

# Quercetin and a plant substance, identified using a multi-copy number of DNA topoisomerase I in recombinant *Pichia pastoris*, inhibited MDA-MB-231 proliferation *via* different manners.

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## Research Article

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# Abstract

The study aimed to employ an *in vivo* strategy to construct a multi-copy number of human DNA topoisomerase I (hTopI) gene using pPIC3.5K vector in GS115 strain of *Pichia*. The yeast transformant (GS115-pPIC3.5K-hTopI) was then used to investigate the preliminary growth effect of a pure compound (quercetin) and a standardised subfraction of ethanolic red onion peel extract (F1). The underlying mechanisms of quercetin and F1 were then tested on MDA-MB-231 for the cell cycle profile and apoptosis by flow cytometry, and the mRNA expression of CYP genes by real-time PCR. His<sup>+</sup> yeast transformants (clones) with multi-copy inserts resistant to various concentrations of Geneticin were successfully selected in the study. The clones' cell density was likely to be unaffected; only the total protein expression and enzyme activity were increased following the increased copy number of hTop1 in the host. The clone that showed the target enzyme's highest activity is said to respond specifically to growth inhibitors, whereby both quercetin and F1 were proven to be potential growth inhibitors as assessed by the MTT assay. In the process, quercetin reduced cell proliferation by inducing apoptosis and cell cycle arrest (S phase only), whereas F1 reduced cell proliferation by inducing cell cycle arrest only (S and G2 phases). Quercetin and F1 induced CYP1A1 and CYP1B1 (carcinogenicity) gene mRNA expression, but only F1 induced CYP2S1 (cytotoxicity) gene mRNA expression in the treated cells, suggesting that both quercetin and F1 inhibited the cell proliferation of MDA-MB-231 *via* different manners. The newly developed GS115-pPIC3.5K-hTopI can be used to select various potential substances for breast cancer treatment in the future.

# Introduction

DNA topoisomerases play important roles in cellular processes by regulating the topological state of DNA [1]. The inhibition of DNA topoisomerase activities is a successful approach in screening anticancer agents, in which agents that react to the enzymes are predicted to inhibit the growth of cancer cells. Therefore, the enzymes can be exploited as scientific and clinical tools to rapidly screen new inhibitors *in vitro* as anticancer agents [2–5]. The expression of DNA topoisomerases has been previously demonstrated in a few works [6–8]. However, the use of multi-copy number gene strategies to express a large yield of specific enzymes, e.g., human DNA topoisomerase I (hTopI), which is said to increase the host's sensitivity towards growth inhibitors, has never been demonstrated previously. In many cases, expression using single-copy gene transformants has been disappointingly low. A multi-copy number of gene expression strategy that produces tandem inserts of a gene by ligation (*in vitro* strategy) has been utilised, which was shown to be one of the most effective strategies to increase gene expression [9], whereas a more popular *in vivo* strategy, which utilises hyper-resistance to an antibiotic that allows users to screen the multi-copy inserts, conferring resistance to the antibiotic Geneticin, has not yet been used to express hTopI.

More than 500 recombinant proteins have been successfully expressed using *Pichia pastoris* [9], where *Pichia* is a faster and cheaper eukaryotic expression system than baculovirus or mammalian tissue culture [10]. The increasing popularity of the *Pichia* expression system is due to a few factors, including

the availability of different strains commercially that can be used as expression systems, the simplicity of the techniques needed for molecular genetic manipulation and the similarity to those of *S. cerevisiae*. Second, *Pichia* can produce foreign proteins at high levels, both intracellularly and extracellularly. It can perform many eukaryotic post-translational modifications while being as easy to manipulate as *E. coli* and *S. cerevisiae* [11]. Taken together, the tightly regulated alcohol oxidase I promoter [12] and robust respiratory growth of this system that facilitates high cell densities [13–15] are also factors contributing to the rapid acceptance of this system over other expression systems. The preliminary screening of anticancer agents using cell-based assays is not encouraging because the assays are costly and require specific equipment, expertise, and specialised facilities. Therefore, it is better to have other alternatives to the screening process rather than relying on expensive and commercially available cell-based assays alone [16]; only candidate compounds that exhibit a positive effect at the early discovery stages will then proceed to the next drug discovery steps, to faster the preliminary screening process. In this study, a multi-copy number of the hTop1 gene was produced using the pPIC3.5K vector in the GS115 strain of *Pichia* to enhance the protein yield of interest in the host. It is hypothesised that the transformation of *Pichia* with multi-copy number genes may increase the target enzyme expression in the host to increase the sensitivity of the host to the testing compounds during the screening process.

This study also aimed to investigate the effects of the multi-copy number of hTop1 in *Pichia* (multi-copy insert transformants) on its cell density, total protein expression, and enzyme activity. The multi-copy insert transformants were then used to a preliminary screen of the growth inhibitory effect of quercetin (main flavonoid of red onion peel ethanolic extract) and a red onion peel ethanolic extract subfraction (F1). The F1, which was produced in-house, contains high quercetin levels that can be considered a potential anticancer. Numerous epidemiological studies have revealed that dietary intake of onion reduces the risk of developing breast cancer [17–18]. A large number of *in vitro* and *in vivo* studies have also been conducted to confirm the anticancer properties of red onion peels. However, the cellular mechanism and cell metabolism induced by F1 has not been studied in detail. The cytotoxic effect of both pure and plant substances was further confirmed in breast cancer using MDA-MB-231. This study also investigated the cell cycle profile, apoptosis induction and mRNA expression of CYP genes in quercetin- and F1-treated MDA-MB-231. The findings will facilitate multi-copy insert yeast transformants to discover more viable therapeutic agents for breast cancer treatment from natural products with bioavailable, safe, cost-effective and minimal side effect properties in the future.

## Materials And Methods

### Production of a multi-copy number insert in *Pichia* by an *in vivo* strategy

*Pichia*, which had a multi-copy number of hTop1 (target gene; insert), was first produced *via* an *in vivo* strategy. Each copy of hTop1 was carried by pPIC3.5K plasmid (9.0 kb; Invitrogen, Carlsbad, USA). In the process, the complete nucleotide sequence encoded hTop1 that had been generated in a previous study

[19] was excised from a pPICZ $\alpha$ -A-hTopI plasmid with the restriction endonucleases *EcoRI* (Thermo Fisher Scientific, Waltham, USA) and *NotI* (Thermo Fisher Scientific, Waltham, USA) in two separate enzymatic reactions. The sequence was then inserted into the pPIC3.5K plasmid at the *EcoRI* and *NotI* sites. The successful construction of the recombinant plasmid was then viewed by agarose gel electrophoresis and confirmed by sequencing. The extracted recombinant pPIC3.5K-hTopI plasmid was then linearised by digestion with restriction endonuclease *SalI* (Thermo Fisher Scientific, Waltham, USA) prior to transformation into *Pichia* strain GS115. The yeast transformants were then screened on agar plates containing various concentrations of Geneticin<sup>®</sup> (Invitrogen, Carlsbad, USA). The transformants that survived at a higher concentration of Geneticin<sup>®</sup> are said to have more copies of recombinant plasmids transferred into yeast cells. The yeast colonies were subjected to copy number insert screening as follows. A brief schematic of the overall construction is shown in Fig. 1.

## Screening the multi-copy number insert *Pichia* transformants

A 200  $\mu$ l aliquot of sterile yeast extract peptone dextrose (YPD; Merck, Darmstadt, Germany) broth was added to each well of a microtiter plate using the aseptic technique. Each well of the first microtiter plate was inoculated with a single colony of the selected transformant (from the above section) using sterile toothpicks to resuspend the cells. The cell suspension in the microtiter plate was incubated at 30°C without shaking. After 2 days, 10  $\mu$ l of each culture was added to 190  $\mu$ l sterile YPD broth in a new microtiter plate with the orientation marked as the first microtiter plate to keep track of the wells. The microtiter plate was incubated again as above. The process was repeated using the third microtiter plate to adjust the culture to be approximately similar visually to ensure equivalent numbers of cells were spotted on Geneticin<sup>®</sup> contained YPD plates. After overnight incubation, 10  $\mu$ l of cell suspension from each well of the third microtiter plate was spotted on YPD plates containing 0 (control), 0.25, 0.50, 0.75, and 1.00 mg/ml Geneticin<sup>®</sup> (Invitrogen, Carlsbad, USA). The cells were spotted on the agar in a regular pattern with a grid underneath the plate to ensure that an equal intensity of cells was spotted on the grid. The liquid of the cell suspension was allowed to soak in the gel, and then the plates were incubated at 30°C for 2–5 days to allow the colonies of Geneticin-resistant transformants to grow on the respective plates in the grid. Single colonies that could survive at different concentrations of Geneticin<sup>®</sup> were picked and re-streaking the selected colonies on respective Geneticin<sup>®</sup> contained YPD plates.

## Growth induction of the multi-copy number insert *Pichia* transformants

A single antibiotic-resistant colony of *Pichia* transformants (GS115-pPIC3.5K-hTopI) from each Geneticin<sup>®</sup> contained YPD plate (as above) was inoculated in 5 ml YPD medium. The cells were grown at 30°C overnight with shaking at 200 rpm. In a 1 L flask, 250 ml fresh buffered glycerol complex medium (BMGY; Merck, Darmstadt, Germany) was inoculated with 250  $\mu$ l of the overnight culture. The culture was grown again overnight until the optical density (OD) at 600 nm reached 2.0. The culture was harvested by centrifugation at 3,000  $\times$  g for 5 min at room temperature. Removed the sample's supernatant, and the

cell pellet was resuspended in 10 ml buffered methanol-complex medium (BMMY; Merck, Darmstadt, Germany). The cell suspension was diluted with BMMY to a final volume of 25 ml or until the culture reading at 600 nm reached 1.0. The cells were induced with 0.5% methanol every 24 h at 30°C for 96 h. The cells were collected every 12 h to measure the cell density that represented growth. The OD<sub>600</sub> was adjusted to 2.0 at 0 h of cultivation. The cells were also collected on ice for protein sample preparation.

## **Analysis of specific protein expression in the *Pichia* transformants by Western blotting**

For the cell pellets collected from each ml of sample on ice (as above), 100 µl of breaking buffer and an equal volume of acid-washed glass beads were added. The mixture was vortexed for 30 sec and then incubated for another 30 sec at room temperature for a total of 10 cycles. The mixture was then centrifuged at maximum speed for 10 min at 4°C. The clear supernatant was transferred to a fresh microcentrifuge tube for protein concentration analysis using the Protein Assay Kit (Bio-Rad Life Sciences, Hercules, USA) or stored at -80°C until use. Twenty (20) µg of protein from each sample was then used for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the distributed proteins on the gel were transferred onto a nitrocellulose membrane (GE Healthcare, Munich, Germany) by the semidry transfer method using a Trans-blot SD Semidry Transfer Cell (Bio-Rad Life Sciences, Hercules, USA). After protein transfer, nonspecific proteins on the membrane were blocked with 5% blocking solution for 2 h on a shaker. Then washed the membrane with TBST 3 times for 10 min each. The membrane was incubated with purified mouse anti-hTopI (1:5,000 dilution in TBST; BD Pharmingen, San Jose, USA) at 4°C overnight. The next morning, the membrane was washed again with TBST as above. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000 dilution in TBST; BD Pharmingen, San Jose, USA) for 1 h on a shaker. The membrane was then washed again with TBST as above. The protein signal development process was carried out in the darkroom, whereby the membrane was first overlaid with chemiluminescence substrate (Thermo Fisher Scientific, Waltham, USA). Cling wrap was then overlaid on the wet membrane, followed by X-ray film (GE Healthcare, Munich, Germany) exposure for approximately 5 min. The film was then immersed in developer solution (Sigma-Aldrich, Saint Louis, USA) for a few seconds, rinsed with tap water and then fixed with fixer solution (Sigma-Aldrich, Saint Louis, USA) for a few seconds. The developed film was then air-dried and photographed the signals on the X-ray film.

