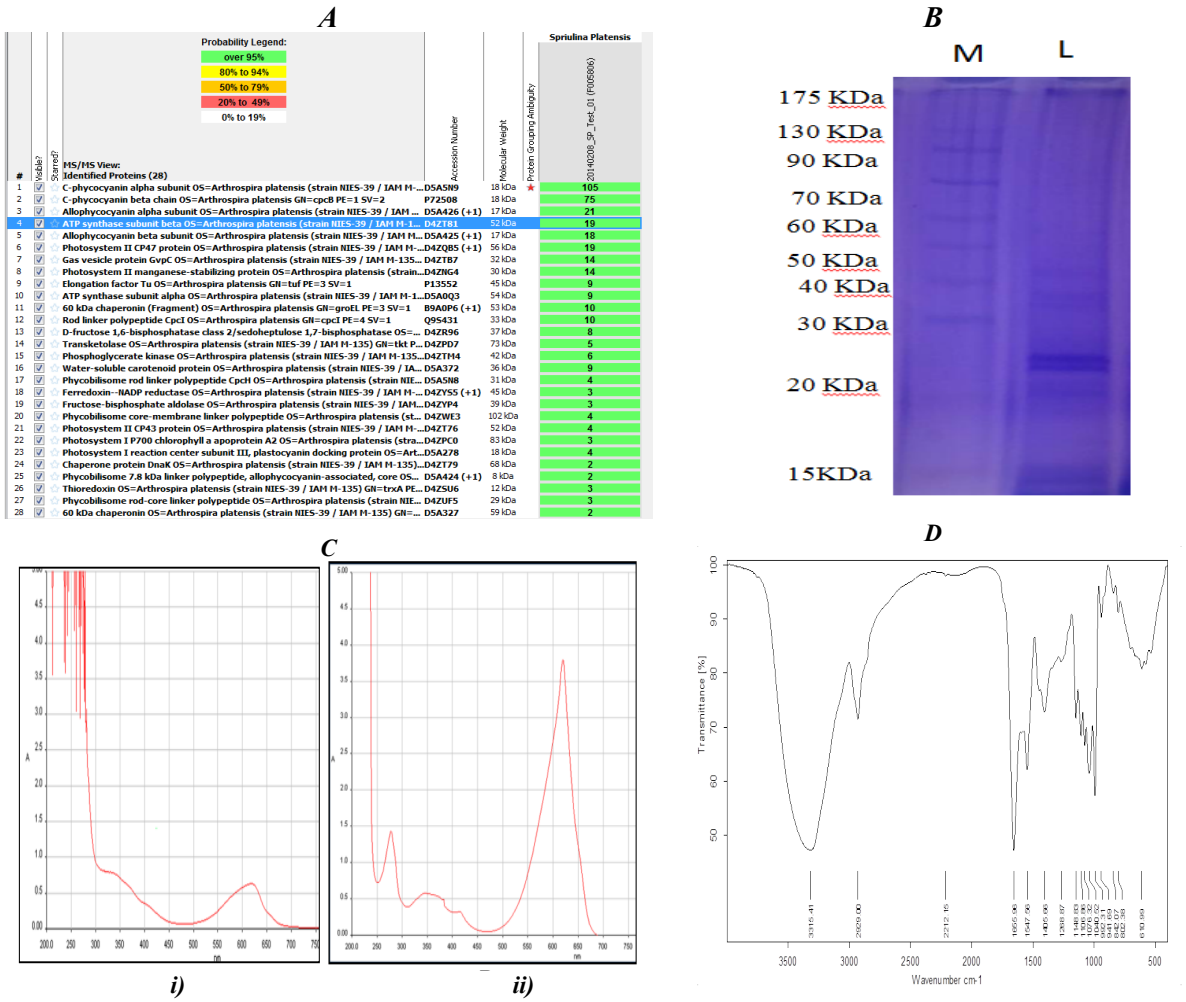


Figure 1. (A) LC-MS of *S. platensis*, (B) SDS-PAGE purification of PC, (C) absorption spectrum of (i) PC crude extract, (ii) purified PC, (D) FT-IR of purified PC.