## **Determination of recombinant enzyme activity by DNA relaxation assay**

The protein samples, as prepared above, were also used for enzyme activity determination by DNA relaxation assay. The assay was performed in a total volume of 20 µl of reaction mixture containing 4 µl of 5X hTopI reaction buffer, 1 µl of 0.25 µg pBR322 plasmid DNA, and 1 µl of the protein sample [20]. Water was used instead of the protein sample in the reaction mixture for the negative control. All reactions were incubated at 37°C for 30 min. Then, 4 µl of 6X hTopI stopping buffer was added to the

reaction mixture to stop the reactions. The reaction mixture was then briefly centrifuged and electrophoresed on a 1% (w/v) agarose gel in 0.5X TBE at 30 V for 200 min. The gel was then stained with ethidium bromide for 30 min and destained by soaking it in distilled water for another 30 min. The gel was viewed under ultraviolet (UV) light and photographed with an Alpha Innotech Fluorchem FC2 (Fisher Scientific, Edmonton, Canada). The enzyme action is expressed as  $B/A \times 10^6 \text{ UL}^{-1}$ , whereby A is an appropriate volume of supernatant added to the reaction. In contrast, B is the dilution factor of the supernatant required to complete the relaxation of 0.25  $\mu\text{g}$  of pBR322 plasmid DNA.

## Screening quercetin and F1 effects by yeast-based assay

The *Pichia* transformants that exhibited the highest enzyme activity were retrieved with 5 ml of BMGY. The inoculated BMGY was grown at 30°C with shaking at 250 rpm for 18 h. After overnight incubation, the transformants' cell density was adjusted by consecutive serial dilution with a dilution factor of 1:20. Approximately 5  $\mu\text{l}$  of the transformants with a dilution factor of  $7.81 \times 10^{-10}$  was then spotted in triplicate on the microbiological agar plates of YPD that contained 0  $\mu\text{g/ml}$  (control), 25  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$ , 75  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$  quercetin (Cayman Chemical, Michigan, USA) or F1 [21]. The control contained < 1% of dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). The cells were then spotted on the agar in a regular pattern with a grid underneath the plate to ensure the equal intensity of the cell spotting on the grid. The cell suspension liquid was allowed to soak in the gel, and the plates were incubated at 30°C for 3 days. Pictures of the colonies were taken consecutively for 3 days to observe the transformants' growth in the plates.

## Breast cancer cell culture, seeding and treatment

The MDA-MB-231 (ATCC®HTB-26™) cell line was purchased from the American Type Culture Collection (ATCC, Virginia, USA). The cells were cultured in the complete growth medium in T-25 or T-75 tissue culture flasks (Nunc, Roskilde, Denmark). The growth medium contained high glucose Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific Inc.) supplemented with 10% (v/v) foetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100 units/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin. The MDA-MB-231 was incubated at 37°C in a humidified atmosphere of 5% (v/v) carbon dioxide ( $\text{CO}_2$ ) and grown with a doubling time of approximately 38 h. The MDA-MB-231 was trypsinised, when the number of cells in the culture flasks had reached sufficient growth. The cells were seeded in 96-well plates at a cell density of  $2 \times 10^4$  cells/ml for growth inhibitory effect analysis or in 6-well plates at a cell density of  $1.2 \times 10^6$  cells/ml for cell cycle, apoptotic effect and CYP mRNA expression analyses. The cells were then subjected to treatment with 0.5% DMSO (control) or 60  $\mu\text{g/ml}$  quercetin or 50  $\mu\text{g/ml}$  F1 in growth media that contained only 2% FBS. The treated cells were incubated at 37°C in a 5% (v/v)  $\text{CO}_2$  incubator for 24, 48 and 72 h prior to further analysis.

## Growth inhibitory effect analysis of quercetin and F1 by MTT assay

The cells treated with quercetin and F1 in 96-well plates, as described above, at the end of each incubation period, were added with 24 µl of 5 mg/ml MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) in each well. The reaction was incubated for 4 h. Carefully removed the solution without disturbing the formazan crystals formed in each well. Next, 100 µl acidified isopropanol (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and agitated to promote homogeneous colour development. Following colour development, the colour's intensity in each well was read at 570 nm using an ELISA plate reader (Tecan, Männedorf, Switzerland).

## **Cell cycle analysis of quercetin- and F1-treated MDA-MB-231 by flow cytometry**

The cells treated with quercetin and F1 in 6-well plates, as described above, were trypsinised at the end of each incubation period, and the cell suspension was transferred into falcon tubes. The cell suspension with the old medium in the tube was centrifuged at 1,000 g for 5 min. The supernatant was then discarded, and the cell pellet was washed with PBS. After washing, a  $1 \times 10^6$  cell suspension was transferred into new falcon tubes, and the cell suspension was then centrifuged as above. The cells were then fixed and permeabilised by adding 70% ice-cold absolute ethanol dropwise with gently mixing. The cell suspension was then stored at 4°C overnight to allow fixation. The ethanol-fixed cells were centrifuged at 1,000 g for 10 min and washed by resuspending the cells in PBS. The washed cells were then stained with 500 µl FxCycle™ propidium iodide (PI)/RNase Staining Solution (Thermo Fisher Scientific, Massachusetts, USA) in the dark for 30 min. The stained samples were transferred into new sterile flow tubes and kept on ice until subjected to flow cytometry analysis using a BD FACSCalibur (BD Biosciences, New Jersey, USA). Analysed cell cycle distribution from a total of 15,000 events with Cell Quest software 3.3 (BD Biosciences, New Jersey, USA). The percentage of cells in each phase was analysed and plotted as a DNA histogram using ModFit LT™ software (BD Biosciences, New Jersey, USA). The entire analysis was performed at the Advanced Medical and Dental Institute (AMDI), USM in Bertam, Penang.

## **Apoptotic effect analysis of quercetin- and F1-treated MDA-MB-231 by flow cytometry**

The cells treated with quercetin and F1 in 6-well plates, as described above, were also subjected to apoptotic effect analysis using the Annexin V-FITC Apoptosis Detection Kit (Thermo Fisher Scientific, Massachusetts, USA). Briefly, the trypsinised cells collected in 15 ml falcon tubes were centrifuged at 400 g for 4 min at 4°C to remove the old medium. Then, the cells were washed twice with 3 ml of cold PBS. The cell suspension was counted, and  $5 \times 10^5$  cells/ml was transferred into new falcon tubes. The cells were centrifuged to remove the supernatant, and then the cell pellet was resuspended in 200 µl of 1X binding buffer. A volume of 5 µl Annexin V-FITC was then added to 195 µl of the cell suspension, mixed well, and incubated in the dark at room temperature for 10 min. The cells were then washed with 200 µl of 1X binding buffer. The cells were resuspended in 190 µl of 1X binding buffer prior to the addition of 10 µl of PI (20 µg/ml). The mixture was transferred to a new sterile tube for flow cytometry analysis using a BD

FACSCalibur (BD Biosciences, New Jersey, USA) with 488 nm excitation, 655–730 nm emission for PI, and 525 nm emission for FITC.

## **CYP expression analysis in quercetin- and F1-treated MDA-MB-231 by real-time PCR**

The cells treated with quercetin and F1 in 6-well plates, as described above, were also subjected to total RNA extraction using the TRIzol Total RNA Isolation Reagent (Life Technologies Corporation, Carlsbad, CA, USA). The extracted total RNA pellet was then air-dried for 15 min and resuspended in 25 µl RNase-free water. Only the extracted total RNA with an absorbance A260/A230 ratio within 1.8-2.0 and an A60/280 ratio within 2.0-2.2, assessed using a NanoDrop™ 2000C spectrophotometer (Thermo Fisher Scientific, USA), was used for cDNA conversion. The bands that indicated a good quality of RNA, assessed by 1% (w/v) agarose gel electrophoresis, were visualised and captured under UV light by an image analyser (Syngene, Cambridge, UK). The extracted total RNA was then reverse transcribed to cDNA using a Tetro cDNA Synthesis Kit (Bioline, London, UK). Kept the reverse transcription products (cDNA) at -20°C until the products were used for real-time PCR gene expression analysis. All primers were designed using Primer Express Software v3.0.1 (Thermo Fisher Scientific, Massachusetts, USA; Table 1). All oligonucleotide primers ordered from Integrated DNA Technologies (Coralville, USA) were packed in desalted lyophilised form. The primers' stocks were dissolved in RNase-free water (Sigma-Aldrich, St. Louis, USA) to generate a final 100 µM concentration of each primer solution. The primer solutions were stored at -20°C until use. Real-time PCR was performed using an Agilent AriaMx Real-Time PCR System (Agilent, California, USA). The PCR cocktail was prepared by adding 10 µl iTaq™ Universal SYBR Green Supermix (Bio-Rad, California, USA), 0.8 µl of 10 µM forward and reverse primer each for the gene of interest (GOI) and 50 ng cDNA diluted in nuclease-free water to a total volume of 20 µl in each well of 0.1 ml, 8-tube qPCR strips (AITbiotech, Singapore). The reactions were initiated with a hot start at 95°C for 30 sec, followed by 40 cycles of amplification at 95°C for 15 sec as the denaturation step and 60°C for 1 min as the annealing and extension step. The reactions were continued at 1 cycle of thermal profile consisting of 95°C for 15 sec, followed by 60°C for 1 min, 95°C for 30 sec and 60°C for 15 sec to generate a melting curve. The expression value of GOI was normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whereby the relation between the normalised GOI in treated and vehicle control samples was determined by the fold change using the following formula:  $2^{(-\Delta\Delta Ct)}$  [22]. The fold change value for the vehicle control was set as 1, whereas an expression change value > 1 represented upregulation and that of < 1 represented downregulation of the gene.

Table 1  
The list of primers used for real-time PCR.

| Gene   | Primer Sequence                         | Size amplicon |
|--------|---|---------------|
| CYP1A1 | Forward 5'-TCAGGAGAAGCAGCTGGATGA-3'     | 76 bp         |
|        | Reverse 5'-GAGGTCCAAGACGATGTTAATGATC-3' |               |
| CYP1B1 | Forward 5'-ATCAGGTGAGGTGTGCTCCAT-3'     | 70 bp         |
|        | Reverse 5'-TCTCCCAGAAGCTCCTGCATA-3'     |               |
| CYP2S1 | Forward 5'-GACAGGGTTAATGTCTCCAGAGTGT-3' | 78 bp         |
|        | Reverse 5'-GGACAGACTCCGGAAAACAACT-3'    |               |
| GAPDH  | Forward 5'-ACAGCCTCAAGATCATCAGCA-3'     | 137 bp        |
|        | Reverse 5'-AGTCTTCTGGGTGGCAGTGAT-3'     |               |

## Statistical analysis

The dose-response growth curves were generated using GraphPad Prism version 8.2.1 for Windows (GraphPad Software Inc., La Jolla, CA, USA). The statistical analysis was presented as the mean  $\pm$  SD of triplicate determinations. The Student's *t*-test was used to analyse the cell density, total protein expression and enzyme activity to compare mean values between 2 datasets. In contrast, one-way analysis of variance (ANOVA) was used to analyse the mRNA gene expression with an additional Dunnett's multiple comparisons test to compare 2 among 3 or more datasets. Data analysis was also performed for photographs using ImageJ scientific software. The level of significance was set at  $\alpha = 0.05$  (95% confidence interval), where the confidence levels were indicated as statistically significant by asterisks \* for *p*-values < 0.05, \*\* for *p*-values < 0.01 and \*\*\* for *p*-values < 0.001.