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

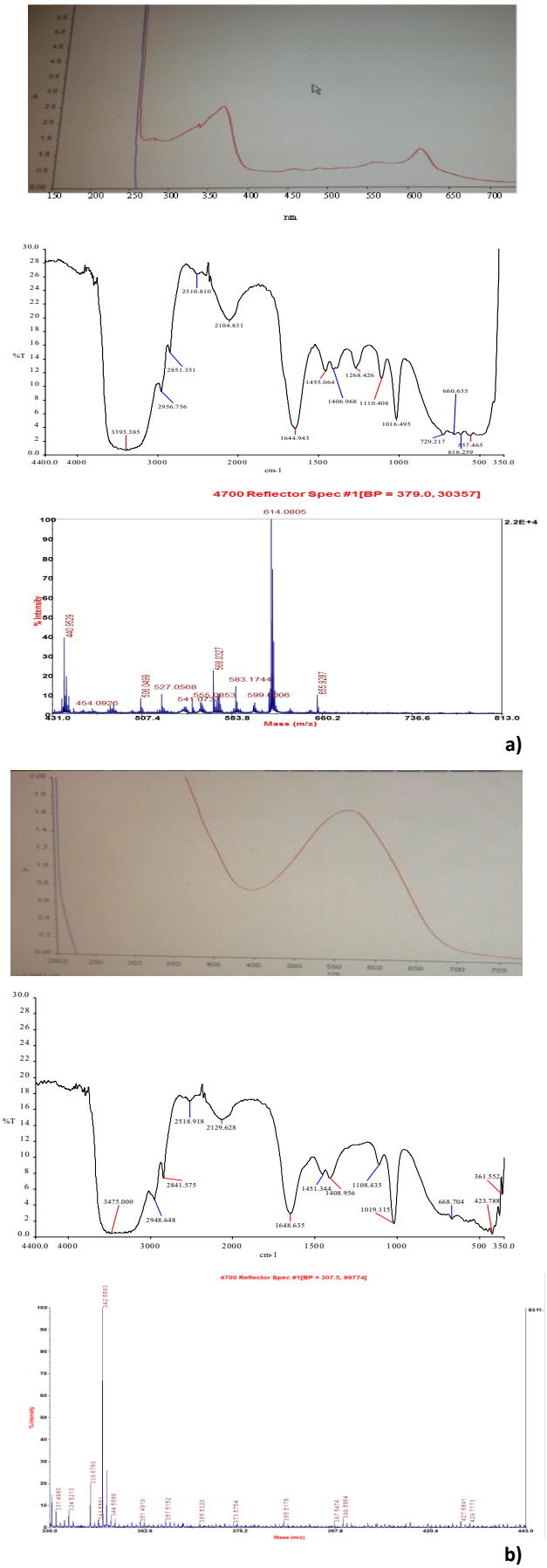


Figure 2. UV-Visible scanning, FT-IR, and mass spectrum for (a) PCB, (b) violacein

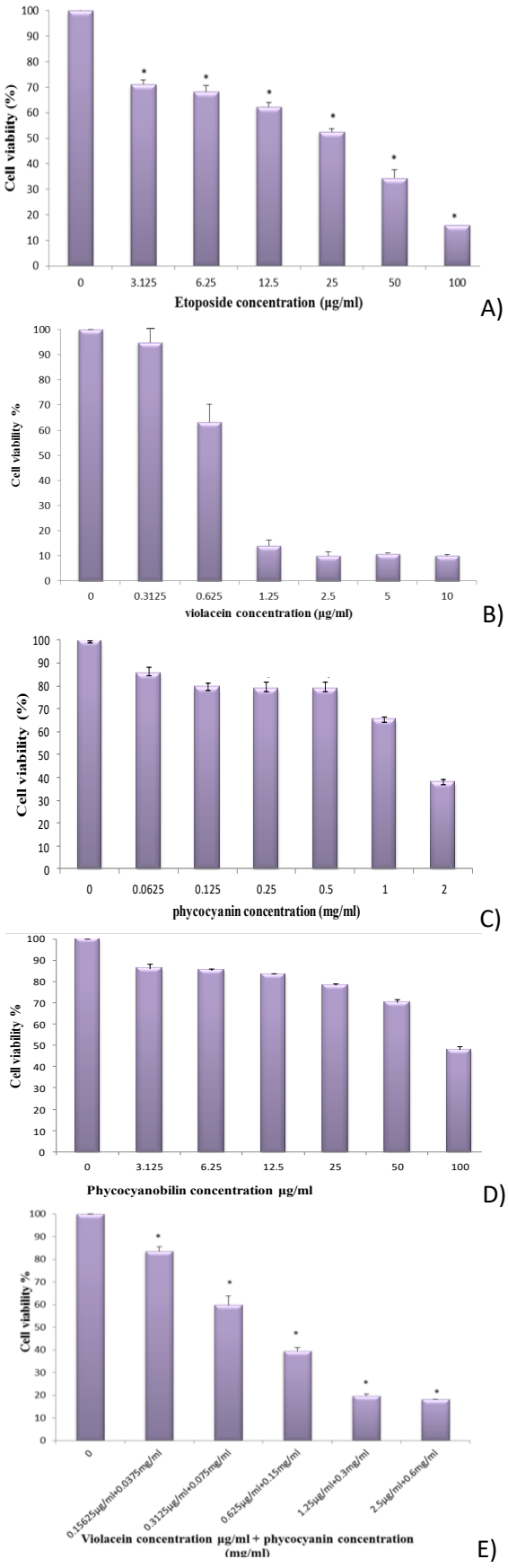
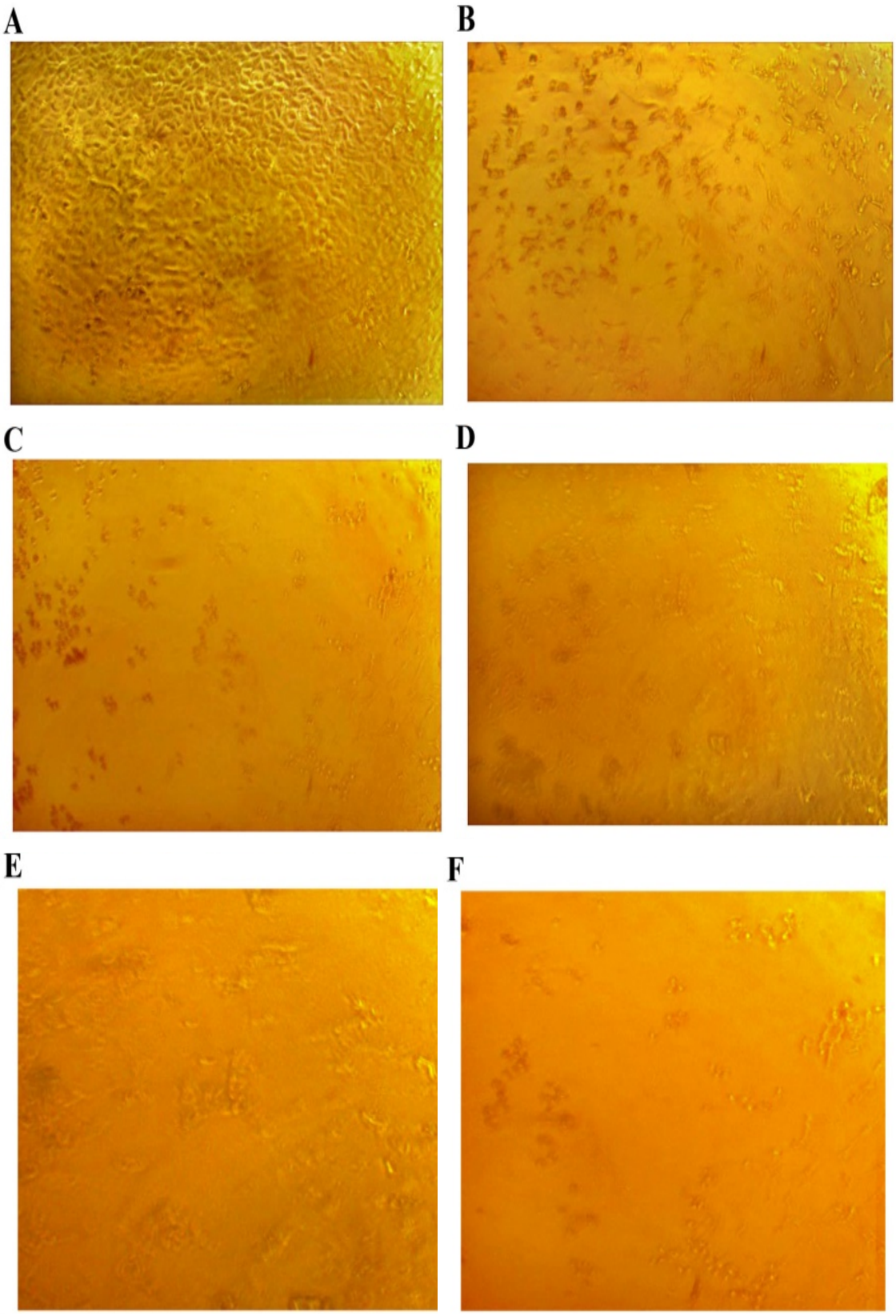


Figure 3. Viability % of untreated and treated MCF-7 cells with different concentration (A) etoposide, (B) violacein, (C) PC, (D) PCB, (E) combined therapy (violacein-PC).

1)



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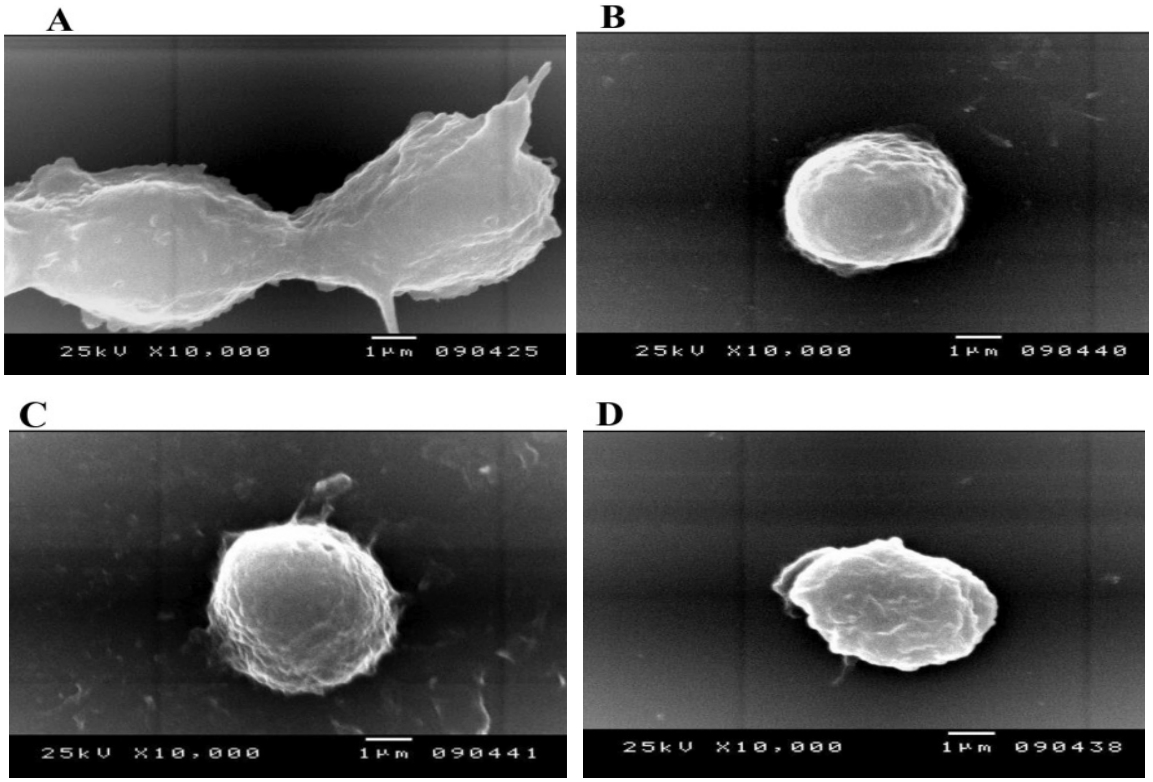