## Results

### The constructed recombinant plasmid for in vivo strategy yeast transformation

Figure 2 shows a single copy of the hTop1 fragment (~ 2,717 bp) that was ligated into the pPIC3.5K plasmid (~ 8,575 bp) at the *EcoRI* and *NotI* sites, producing the pPIC3.5K plasmid containing a single copy of hTop1 (pPIC3.5K-hTop1). The recombinant pPIC3.5K-hTop1 plasmid, which was expected to be ~ 11,292 bp in length, was then linearised with the *SalI* prior to the transformation *Pichia* via an *in vivo* strategy to determine the effect of gene copy number on cell growth and total protein expression in the yeast transformants. The pPIC3.5K-hTop1 plasmid was digested with *EcoRI* and *NotI* to confirm that all extracted plasmids harboured the correct insert. The extracted plasmid DNAs were also sent for

sequencing to verify that each plasmid's correct insert was cloned. The nucleotide sequence (Submission # 2391932) of the insert in the plasmid is shown in Fig. 3. The results indicated that the recombinant pPIC3.5K-hTop1 plasmid was successfully constructed.

## In vivo screening of multi-copy insert transformants

The multi-copy inserts of His<sup>+</sup> transformants were produced post linearised pPIC3.5K-hTop1 plasmid DNA was transformed into the *Pichia* strain of GS115. As many as 68 yeast transformants (GS115-pPIC3.5K-hTop1) were selected for subculturing from the microtiter plate. From our observations, clones 46, 35, 19 and 50 were selected from the plates that contained 0.25, 0.50, 0.75, and 1.00 mg/ml Geneticin, respectively (Fig. 4A-D). The selection was based on the clones' ability to survive on agar plates containing various antibiotic concentrations. The *Pichia* transformants (GS115-pPIC3.5K-hTop1), which were successfully constructed *via* an *in vivo* strategy and resistant to various Geneticin concentrations, were subjected later to cell growth (density) and total protein expression analyses.

## Cell growth and total protein expression of the transformants

Figure 5A shows the cell density of the collected cultures every 12 h, whereby the cell density increased with the incubation times. The highest cell density was observed in the GS115-pPIC3.5K-hTop1 resistant to 0.50 mg/ml Geneticin at 96 h of incubation. The absorbance of the cell density of the His<sup>+</sup> transformants at this time point was 11.39 units at OD<sub>600</sub>. However, increasing the number of inserts and incubation time did not increase the transformants' cell density. The transformants resistant to 0.25 mg/ml Geneticin at 60 and 84 h of incubation times showed similar cell densities: 11.19 units and 11.33 units at OD<sub>600</sub>, respectively. Similarly, the increase in insert copy number and incubation time did not increase the expression level of total protein in the transformants. In brief, the highest total protein expression was obtained in the clone that was resistant to 1.00 mg/ml Geneticin at 48 h of incubation and 0.75 mg/ml Geneticin at 60 h of incubation (Fig. 5B). The expression levels of these transformants' total protein were 1.76 and 1.75 mg/ml, respectively. Other GS115-pPIC3.5K-hTop1 strains that showed similar total protein expression levels were also observed in the transformants resistant to 1.00 mg/ml Geneticin at 24 h of incubation (1.70 mg/ml) and 1.00 mg/ml Geneticin at 84 h of incubation (1.72 mg/ml). In summary, the level of total protein expression in the clones of GS115-pPIC3.5K-hTop1, which were resistant to various concentrations of Geneticin, showed the GS115-pPIC3.5K-hTop1 that showed the highest level of total protein expression and resistance to 0.25 mg/ml Geneticin was the clone collected at 24 h of incubation (1.60 mg/ml). The GS115-pPIC3.5K-hTop1 that showed the highest level of total protein expression and resistance to 0.50 mg/ml Geneticin was the clone collected at 36 h of incubation (1.68 mg/ml), the GS115-pPIC3.5K-hTop1 that showed the highest level of total protein expression and resistance to 0.75 mg/ml Geneticin was the clone collected at 60 h of incubation and (1.75 mg/ml), and the GS115-pPIC3.5K-hTop1 that showed the highest level of total protein expression and resistance to 1.00 mg/ml Geneticin was the clone collected at 48 h of incubation (1.76 mg/ml) (Table 2). These four clones were selected for comparison for subsequent experiments. Although the highest expression level

of total protein was found in the selected transformants, normalisation of the increased total protein expression level in all transformants with cell density per se did not show any statistical significance.

Table 2

Cell density and total protein expression by GS115-pPIC3.5K-hTop1 were resistant to various Geneticin concentrations by spectrophotometry and Bio-Rad protein assays. All clones were cultured on antibiotic contained agar plates for 96 h.

| Time Point (hour) | Geneticin® Concentration (mg/ml) | Cell Density (Absorbance OD <sub>600</sub> ) | Total Protein (mg/ml) | Total protein per hour (mg/ml/hour) | Total Protein per cell density (mg/ml/unit) |
|-------------------|----------------------------------|--|-----------------------|-------------------------------------|---|
| 12                | 0.25 mg/ml                       | 7.38   | 1.02                  | 0.08                                | 0.14  |
|                   | 0.50 mg/ml                       | 5.14   | 1.40                  | 0.12                                | 0.27  |
|                   | 0.75 mg/ml                       | 8.33   | 0.69                  | 0.06                                | 0.08  |
|                   | 1.00 mg/ml                       | 7.60   | 1.68                  | 0.14                                | 0.22  |
| 24                | 0.25 mg/ml                       | 7.75   | 1.60                  | 0.07                                | 0.21  |
|                   | 0.50 mg/ml                       | 5.45   | <b>1.60</b>           | 0.07                                | 0.29  |
|                   | 0.75 mg/ml                       | 8.15   | 1.35                  | 0.06                                | 0.17  |
|                   | 1.00 mg/ml                       | 7.48   | 1.70 #                | 0.07                                | 0.23  |
| 36                | 0.25 mg/ml                       | 9.09   | 1.15                  | 0.03                                | 0.13  |
|                   | 0.50 mg/ml                       | 7.57   | <b>1.68</b>           | 0.05                                | 0.22  |
|                   | 0.75 mg/ml                       | 9.72   | 1.52                  | 0.04                                | 0.16  |
|                   | 1.00 mg/ml                       | 9.70   | 1.50                  | 0.04                                | 0.15  |
| 48                | 0.25 mg/ml                       | 8.65   | 0.99                  | 0.02                                | 0.11  |
|                   | 0.50 mg/ml                       | 7.56   | 1.59                  | 0.03                                | 0.21  |
|                   | 0.75 mg/ml                       | 9.49   | 1.39                  | 0.03                                | 0.15  |
|                   | 1.00 mg/ml                       | 9.16   | <b>1.76 #</b>         | 0.04                                | 0.19  |
| 60                | 0.25 mg/ml                       | 11.19 #                                      | 0.86                  | 0.01                                | 0.08  |
|                   | 0.50 mg/ml                       | 9.02   | 1.20                  | 0.02                                | 0.13  |
|                   | 0.75 mg/ml                       | 10.69  | <b>1.75 #</b>         | 0.03                                | 0.16  |
|                   | 1.00 mg/ml                       | 10.50  | 1.21                  | 0.02                                | 0.12  |
| 72                | 0.25 mg/ml                       | 10.72  | 1.04                  | 0.01                                | 0.10  |
|                   | 0.50 mg/ml                       | 9.39   | 1.16                  | 0.02                                | 0.12  |

" # " High level of cell density and total protein expression in the cultures. "Bold" Clones that were selected for subsequent experiments.

| Time Point (hour) | Geneticin® Concentration (mg/ml) | Cell Density (Absorbance OD <sub>600</sub> ) | Total Protein (mg/ml) | Total protein per hour (mg/ml/hour) | Total Protein per cell density (mg/ml/unit) |
|-------------------|----------------------------------|--|-----------------------|-------------------------------------|---|
|                   | 0.75 mg/ml                       | 9.44   | 1.53                  | 0.02                                | 0.16  |
|                   | 1.00 mg/ml                       | 9.78   | 1.59                  | 0.02                                | 0.16  |
| 84                | 0.25 mg/ml                       | 11.33 #                                      | 0.82                  | 0.01                                | 0.07  |
|                   | 0.50 mg/ml                       | 10.18  | 1.05                  | 0.01                                | 0.10  |
|                   | 0.75 mg/ml                       | 10.23  | 1.11                  | 0.01                                | 0.11  |
|                   | 1.00 mg/ml                       | 9.78   | 1.72 #                | 0.02                                | 0.18  |
| 96                | 0.25 mg/ml                       | 10.93  | 1.48                  | 0.02                                | 0.14  |
|                   | 0.50 mg/ml                       | 11.39 #                                      | 1.45                  | 0.02                                | 0.13  |
|                   | 0.75 mg/ml                       | 9.59   | 1.57                  | 0.02                                | 0.16  |
|                   | 1.00 mg/ml                       | 9.08   | 1.18                  | 0.01                                | 0.13  |

“ # ” High level of cell density and total protein expression in the cultures. “Bold” Clones that were selected for subsequent experiments.

## Specific protein expression and enzyme activity of hTopI on selected clones

The expression analysis showed that a protein band of approximately 91 kDa was observed in the total protein of all four selected clones (Fig. 6A). The 91 kDa protein was detected by the antibody purified from mouse anti-human DNA TopI, indicating that the protein of interest was successfully expressed in the multi-copy gene of His<sup>+</sup> transformants using the *in vivo* strategy (Fig. 6B). The clones of GS115-pPIC3.5K-hTopI resistant to 0.25 mg/ml Geneticin and were collected at 24 h of incubation, resistant to 0.50 mg/ml Geneticin and were collected at 36 h of incubation, resistant to 0.75 mg/ml Geneticin and were collected at 60 h of incubation, and resistant to 1.00 mg/ml Geneticin and were collected at 48 h of incubation, were then subjected to the determination of the activity of hTopI. The clones' hTopI activity was assayed based on the ability of hTopI to relax pBR322 supercoiled DNA. Figure 6C shows that hTopI expressed by GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations could successfully relax the pBR322 supercoiled DNA compared with the supercoiled DNA in the reaction without hTopI. The supercoiled DNA migrated faster than the relaxed form of DNA due to the smaller size of the DNA, and hence, the band of supercoiled DNA appeared lower than the relaxed form of the DNA on the same agarose gel. The enzyme activity of hTopI in GS115-pPIC3.5K-hTopI resistance to various Geneticin concentrations is also summarised in Table 3. The highest enzyme activity of hTopI was observed in the

culture expressed by GS115-pPIC3.5K-hTop1 resistant to 1.00 mg/ml Geneticin ( $19.7 \times 10^4 \text{ UL}^{-1} \text{ OD}_{600}^{-1}$ ). The enzyme activity was approximately 3 times greater than the enzyme activity of hTop1 in GS115-pPIC3.5K-hTop1 resistant to 0.25 mg/ml Geneticin ( $7.74 \times 10^4 \text{ UL}^{-1} \text{ OD}_{600}^{-1}$ ). The enzyme activities of hTop1 in GS115-pPIC3.5K-hTop1 resistant to 0.50 and 0.75 mg/ml Geneticin were  $10.6 \times 10^4 \text{ UL}^{-1} \text{ OD}_{600}^{-1}$  and  $9.35 \times 10^4 \text{ UL}^{-1} \text{ OD}_{600}^{-1}$ , respectively. This phenomenon demonstrated that the enzyme activity of hTop1 produced in GS115-pPIC3.5K-hTop1 was likely to be proportional to the level of antibiotic resistance or increased with the target gene's increment copy number in each clone.