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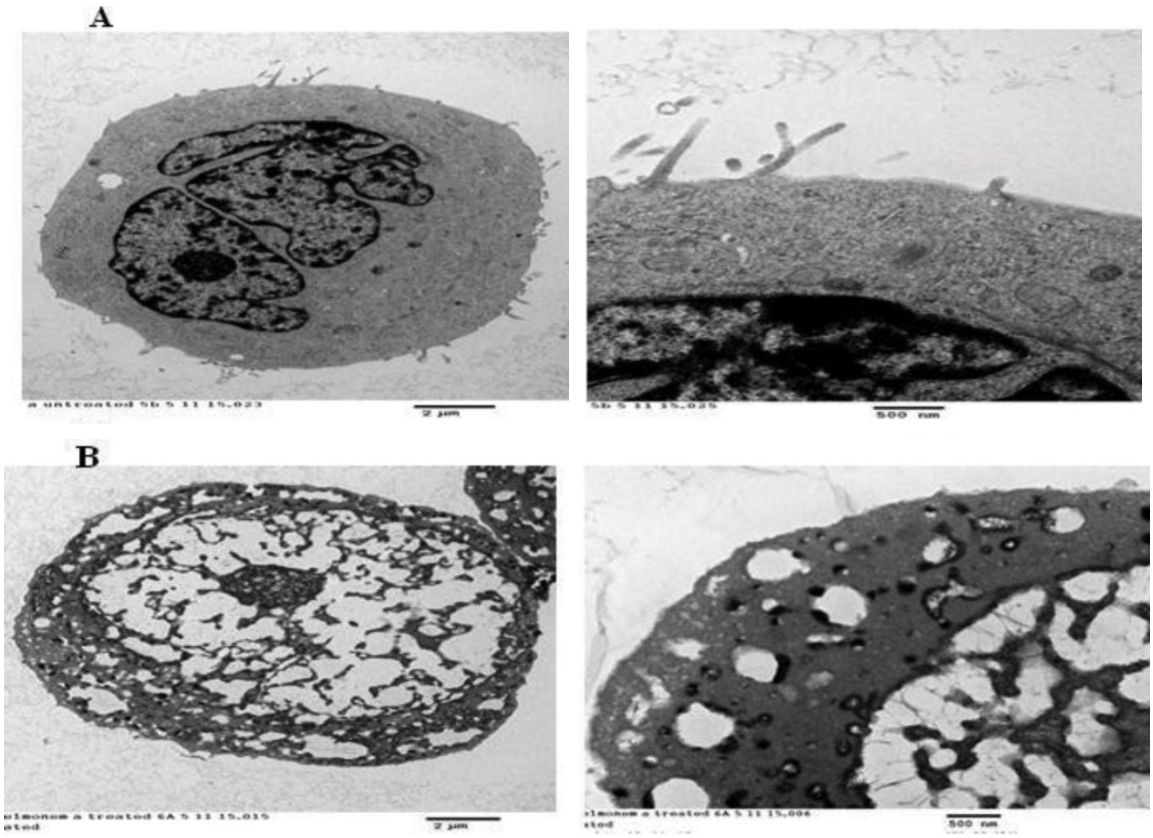

Figure 4. (1) The morphological change of (A): untreated cells, (B): etoposide, (C): violacein, (D): PC (E): PCB, (F): combined-treated MCF-7 cells. (2) Scanning electron microscope of (A): untreated cells, (B): violacein, (C) PC, (D) combined-treated MCF-7 cells. (3) Transmission electron microscope of (A): untreated cells. (B): combined- treated cells