Table 3  
The activity of hTop1 in the clones of GS115-pPIC3.5K-hTop1 resistant to various Geneticin concentrations.

| Clone resistant to Geneticin® (mg/ml) | OD <sub>600</sub> of culture | Intracellular enzyme activity (UL <sup>-1</sup> OD <sub>600</sub> <sup>-1</sup> ) | Total enzyme activity (UL <sup>-1</sup> ) |
|---------------------------------------|------------------------------|---|---|
| 0.25                                  | 7.75                         | $7.74 \times 10^4$  | $3.02 \times 10^6$                        |
| 0.50                                  | 7.57                         | $10.6 \times 10^4$  | $4.02 \times 10^6$                        |
| 0.75                                  | 10.69                        | $9.35 \times 10^4$  | $5.02 \times 10^6$                        |
| 1.00                                  | 9.16                         | $19.7 \times 10^4$  | $9.04 \times 10^6$ *                      |

Remark: The enzyme activity is expressed as the volume of supernatant induced in the reaction over the dilution factor of the supernatant required to complete the relaxation assay  $\times 10^6 \text{ UL}^{-1}$ . \*  $p < 0.05$  indicates statistically significant

## The activity of Pichia transformants for visual screening purposes

Visual screening using microbiological agar plates containing various concentrations of quercetin or F1 showed a decrease in intensity (growth) of the *Pichia* transformants (GS115-pPIC3.5K-hTop1) that expressed the highest enzyme activity of hTop1 and were resistant to 1.00 mg/ml Geneticin, after three days of incubation. The average intensities of the transformants after  $7.81 \times 10^{-10}$  dilution on agar plates containing various quercetin concentrations showed no significant difference after three days of incubation (Fig. 7A). In contrast, the average intensity of the transformants at the same dilution on agar plates containing 25 µg/ml F1 were  $145.01 \pm 0.45$ ,  $141.12 \pm 0.42$ , and  $139.84 \pm 2.23$  ( $p < 0.05$ ) at day 1, 2, and 3 of incubation, respectively (Fig. 7B). Therefore, the yeast-based screening assays able to differentiate the growth inhibitory activity of quercetin and F1.

# Cytotoxic effects of quercetin- and F1-treated MDA-MB-231

The ideal dose-response of quercetin was not observed on the cancer cells at 24 h of treatment (Fig. 8A). The ideal dose-response of quercetin was only observed on the cancer cells at 48 (hillslope=-2.247; maximal response  $\leq 75\%$ ) (Fig. 8B) and 72 h of treatment (hillslope=-2.785; maximal response  $\leq 50\%$ ) (Fig. 8C). The cytotoxic effect in MDA-MB-231 treated with F1 resulted in the ideal dose-response of the test compound on the cancer cells at 24 (hillslope=-0.49; maximal response  $\leq 25\%$ ) (Fig. 8D) and 48 h of treatment (hillslope=-0.76; maximal response  $\leq 25\%$ ) (Fig. 8E). However, the ideal dose-response of F1 on the cancer cells did not persist until 72 h of treatment (Fig. 8F). Although quercetin showed a good later growth inhibitory effect on the cancer cells following 72 h of treatment, F1 showed a good early growth inhibitory effect on MDA-MB-231. As for the half-maximal effective concentration (EC<sub>50</sub>) value, only MDA-MB-231 post F1 treatment for 48 h showed the EC<sub>50</sub> value  $\leq 50$   $\mu\text{g/ml}$  (10.22  $\mu\text{g/ml}$ ); compounds that exhibited EC<sub>50</sub> value  $\geq 50$   $\mu\text{g/ml}$  are considered as inactive. The differences in growth inhibitory activity of quercetin and F1 could be predicted by the yeast-based screening assay and further confirmed using the MTT assay.

## Cell cycle profile of quercetin- and F1-treated MDA-MB-231

The cell cycle profile of MDA-MB-231 treated with 0.5% DMSO (control), 60  $\mu\text{g/ml}$  quercetin, and 50  $\mu\text{g/ml}$  F1 for 24, 48, and 72 h showed changes in cell cycle phases. The distribution of cell cycle phases in MDA-MB-231 treated with quercetin for 24 h showed no significant difference compared to the controls (Fig. 9A). Conversely, the cell population in S phase for F1-treated cells increased significantly to 53.13% ( $p < 0.001$ ), accompanied by a decrease in the cell population in G<sub>0</sub>/G<sub>1</sub> phase to 39.68% ( $p < 0.001$ ) compared to respective controls (DMSO-treated MDA-MB-231), but with no significant effect on the cell population in G<sub>2</sub>/M phase at 24 h of treatment. At 48 h of treatment, quercetin only caused a reduction in the cell population in G<sub>0</sub>/G<sub>1</sub> phase to 70.53% ( $p < 0.001$ ) and an increase in the cell population in S phase to 25.72% ( $p < 0.001$ ), but it had no significant effect on the cell population in G<sub>2</sub>/M phase (Fig. 9B). A significant increase in the cell population in both S (39.34%;  $p < 0.001$ ) and G<sub>2</sub>/M (39.70%;  $p < 0.001$ ) phases was observed in F1-treated cells, where a drop in the cell population to only 20.96% ( $p < 0.001$ ) in G<sub>0</sub>/G<sub>1</sub> phase compared with the controls. At 72 h of treatment, quercetin-treated cells constantly underwent cell cycle progression, shifting only from G<sub>0</sub>/G<sub>1</sub> (61.02%,  $p < 0.001$ ) to S (36.88%,  $p < 0.001$ ) phases with no effect on G<sub>2</sub>/M phase (Fig. 9C). Similarly, F1-treated cells showed a further reduced cell distribution in G<sub>0</sub>/G<sub>1</sub> phase (27.09%,  $p < 0.001$ ) with increased distribution in both S (31.52%,  $p < 0.001$ ) and G<sub>2</sub>/M (41.39%,  $p < 0.001$ ) phases at 72 h of treatment. This phenomenon implied that F1 induced cell cycle arrest at both S and G<sub>2</sub>/M phases, but quercetin induced cell cycle arrest at S phase only in MDA-MB-231.

## Apoptosis induction of quercetin- and F1-treated MDA-MB-231

Analysis of the effect revealed that apoptosis was induced in quercetin-treated MDA-MB-231, but F1 did not induce apoptosis in MDA-MB-231 for 72 h of treatment compared to the controls (DMSO-treated

MDA-MB-231). There was a significant increase in the cell population at the early apoptosis stage, as indicated by an increase in the cell population from 2.99% in control to 6.61% ( $p < 0.001$ ) in quercetin-treated cells (Fig. 10A). When prolonged quercetin treatment to 48 h, 7.71% ( $p < 0.001$ ) and 5.26% ( $p < 0.01$ ) of cells were observed in the early apoptosis stage and late apoptosis stage, respectively, compared to 2.43% of the control in the early apoptosis stage and 2.42% of the control in the later apoptosis stage (Fig. 10B). The apoptotic effect induced by quercetin at 72 h of treatment was most significant, as 18.48% ( $p < 0.001$ ) was found in the early apoptosis stage and 8.13% ( $p < 0.05$ ) in the late apoptosis stage (Fig. 10C). A significant reduction in cell viability was also observed in the quercetin-treated cells after 72 h of treatment, suggesting that quercetin was capable of inducing apoptosis compared to F1 in MDA-MB-231.

## mRNA expression of CYP genes in quercetin- and F1-treated MDA-MB-231

Analysis only observed the optimum level of CYP1A1 mRNA expression at 48 h of treatment compared with the control (DMSO-treated MDA-MB-231), where the level of CYP1A1 was upregulated to a much higher extent in quercetin- than in F1-treated MDA-MB-231 (Fig. 11A). The mRNA expression of CYP1A1 in quercetin- and F1-treated cells was upregulated by 20.8- ( $p < 0.001$ ) and 6.8-fold change ( $p < 0.01$ ), respectively, compared with the control at 48 h of treatment. After treatment for 72 h, CYP1A1 mRNA expression in quercetin-treated cells remained upregulated by 13.2-fold change ( $p < 0.001$ ). Both quercetin and F1 also showed a similar optimum profile of CYP1B1 mRNA expression with 4.60- ( $p < 0.01$ ) and 3.15-fold change ( $p < 0.05$ ), respectively, in MDA-MB-231 after 48 h of treatment (Fig. 11B). The results further showed that significant upregulation of CYP1B1 mRNA expression was observed following 72 h of quercetin treatment in MDA-MB-231: 3.65-fold change ( $p < 0.05$ ), but F1 had no discernible effect on CYP1B1 mRNA expression. Quercetin also failed to modify CYP2S1 mRNA expression in MDA-MB-231 following 72 h of treatment. In contrast, F1 increased CYP2S1 mRNA expression in MDA-MB-231 at 24 (1.80-fold change;  $p < 0.05$ ) and 48 h (2.85-fold change;  $p < 0.001$ ) of treatment (Fig. 11C). However, no significant induction of CYP2S1 mRNA expression was observed at 72 h of F1 treatment. In comparison, quercetin- and F1-treated MDA-MB-231 for 72 h showed different mRNA expression levels of CYP1A1, CYP1B1 and CYP2S1 in the cells.

## Discussion

The present results demonstrated that the recombinant pPIC3.5K-hTopI plasmid, which contained a copy of the human DNA topoisomerase I (hTopI), was successfully constructed. The *Pichia* transformants or recombinant yeast (GS115-pPIC3.5K-hTopI), which contained a multi-copy number of hTopI, was also successfully produced *via an in vivo* strategy. The cell density of GS115-pPIC3.5K-hTopI was likely to be unaffected by the copy number of hTopI. However, the total protein expression and the target enzyme activity of the recombinant yeast were increased in accordance with the increased copy number of hTopI in the host, whereby the yeast that was able to survive at the highest concentration of Geneticin expressed the highest level of total protein and had the highest activity of the enzyme. The recombinant

yeast was able to differentiate the growth inhibitory activity of quercetin and F1, indicating that the growth inhibitory activity of pure and plant substances could be predicted by yeast-based screening assay and further confirmed using MTT assay. Quercetin induced cell cycle arrest at S phase only in MDA-MB-231, but the study found F1 induced cell cycle arrest at both S and G2/M phases. Quercetin induced apoptosis in MDA-MB-231, but F1 did not. The F1 showed lower mRNA expression levels of CYP1A1 and CYP1B1 (carcinogenicity). However, the study found a higher mRNA expression level of CYP2S1 (cytotoxicity) in quercetin-treated MDA-MB-231 following 72 h of treatment.

*Pichia* is a widely used host system for the expression of heterologous proteins [23]. In addition to the popularity factors described in the Introduction, this system also offers the strong and highly regulated alcohol oxidase promoter, stable integration events in the host chromosomal DNA and efficient techniques for high-density cultivation to express the protein of interest [24]. Therefore, this study utilised this yeast system to express hTop1, whereby the gene encoding the protein of interest is approximately 2,298 bp. The expression using recombinant yeast containing a single copy number of the target gene was disappointingly low; indeed, the multi-copy number of the gene expression cassette has been one of the most effective strategies to increase the expression of the GOI [9, 25–27]. The recombinant yeast was successfully constructed using the pPIC3.5K vector in this study *via* the *in vivo* strategy to determine the effects of gene copy number on cell density, the expression of total protein, and the target enzyme activity in *Pichia*.

His<sup>+</sup> *Pichia* transformants with multi-copy inserts (recombinant yeast) resistant to various concentrations of Geneticin were also successfully selected in this study. However, the selected clones' cell density was likely not affected by the copy number of the target gene in the host, which may interpret as not affect the downstream metabolic activity of the cells. According to previous studies, gene expression induction resulted in excessive plasmid replication that consequently increased the plasmid copy number in the transformants [28–30]. However, this phenomenon contributed also to the host cell metabolic burden [28, 31]. As a result, the metabolic activity was strongly impaired in the cells, indicated by the decelerated increase in biomass and OD. For the effect of the *in vivo* strategy, the study found the highest expression level of total protein (as much as 1.76 mg/ml) in GS115-pPIC3.5K-hTop1 resistant to 1.00 mg/ml Geneticin at 48 h of incubation. However, normalisation of the total protein level per hour and per cell density in each transformant was statistically insignificant compared to the total protein level in control. Therefore, the study is continued by investigating the target enzyme activity, whereby the study found the increment of gene copy number to increase the enzyme activity of hTop1 produced in GS115-pPIC3.5K-hTop1.

A transformant or clone with two identical copies of a gene under the control of an identical promoter, in theory, should produce twice as much protein. However, in practice, increasing the gene dosage does not necessarily increase protein expression. In some cases, e.g., human trypsinogen [32] and Na-ASPI [33], increased the gene dosage reduced the protein expression. Therefore, an optimal level rather than a maximal copy number should be considered due to other possible protein expression bottlenecks, e.g., protein translation, secretion or degradation [32, 34–36]. Furthermore, an increased copy number of

foreign genes may result in the alteration of normal metabolism in *Pichia*, leading to a negative influence on the normal cell physiology of multiple-copy recombinant yeast, especially in the case of secretory expression, which includes a reduction in methanol consumption capacity and specific growth rate, decreased cell viability, increased instability of integrated foreign genes or diminished cell secretory ability [37]. For this reason, it is suggested to test the transformants with increasing gene copy numbers and later identify the optimal gene copy number for maximum protein production [34, 38]. Although the strategy used in this study did not significantly change the expression of total protein per cell density in each clone, the ability of hTopI expressed by GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations was increased as the resistance towards Geneticin was increased. This event also further showed that the hTopI expression ability in this study was able to relax supercoiled DNA, and the enzyme activity increased with increasing target gene copy number.

Incubation of the recombinant yeast (GS115-pPIC3.5K-hTopI) with quercetin and F1 also showed that both substances had different growth inhibitory activity to reduce the growth of the recombinant yeast, which could be further confirmed when MDA-MB-231 was used for the screening. MDA-MB-231 is a poorly differentiated, highly aggressive and invasive breast cancer cell type. It is a model representing triple-negative breast cancer, which is characterised by the lack of oestrogen receptor, progesterone receptor, E-cadherin and HER2 growth factor receptor, but presenting with mutated p53 gene expression [39]. Therefore, MDA-MB-231 is the ideal cell model for investigating the effectiveness of newly developed chemotherapeutic agents. In this study, quercetin and F1 were found to reduce the proliferation of MDA-MB-231 by inducing different cell cycle arrest profiles. Regulation of the cell cycle is crucial for the development of healthy cells. Nevertheless, cancerous cells exhibit uncontrolled cell proliferation and evasion of apoptosis resulting from dysfunction of the cell cycle's checkpoint and destruction [40]. Uncontrolled cell growth and apoptosis resistance are the major defects in cancer cells; thus, discovering potential compounds targeting cell cycle mechanisms and apoptotic machinery could be effective against uncontrolled cell proliferation in neoplasia. This study also explored the mechanism by which quercetin and F1 inhibited MDA-MB-231 cell proliferation in different manners.

Quercetin and F1 were also found to exhibit different cell cycle arrest profiles. Red onion peel is known to contain high concentrations of quercetin [41–42] and is able to induce cell cycle arrest and apoptosis in different cancer cells [43], whereas F1 is able to obstruct cell cycle progression in both S and G2/M phases, suggesting that different comprehensive effects of quercetin and F1 in MDA-MB-231. One possible explanation is the presence of different compound compositions in F1, which gives rise to different molecular mechanisms of cell cycle regulation induced by F1. Nguyen's study reported that 20  $\mu\text{M}$  quercetin induced cell cycle arrest at the S and G2/M phases in MDA-MB-231 at 48 h of treatment [44]. This finding was linked to the increased signalling activities of p21 and GADD45, which contributed to G1/S and G2/M phase arrest, respectively, regulated by p53 [44]. Another study indicated that cell cycle arrest was observed at G2/M phase after treatment of MDA-MB-231 with 100  $\mu\text{M}$  quercetin for 24 and 48 h [45]. Nevertheless, Rivera's study showed cell cycle arrest at G2/M phase in MDA-MB-231 after 48 h of treatment with 15  $\mu\text{M}$  quercetin [46]. In this study, cell cycle arrest was observed at S phase only after treatment with quercetin for 48 h, which is slightly in contrast to previously published findings. This

difference may be due to variation in the cell treatment concentration, where a higher concentration of quercetin was utilised for the experiments. For apoptosis analysis, the results showed that an apoptotic effect was observed in MDA-MB-231 treated with quercetin, but F1 did not induce apoptosis in MDA-MB-231. The induction of apoptotic effects in MDA-MB-231 cells in this study was consistent with other findings using the same cell line [44–47]. This result suggests that cell proliferation inhibition by F1 in MDA-MB-231 occurred through mechanisms other than the apoptosis pathway.

CYP genes have been extensively confirmed to be involved in the metabolism of pro-carcinogenic compounds [48]. Other studies have also shown that the genes encoding these proteins are linked to other cell signalling pathways critical for cell cycle regulation [49]. For instance, the aryl hydrocarbon receptor (AHR), responsible for activating CYP genes' transcription, is a protein that affects cell cycle regulation [50]. Several findings have shown that dietary flavonoids play a role as AHR ligands with either antagonist or agonist activity to inhibit cancer cell growth [51–52]. Additionally, flavonoids may also undergo CYP1-mediated oxidative metabolism to become anti-proliferative products [51]. A study has demonstrated anti-proliferative and cytostatic effects of a flavonoid lipid molecule, eupatorine, in breast cancer cells due to the involvement of CYP1-mediated metabolism [53]. The studies showed that cell cycle arrest at G2/M phase induced by eupatorine could be reversed when MDA-MB-468 cells were coincubated with the CYP1 inhibitor acacetin. Another finding by Atherton confirmed that metabolites produced from the isoflavones daidzein and genistein *via* CYP1A1, CYP1A2 and CYP1B1 metabolism induced an anti-proliferative response in MCF-7 cells [54].

In this study, the analysis of CYP genes showed that quercetin and F1 induced mRNA expression of CYP1A1 and CYP1B1, with the highest level was observed at 48 h of treatment. This phenomenon corresponded to the initiation of cell cycle arrest at S phase by quercetin in MDA-MB-231. These results also corresponded to the profound changes in cell cycle progression, which was also observed at 48 h of treatment with further induction of cell cycle arrest at G2/M phase by F1. The finding is supported by numerous studies that revealed the role of quercetin in cancer proliferation in relation to its interaction with CYP family enzymes. For instance, quercetin was shown to be an agonist of CYP1A1 in breast cancer cells [55]. A study by Ciolino also showed that quercetin increases CYP1A1 mRNA through mediation by the AHR receptor [56]. Furthermore, the metabolism of quercetin by the CYP1 enzyme, particularly CYP1A1 and CYP1B1, intensifies their anti-proliferative effects in breast cancer cells [55]. Hence, these results suggested that the anti-proliferative effect of F1 on MDA-MB-231 might be due to the metabolic activities of these CYPs resulting in the production of active metabolites, which indirectly modulate the cell cycle progression and survival of MDA-MB-231. For CYP2S1 gene expression analysis, F1 (but not quercetin) induced significantly high gene expression levels in MDA-MB-231 at 24 and 48 h of treatment. The selective expression of CYP2S1 in MDA-MB-231 treated with F1 suggested that CYP2S1 likely plays a role in the regulation of F1 anticancer activity, which is likely regulated by AHR [57].

## Conclusion

In conclusion, the recombinant yeast produced in this study can provide preliminary information on the growth inhibitory activity of quercetin and F1, which can then be further confirmed using the MTT assay. The study also explored the basic mechanism by which quercetin and F1 inhibited cell proliferation in MDA-MB-231 *via* different manners.

## **Declarations**

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### **Conflicts of interest/Competing interests**

The author(s) declare(s) that there is no conflict of interest or competing interests regarding the publication of this article.

### **Availability of data and materials**

The datasets used and/or analysed during the current study, including the nucleotide sequence of hTop1 in the pPIC3.5K-hTop1 plasmid that had been deposited to the GenBank (Submission # 2391932), are available from the corresponding author and can be presented on reasonable request.

### **Code availability**

Not applicable

### **Authors' contributions**

Z.G. and K.B.Y. contributed to the conception and design of the study. C.S.P., N.A.F., and L.S.K. performed the experiments under technical support provided by C.A.L. and K.B.Y. K.B.Y. interpreted the results, drafted and revised the manuscript. All authors have read and agreed to the final version of this manuscript submitted for publication.

### **Ethics approval**

Not relevant

### **Consent to participate**

Not relevant

## Consent for publication

Not relevant

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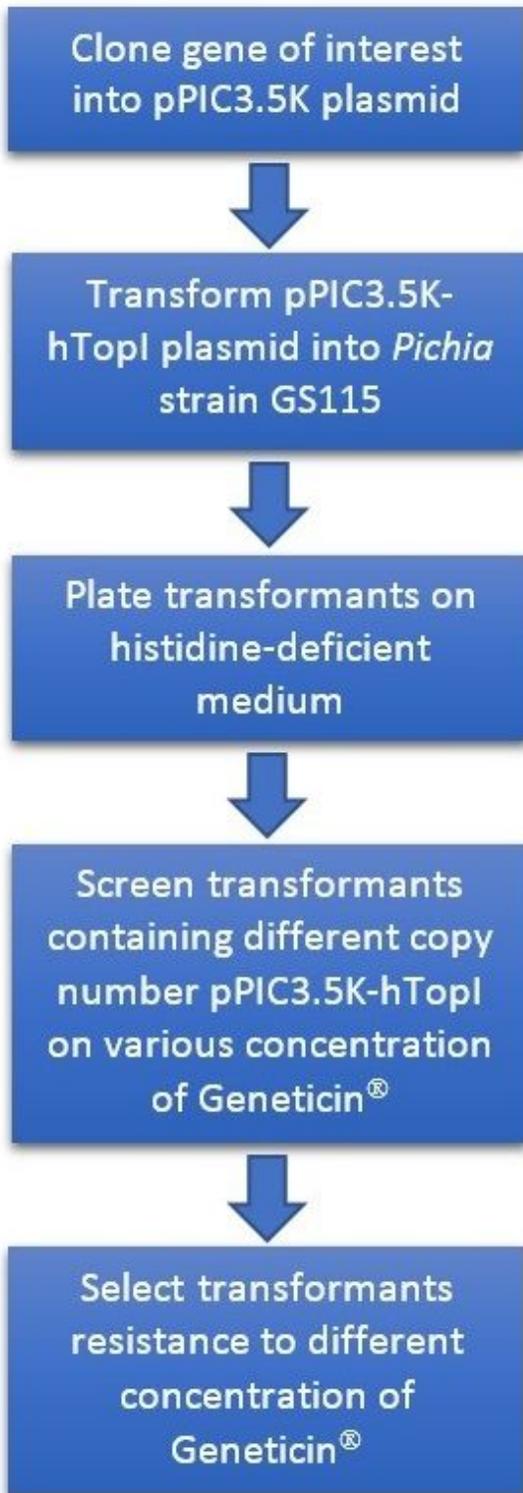
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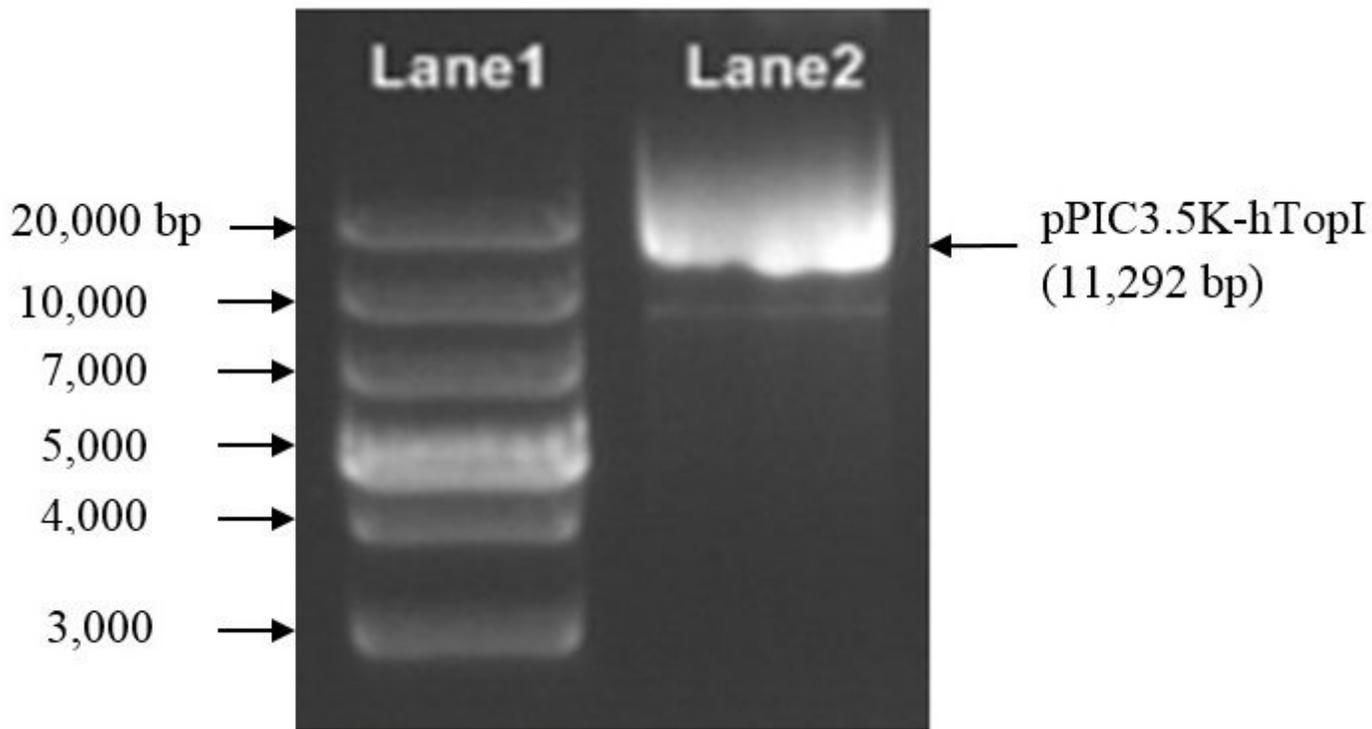
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## Figures