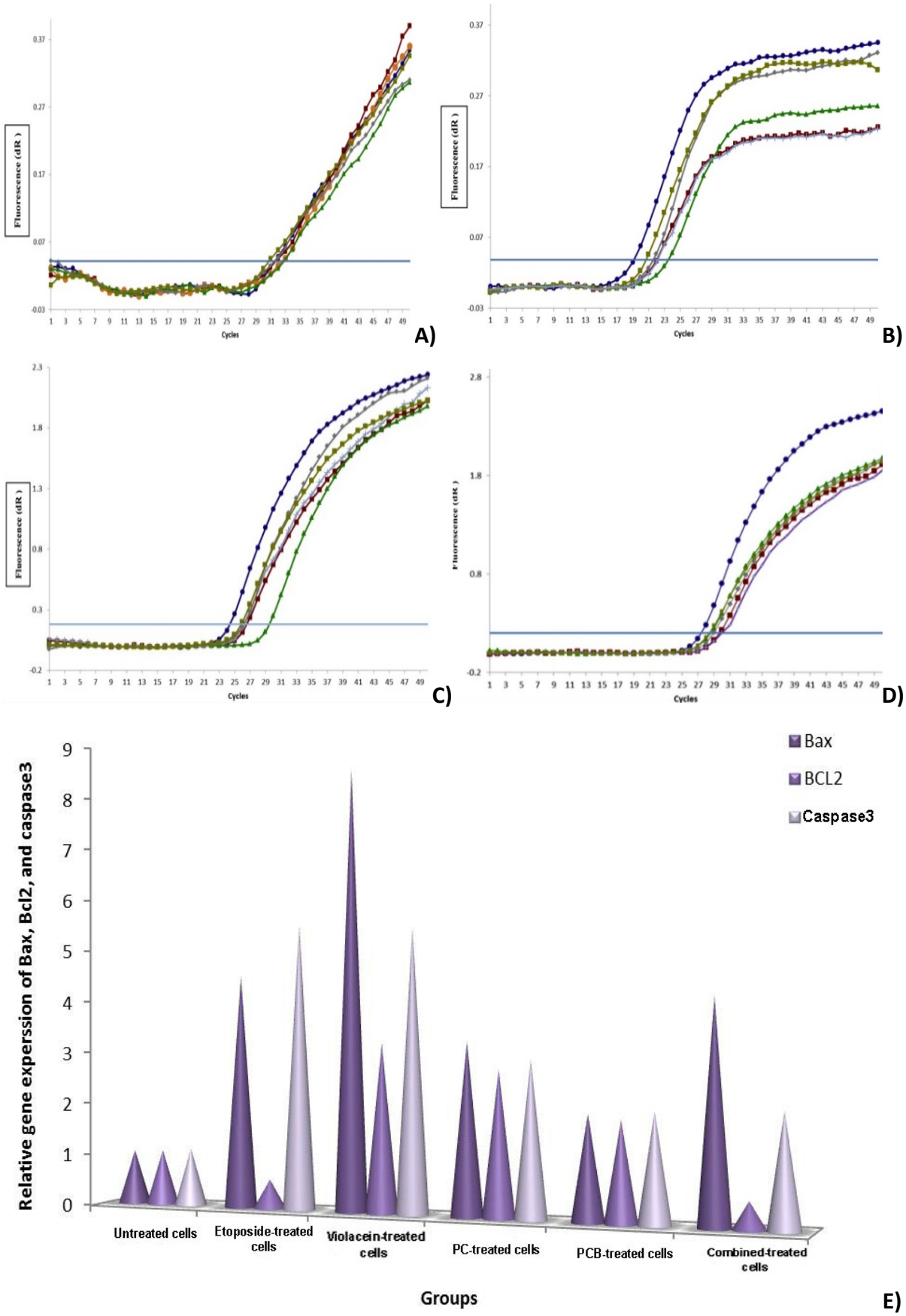

Figure 5. Amplification plot of (A) Bax, (B) Bcl-2, (C) caspase-3, (D) GDPH expression and (E) genes relative expression in different studied groups.

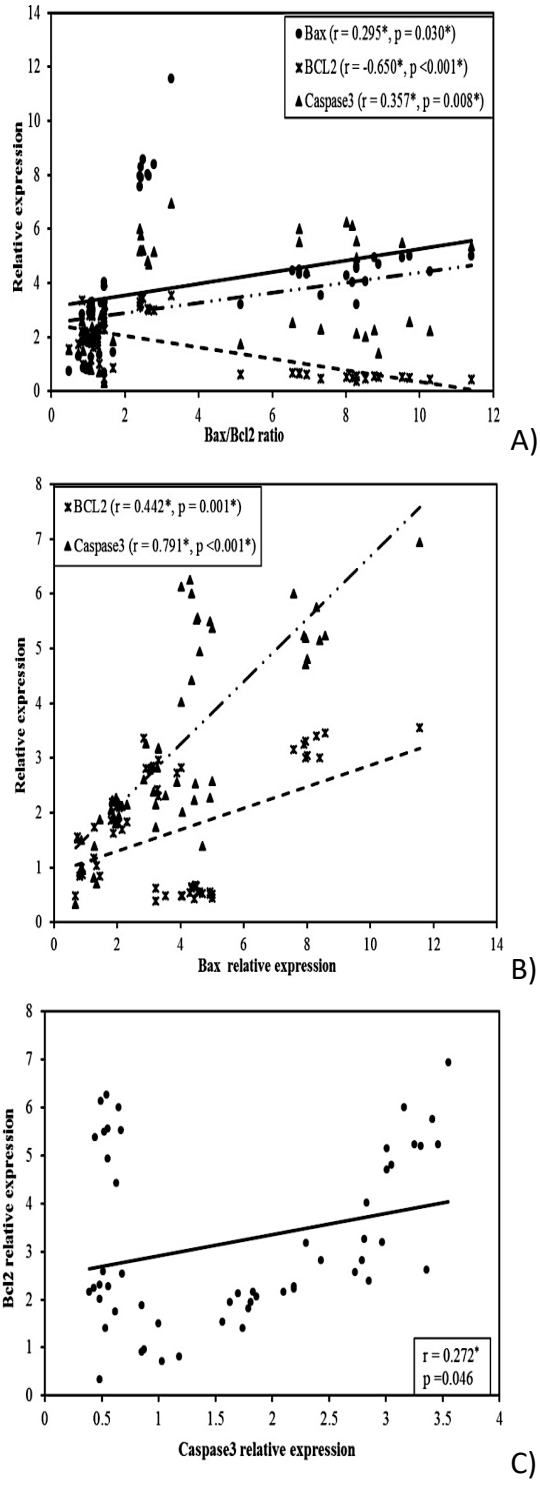

Figure 6. Correlation between the studied genes in total sample (n = 54): (A) Bax/Bcl-2 ratio and Bax, Bcl-2, caspase-3; (B) Bax and Bcl-2, caspase-3; (C) caspase-3 and Bcl-2.

induction of mitochondrial apoptotic pathway (intrinsic pathway). On the contrary, PC and PCB-treated cells exerted a significant decrease in Bax, and caspase-3 expression indicating that PC and PCB have less potent effect than etoposide.