**Figure 1**

Schematic overview of in vivo strategy to produce recombinant yeast with multiple copies of pPIC3.5K-hTop1 in *Pichia*.



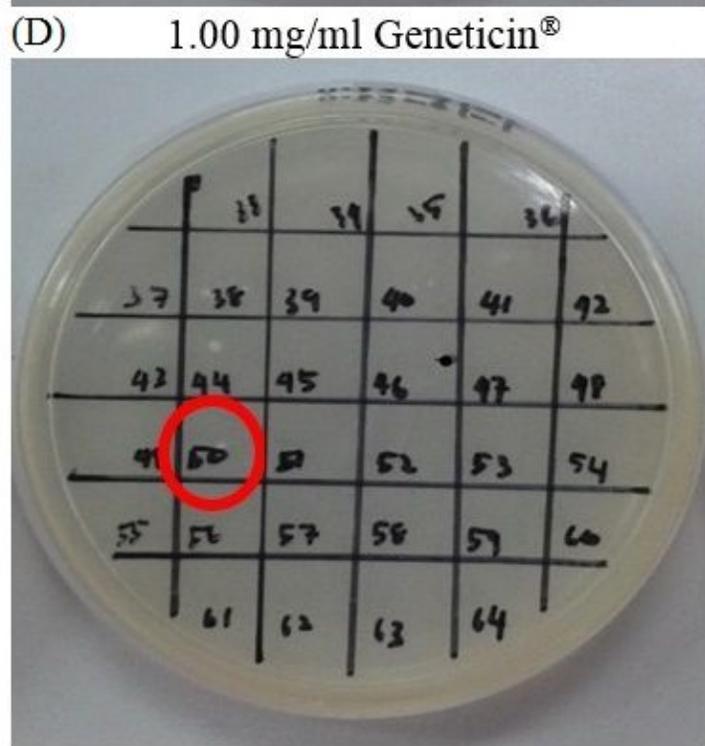
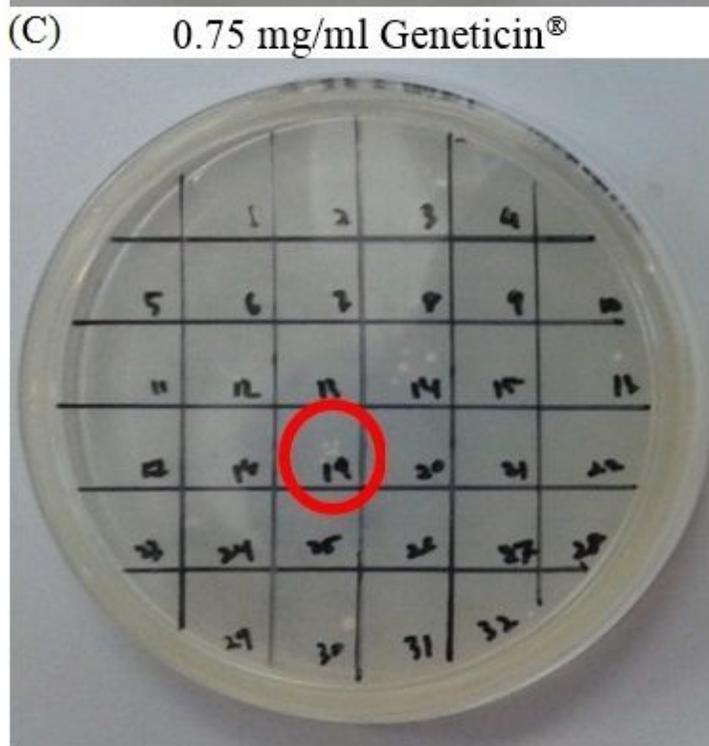
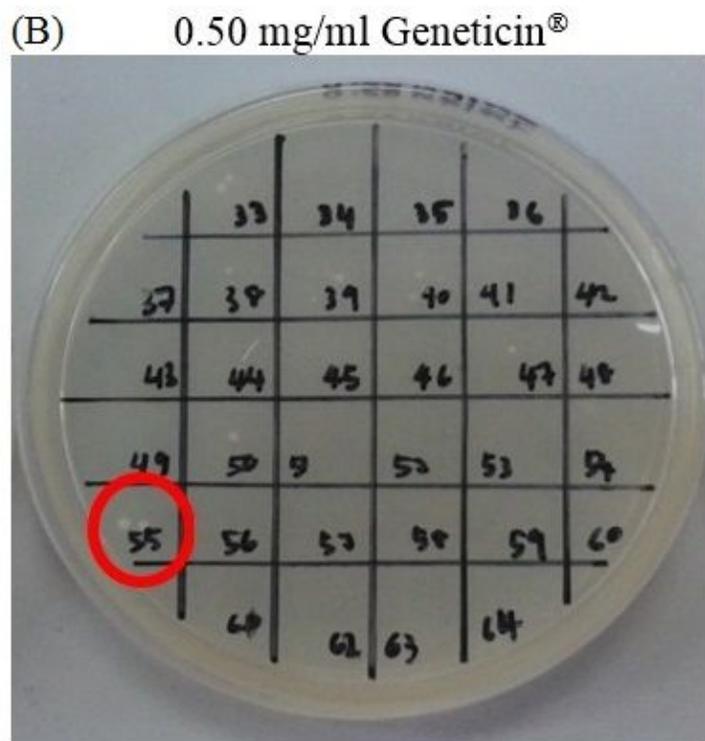
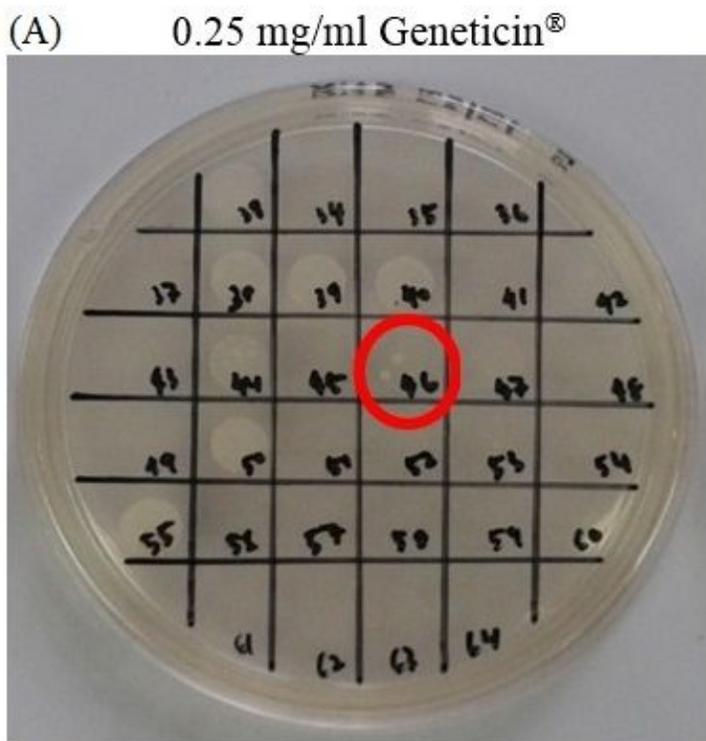
**Figure 2**

Construction of recombinant plasmid via in vivo strategy. The 0.7% agarose gel electrophoresis of constructed recombinant pPIC3.5K-hTopI plasmid DNA. Lane 1: GeneRuler 1 kb Plus DNA Ladder. Lane 2: Recombinant pPIC3.5K-hTopI plasmid. Cropped image of gel; full-length gel can be presented on reasonable request.