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

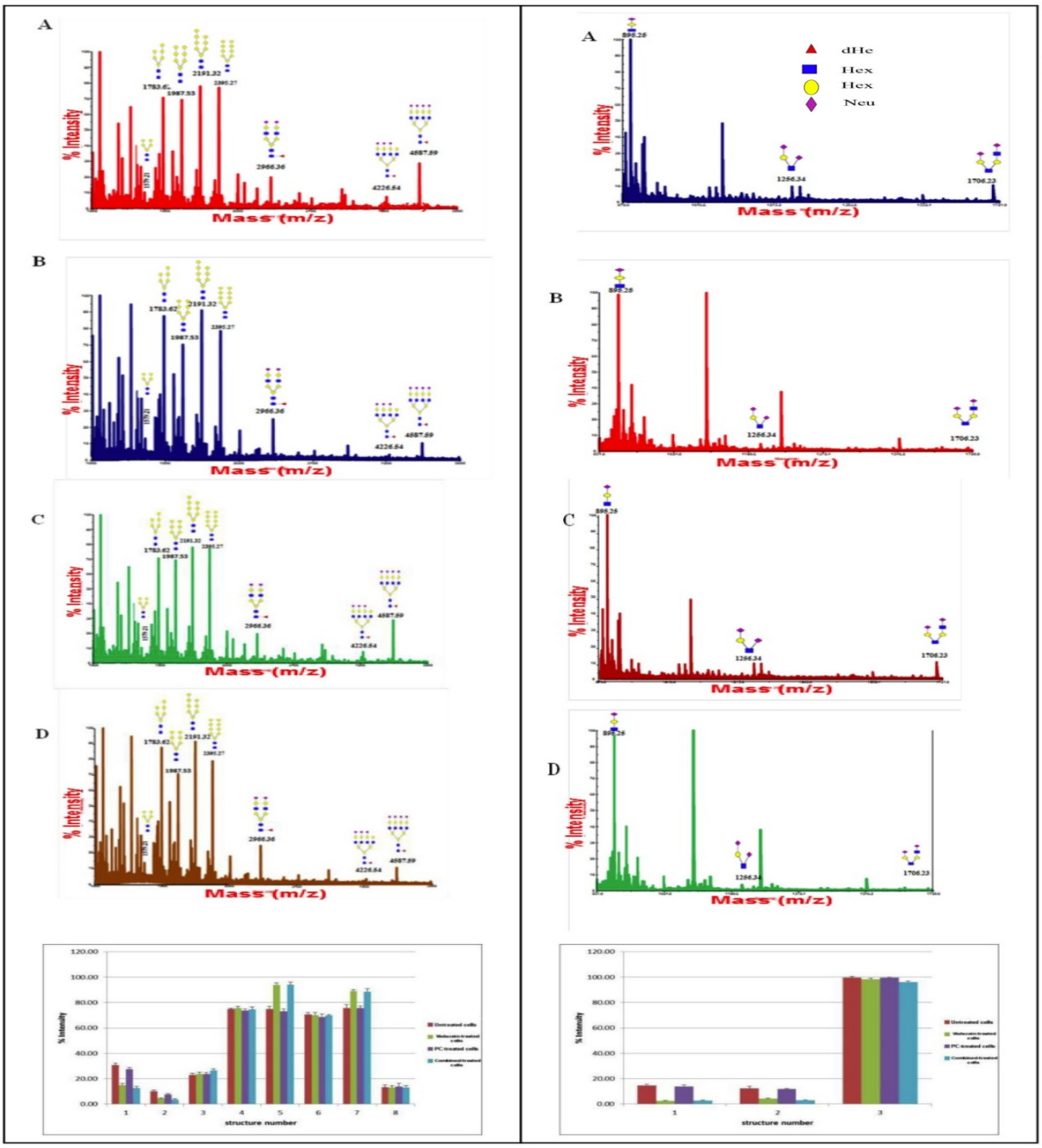

Figure 7. MS profile and intensity % of N-glycan (left) and O-glycan (right): (A) untreated; (B) violacein; (C) PC; (D) combined-treated MCF-7 cells.

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 sialylation prevents this signal amplification loop and disrupts the downstream apoptotic signaling cascades, thus enabling cancer to deactivate the main apoptotic pathway (Büll et al., 2014). Accordingly, treatment of MCF-7 cells with violacein or combined therapy may have an obvious role in reducing sialylation 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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REFERENCES

Ahmad WA, Yusof NZ, Nordin N, Zakaria ZA, Rezali MF (2012). Production and characterization of violacein by locally isolated Chromobacterium

violaceum grown in agricultural wastes. Appl Biochem Biotechnol, 167: 1220-1234.

Alshatwi, A.A., Subash-Babu, P., and Antonisamy, P. (2016). Violacein induces apoptosis in human breast cancer cells through up regulation of BAX, p53 and down regulation of MDM2. Exp Toxicol Pathol; 68: 89-97.

Beuhler, R.J., Pierce, R.C., Friedman, L., and Siegelman, H.W. (1979). Cleavage of phycocyanobilin from C-phycocyanin. Separation and mass spectral identification of the products. J Biol Chem; 251: 2405-2411.

Brockhausen, I., and Stanley, P. (2015). O-GalNAc Glycans. In: rd, Varki A, Cummings RD, Esko JD, Stanley P, Hart GW, Aebi M, Darvill AG, Kinoshita T, Packer NH, et al. editors. Essentials of Glycobiology. Cold Spring Harbor (NY).

Büll, C., Stoel, M.A., den, Brok, M.H.and Adema, G.J. (2014). Sialic acids sweeten a tumor's life. Cancer Res; 74: 3199-3204.

Chou, T.C., and Talalayn, P. (1984). Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul; 22: 27-55.

De Carvalho, D.D., Costa, F.T., Duran, N., and Haun, M. (2006). Cytotoxic activity of violacein in human colon cancer cells. Toxicol In Vitro; 20: 1514-1521.

De Leoz, M.L.A., Young, L.J., An, H.J., Kronewitter, S.R., Kim, J., Miyamoto., et al., (2011). High-mannose glycans are elevated during breast cancer progression. Mol Cell Proteomics; 10: M110.002717.

Demain, L.A., and Vaishnav, P. (2012). Natural products for cancer chemotherapy. Microb Biotechno; 4: 687-699.

Devanathan, J., and Ramanathan, N. (2012). Pigment production from Spirulina platensis using seawater supplemented with dry poultry manure. J Algal Biomass Utln; 3: 66-73.