>Seq1 [organism=homo sapiens] [clone=1] DNA topoisomerase I gene, cds  
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CAAGATGGGCATGCTGAAGAGACGAATCATGCCCGAGGATATAATCATCAACTGTAGCAAAGATGCCAAGG  
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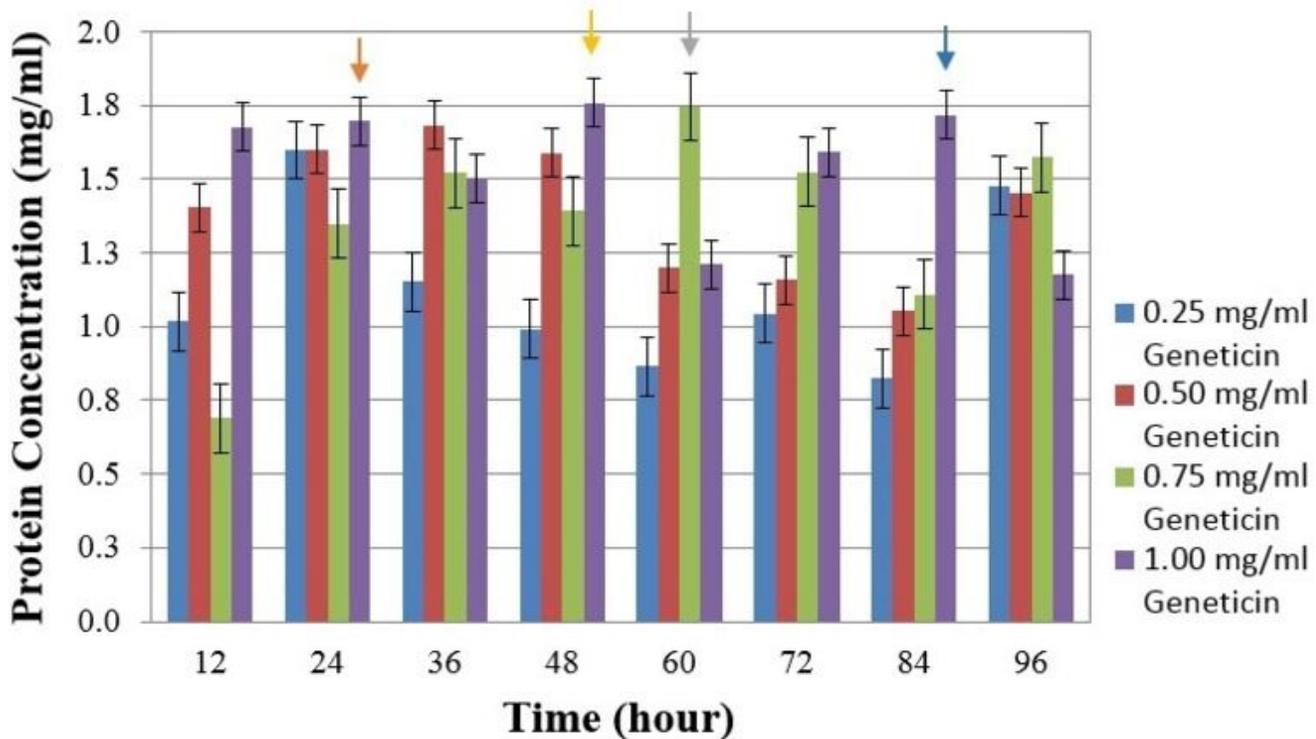
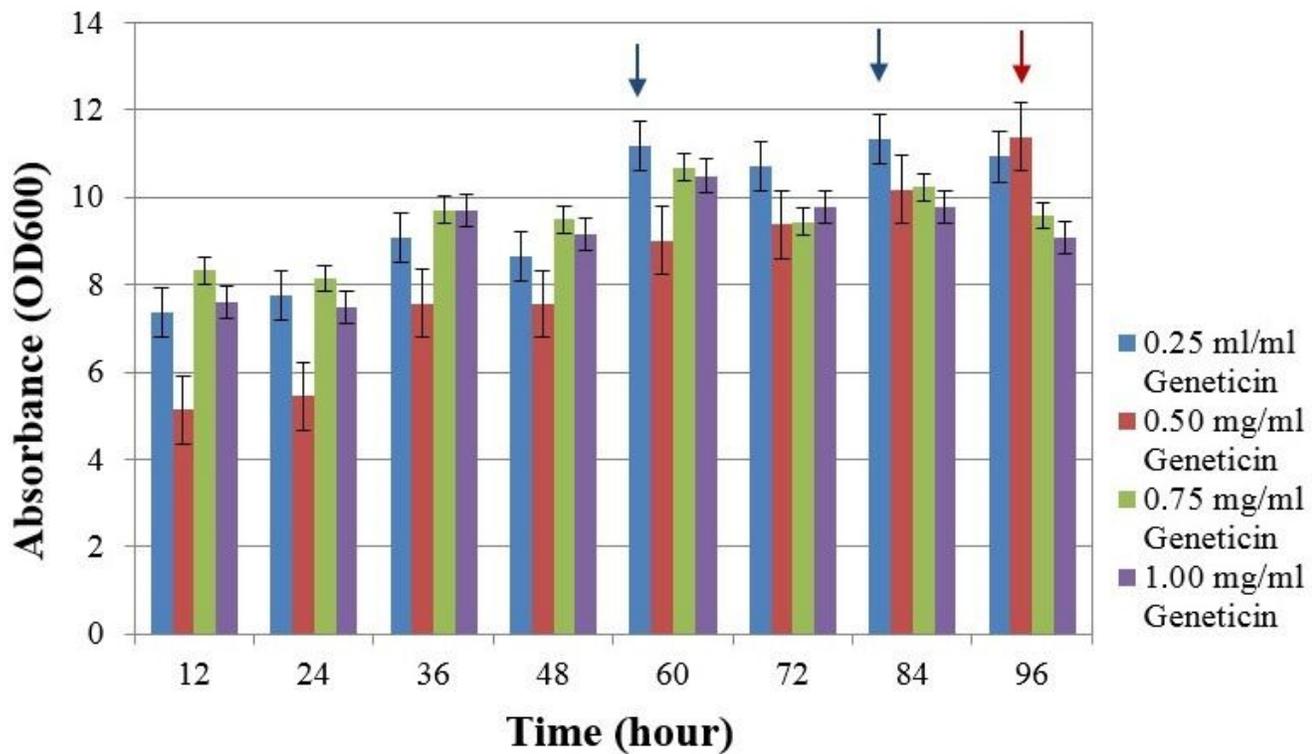
### Figure 3

Nucleotide sequence of hTopI in the pPIC3.5K-hTopI plasmid. The sequence was identified by sequencing and had been deposited to the GenBank (Submission # 2391932). The sequence can be presented at a reasonable request.



**Figure 4**

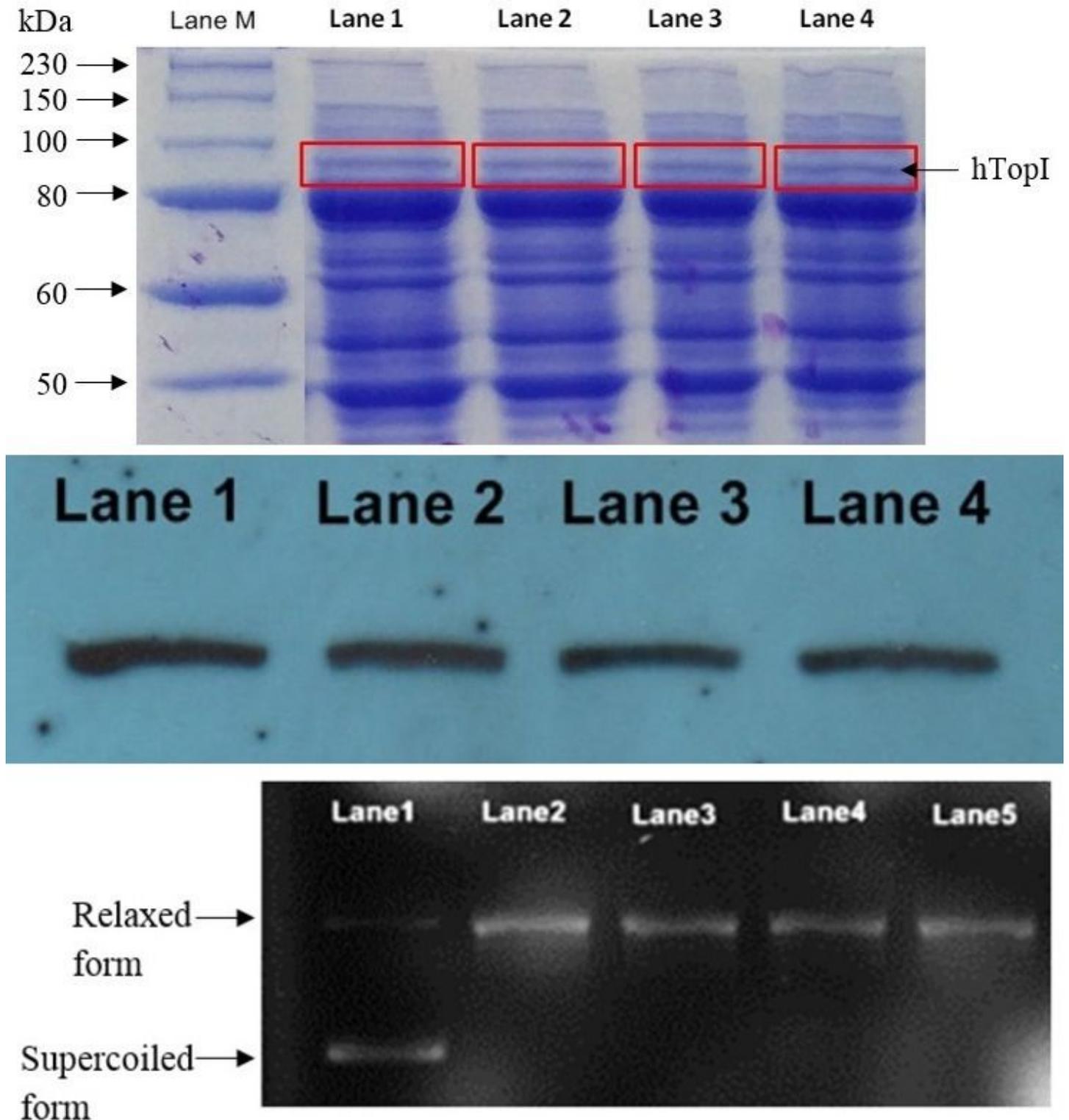
Screening of GS115-pPIC3.5K-hTop1 on YPD agar containing various Geneticin concentrations. The concentrations of antibiotic used on the YPD agar were (A) 0.25, (B) 0.50, (C) 0.75, and (D) 1.00 mg/ml. The grid circled in red indicates the clone resistant to the antibiotic's particular concentration in the agar plate. Images of agar gel were derived from the same experiment and were processed in parallel.



**Figure 5**

Cell growth and total protein expression of GS115-pPIC3.5K-hTopI. (A) Cell growth of the transformants after 96 h of culture. The density of GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations on agar plates was measured by spectrophotometry. (B) Total protein expression of the transformants at 96 h of incubation. The Bio-Rad protein assay measured the expression level of total protein in GS115-

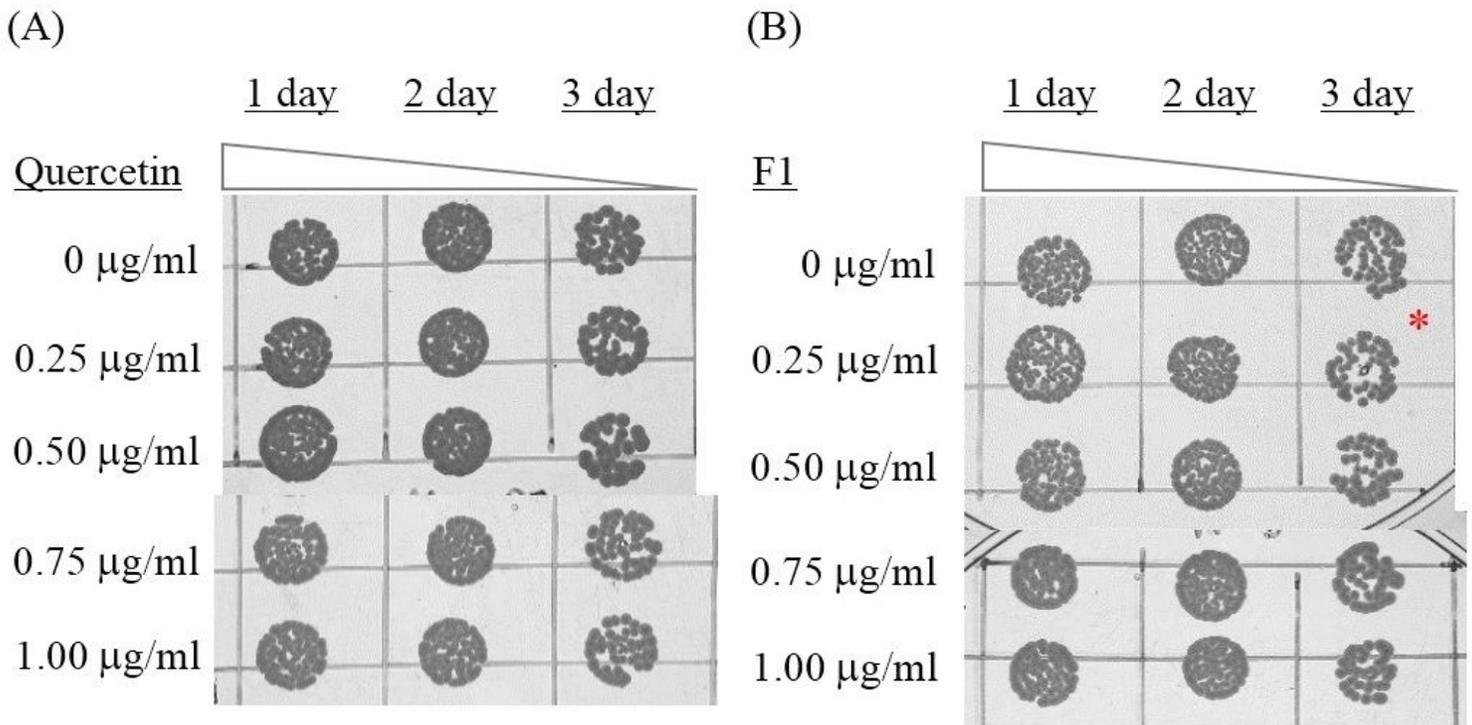
pPIC3.5K-hTop1 resistant to various Geneticin concentrations. All data represent the mean  $\pm$  SD of 3 replications.