Dwarakanath BS, Khaitan D, and Mathur R (2004). Inhibitors of topoisomerases as anticancer drugs: problems and prospects. Indian J Exp Biol; 42: 649-659.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., et al. (20.15). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer;136: 359-86.

Gantar, M., Simović, D., Djilas, S., Gonzalez, W.W., and Miksovska, J. (2012). Isolation, characterization and antioxidative activity of C-phycocyanin from Limnothrix sp. strain 37-2-1. J Biotechnol; 159: 21-26.

Ghoncheh, M., Mohammadian-Hafshejani, A., and Salehiniya, H. (2015). Incidence and Mortality of Breast Cancer and their Relationship to Development in Asia. Asian Pac J Cancer Prev; 16: 6081–6087.

Goetz, J.A., Mechref, Y., Kang, P., Jeng, M.H., and Novotny, M.V. (2009). Glycomic profiling of invasive and non-invasive breast cancer cells. Glycoconj J; 26: 117-131.

Häuselmann, I., and Borsig, L. (2014). Altered tumor-cell glycosylation promotes metastasis. Front Oncol; 4: 28.

Jiang, J., Li, L., Xie, M., Fuji, R., Liu, S., Yin, X., et al. (2015). SPATA4 counteracts etoposide-induced apoptosis via modulating Bcl-2 family proteins in HeLa cells. Biol Pharm Bull; 38: 1458-1463.

Jiang, L., Wang, Y., Liu, G., Liu, H., Zhu, F., Ji, H., and Li, B. (2018). C-Phycocyanin exerts anti-cancer effects via the MAPK signaling pathway in MDA-MB-231 cells. Cancer Cell Int;18: 12.

Jiang, L., Wang, Y., Zhu, F., Liu, G., Liu, H., Ji, H., et al. (2019). Molecular Mechanism of Anti-Cancer Activity of the Nano-Drug C-PC/CMC-CD59sp NPs in Cervical Cancer. J Cancer; 10: 92.

Kang, P., Mechref, Y., Klouckova, I., and Novotny, M.V. (2005). Solid- phase permethylation of glycans for mass spectrometric analysis. Rapid Commun Mass Spectrometry; 19: 3421-3428.

Kang, P., Mechref, Y., and Novotny, M.V. (2008). High-throughput solid-phase permethylation of glycans prior to mass spectrometry. Rapid Commun Mass Spectrom; 22: 721-734.

Konícková, R., Vanková, K., Vaníková, J., Vánová, K., Muchová, L., Subhanová, et al. (2014). Anti-cancer effects of blue-green alga Spirulina platensis, a natural source of bilirubin-like tetrapyrrolic compounds. Ann Hepatol; 13: 273-283.

Madhyastha, H., and Vatsala, T.M. (2010). Cysteine-rich cyanopeptide beta2 from Spirulina fusiformis exhibits plasmid DNA pBR322 scission prevention and cellular antioxidant activity. Indian J Exp Biol. 48: 486-493.

Mechref, Y., Hussein, A., Bekesova, S., Pungpapong, V., Zhang, M., Dobrolecki, L.E., and Novotny, M.V. (2009). Quantitative serum glycomics of esophageal adenocarcinoma and other esophageal disease onsets. J Proteome Res. 8: 2656-2666.

Mohan, J. (2005). Organic spectroscopy principles and applications, 2nd Edn. Narosa publishing House, Daryagani, Delhi.

Mokhtari, B., Homayouni, T.S., Baluch, N., Morgatskaya, E., Kumar, S., Das, B., and Yeger, H. (2017). Combination therapy in combating cancer. Oncotarget. 8: 38022-38043.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65: 55-63.

Patel, A., Mishra, S., Pawar, R., and Ghosh, P.K. (2005). Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. Protein Expr Purif. 40: 248-255.

Pfeffer, C., and Singh, A. (2018). Apoptosis: a target for anticancer therapy. Int J Mol Sci. 19: 448.

Romay, C., and Gonzalez, R. (2000). Phycocyanin is an antioxidant protector of human erythrocytes against lysis by peroxyl radicals. J Pharm Pharmacol. 52: 367-368.

Sagar, S., Esau, L., Moosa, B., Khashab, N.M., Bajic, V.B., and Kaur, M. (2014). Cytotoxicity and apoptosis induced by a plumbagin derivative in estrogen positive MCF-7 breast cancer cells. Anticancer Agents Med Chem. 14: 170-180.

Santhosh, T.V., and Muthusamy, P. (2017). Evaluation of in vitro cytotoxic effect of violacein produced by novel isolate Chromobacterium Vaccinii CV5 against the cervical and lung cancer cell. Asian J Pharm Clin Res. 10: 227-229.

Saran, S., Puri, N., Dut, Jasuja, N., kumar, M., and Sharma, G. (2016). Optimization, Purification and characterization of Phycocyanin from Spirulina platensis. Int J Appl Pure Sci Agri. 2: 2394-5532.

Schram, B.L., and Kroes, H.H. (1979). Structure of phycocyanobilin. Eur J Biochem. 19: 581–594.

Song, E., Hu, Y., Hussein, A., Yu, CY., Tang, H., and Mechref, Y. (2015). Characterization of the glycosylation site of human PSA prompted by missense mutation using LC–MS/MS. J proteome Res. 14: 2872-2883.

Swindall, A.F., and Bellis, S.L. (2011). Sialylation of the Fas death receptor by ST6Gal-I provides protection against Fas-mediated apoptosis in colon carcinoma cells. J Bio Chem. 286: 22982-22990.

Talabnin, K., Talabnin, C., Ishihara, M., Azadi, P., Wongkham, S., and Sripa, B. (2016). Differential expression of O-glycoprotein glycans in cholangiocarcinoma cell lines. Asian Pac J Cancer Prev. 17: 691–695.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J., and Jemal, A. (2015). Global cancer statistics, 2012. CA: a cancer J Clin. 65: 87-108.

Varki, A. (2017). Biological roles of glycans. Glycobiol. 27: 3-49.

Vitek, L., and Schwertner, H.A. (2007). The heme catabolic pathway and its protective effects on oxidative stress-mediated diseases. Adv Clin chem. 43: 1-57.

Wen, P., Hu, T.G., Wen, Y., Linhardt, R.J., Zong, M.H., Zou, Y.X., and Wu, H. (2019). Targeted delivery of phycocyanin for the prevention of colon cancer using electrospun fibers. Food Funct. 19: 1816-1825.

Yang, B., Johnson, T.S., Thomas, G.L., Watson, P.F., Wagner, B., Furness, P.N., and El Nahas, A.M. (2002). A shift in the Bax/Bcl-2 balance may activate caspase-3 and modulate apoptosis in experimental glomerulonephritis. Kidney Int. 62: 1301-1313.

Yue, Z., Zhang, W., Lu, Y., Yang, Q., Ding, Q., Xia, J., and Chen, Y. (2015). Prediction of cancer cell sensitivity to natural products based on genomic and chemical properties. Peer J. 3: e1425.

Zhang, S.H., and Huang, Q. (2013). Etoposide induces apoptosis via the mitochondrial-and caspase-dependent pathways and in non-cancer stem cells in Panc-1 pancreatic cancer cells. Oncol Rep. 30: 2765-2770.

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Material and methods*:* Material and methods should be described in sufficient details so that others can repeat the experiment. Reference to previously published work may be used to give methodological details, provided that said publications are readily accessible and in English. The code of ethics should be followed for all experiments use animals or human samples.

Statistical analysis of results*:* The statistical design and the models of statistical analysis must be described, as well as each of the statistical methods used. Sufficient statistical details must be given to allow replication of the statistical analysis. The experimental unit should be defined (e.g., individual or group of animals).

Results*:* Data are presented as tables and figures. Brief description of the results for each table and figure should be presented. Unpublished data can be mentioned when necessary.

Discussion*:* Should be separate from the Results section and should focus only on intra- and inter-data discussion (the data in the results section) as well as with the relative data in the literature. Don't repeat information already presented in the Introduction section. Start the first paragraph in the Discussion with a paragraph stating the rationale behind the study, the objectives, and the main findings. End Discussion with a short conclusion.

Acknowledgements*:* In this section, the authors may acknowledge (briefly) their support staff.

Conflict of interest*:* All papers with a potential conflict of interest must include a description/explanation in a separate heading.

Funding details: The authors should state the source of findings of the study (with research funder and/or grant number). If no fund, the authors should state that the study is self-funded.

References

Citation of references: In the text, references should be cited by the author(s) surname(s) and the year of publication (e.g. Salem, 2020). References with two authors should be cited with both surnames (e.g. Salem and Meshrif, 2021). References with three or more authors should be cited with the first author followed by et al. (in italics; e.g. Salem et al., 2021). Names of organizations used as authors (e.g. Food and Drug Administration) should be written out in full in the list of references and on the first mention in the text. Subsequent mentions may be abbreviated (e.g. FDA).

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- References from journal articles are formatted in APA as this example: Al-Amoudi WM (2018). Toxic effects of Lambda-cyhalothrin on the rat thyroid. Involvement of oxidative stress and ameliorative effect of ginger extract. Toxicology Reports, 5: 728-736.
- References from books or official reports are formatted as this example. Kebreab E, Dijkstra ANM, Bannink A, Gerrits WJJ, & France J (2006). Nutrient digestion and utilization in farm animals. CABI Publishing. Wallingford, UK.
- References from chapters or parts of books are formatted as this example. Nozière P, & Hoch T (2006). Modelling fluxes of volatile fatty acids from rumen to portal blood. In: Nutrient digestion and utilization in farm animals (Kebreab E, Dijkstra ANM, Bannink A, Gerrits WJJ & France J, eds.), pp. 40–47. CABI Publishing. Wallingford, UK.

Tables: The data should be presented in tables or in graphs, not both.

- Each table should be placed on a separate page at the end of the main text.
- Tables are numbered consecutively using Arabic numbering. They are referred to as Table 1, Table 2, etc., with capital 'T', no italics
- Each table has its explanatory caption. The caption is sufficient to permit the table to be understood without reference to the text.
- Abbreviations used in tables/figures have to be defined either as footnotes or in the caption.

Figures

- Package the figures in a single PowerPoint file. Each figure in a separate slide.
- Figure size should be readable in a width of approximately 8-175 mm (i.e. the maximum size of printing over two columns).
- Ensure that the font size is large enough to be readable at the final print size, use Calibri font to ensure that they are consistent throughout the figures.
- The figures should preferably be provided as TIFF or EPS files.
- The resolutions of figures must be at least 300 dpi.
- Preparation of images for a manuscript: For guidance, we refer to the Journal of Cell Biology's instructions to authors (http://jcb.rupress.org/site/misc/ifora.xhtml#image_aquisition).
- If a cropped image is included in the main text of a paper (e.g. a few lanes of a gel), display the full original image, including the appropriate controls, the molecular size ladder and/or the scale as relevant, as a single figure in a Supplementary Material file to facilitate peer-review and for subsequent online publication.
- Supplementary material is submitted along with the main manuscript in a separate file and identified at uploading as "Supplementary File – for Online Publication Only" The title of the article is included at the top of the supplementary material.

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