**Figure 6**

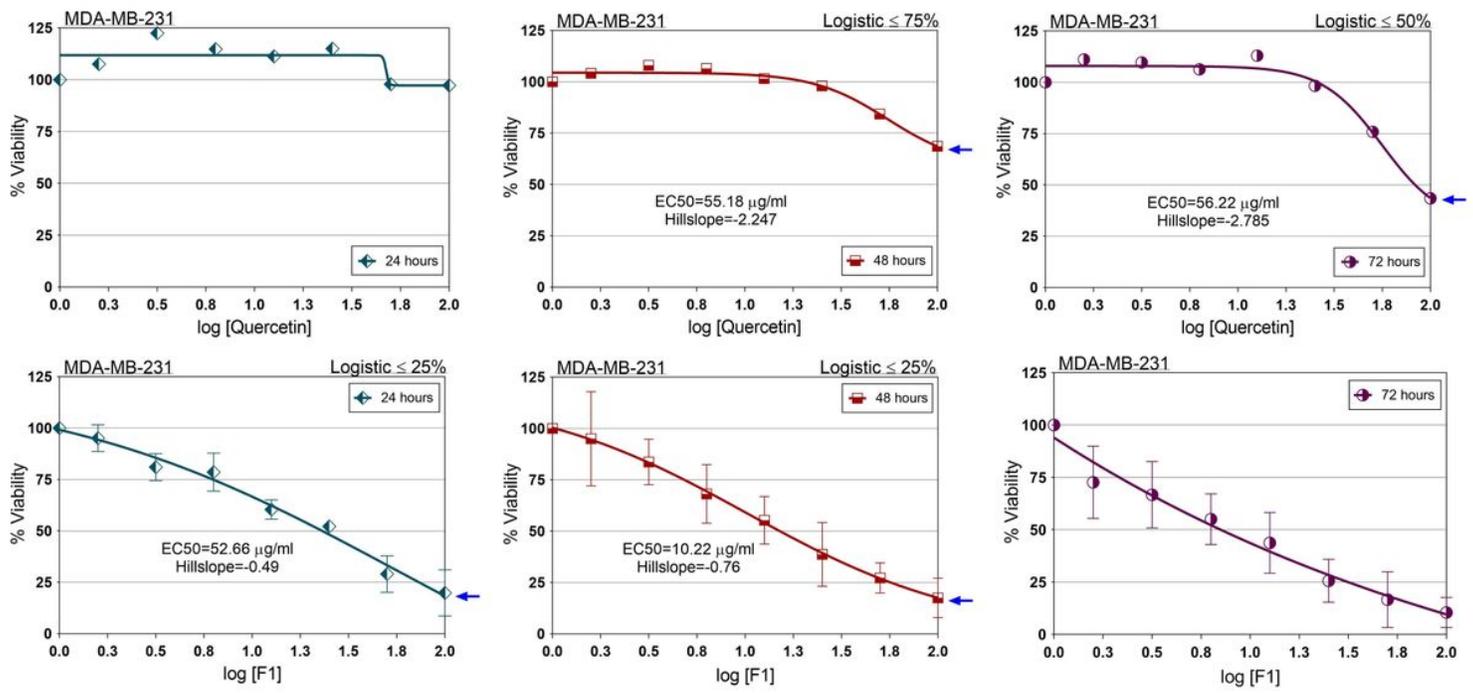
Specific protein expression for selected clones. (A) SDS-PAGE of total protein extracted from GS115-pPIC3.5K-hTop1 resistant to various Geneticin concentrations. Lane M: Protein molecular weight marker (Bio-Rad, USA). Lane 1: Clone resistant to 1.00 mg/ml Geneticin, collected at 24 h of incubation. Lane 2:

Clone resistant to 1.00 mg/ml Geneticin, collected at 48 h of incubation. Lane 3: Clone resistant to 0.75 mg/ml Geneticin, collected at 60 h of incubation. Lane 4: Clone resistant to 1.00 mg/ml Geneticin, collected at 84 h of incubation. (B) Western blotting for hTopI protein detection in GS115-pPIC3.5K-hTopI resistant to various Geneticin concentrations. Lane 1: Clone resistant to 1.00 mg/ml Geneticin, collected at 24 h of incubation. Lane 2: Clone resistant to 1.00 mg/ml Geneticin, collected at 48 h of incubation. Lane 3: Clone resistant to 0.75 mg/ml Geneticin, collected at 60 h of incubation. Lane 4: Clone resistant to 1.00 mg/ml Geneticin, collected at 84 h of incubation. (C) The 1.0% agarose gel electrophoresis of the enzyme activity of hTopI in the clones that resistant to various Geneticin concentrations. Lane 1: Supercoiled DNA of pBR322 incubated without hTopI. Lane 2: Supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 0.25 mg/ml Geneticin (24 h). Lane 3: Supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 0.50 mg/ml Geneticin (36 h). Lane 4: Supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 0.75 mg/ml Geneticin (60 h). Lane 5: Supercoiled DNA of pBR322 incubated with hTopI from the clone resistant to 1.00 mg/ml Geneticin (48 h). Cropped images of gels/blots; full-length gels/blots can be presented on reasonable request. Control and marker were run on the same gels/blots.



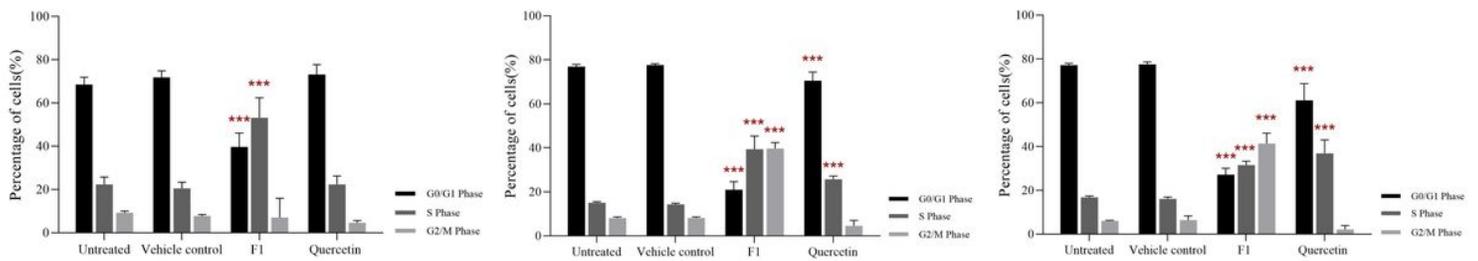
**Figure 7**

Yeast extract agar plates that contained (A) quercetin and (B) F1. The agar plates contained 0 (control), 0.25, 0.50, 0.75, and 1.00  $\mu\text{g/ml}$  quercetin or F1. The growth of the recombinant *P. pastoris* transformants on the plates were incubated for 1, 2, and 3 day. Each recombinant was spotted in triplicates on the agar plate prior  $7.81 \times 10^{-10}$  dilution. Cropped images of agar gel; full-length agar gels can be presented on reasonable request. Controls were derived from the same experiment, and the agar gels were processed in parallel.



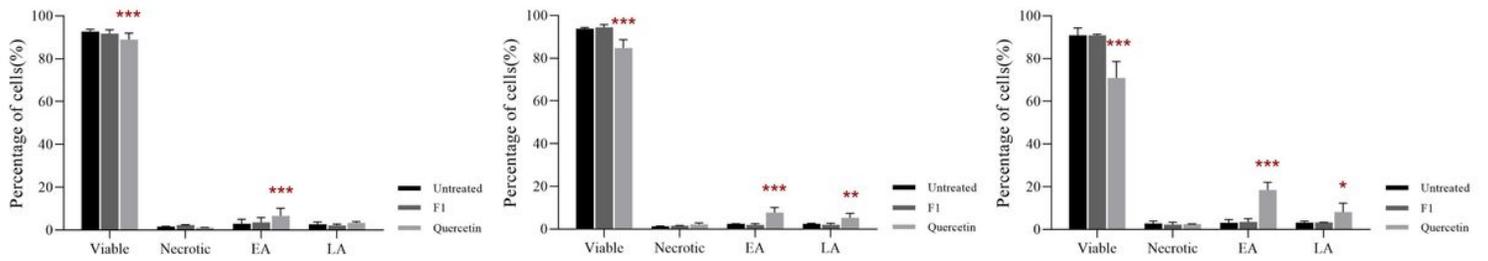
**Figure 8**

Dose-response curves of MDA-MB-231 following treatments with quercetin for (A) 24, (B) 48, and (C) 72 h, and with F1 for (D) 24, (E) 48, and (F) 72 h. The maximal response (inhibition rate at the maximum dose concentration) identified hillslope value (slope at EC50 value) and an EC50 value of each test compound from the curve. The experiments were repeated several times to ensure the repeatability and reproducibility of the results. All values are expressed as the means  $\pm$  SD. A  $*p < 0.05$  was regarded as statistically significant.



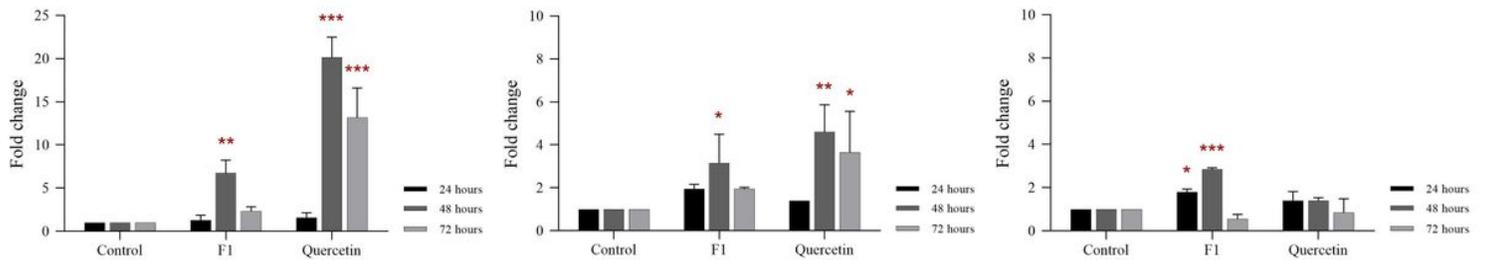
**Figure 9**

Cell cycle profile of quercetin (60  $\mu\text{g/ml}$ ) and F1 (50  $\mu\text{g/ml}$ ) in MDA-MB-231 after (A) 24, (B) 48, and (C) 72 h of treatment. Each data set represents the mean of 2 independent experiments with triplicate readings each. Significant differences were analysed vs untreated sample using one-way ANOVA and Dunnett's multiple comparisons test indicated as  $*p < 0.05$ ;  $**p < 0.01$ ; and  $***p < 0.001$ . Untreated: non-treated cells and vehicle control: DMSO-treated cells.



**Figure 10**

Apoptotic effect of quercetin (60  $\mu\text{g/ml}$ ) and F1 (50  $\mu\text{g/ml}$ ) in MDA-MB-231 after (A) 24, (B) 48, and (C) 72 h of treatment. Each data set represents the mean of 2 independent experiments with triplicate readings each. One-way ANOVA and Dunnett's multiple comparisons test were used to interpret statistically significant differences between treated and untreated cells (control) as \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . EA: early apoptosis, LA: late apoptosis.



**Figure 11**

Effect of quercetin (60  $\mu\text{g/ml}$ ) and F1 (50  $\mu\text{g/ml}$ ) on mRNA gene expression of (A) CYP1A1, (B) CYP1B1, and (C) CYP2S1 in MDA-MB-231. mRNA expression was normalised to the expression of GAPDH in each sample. The bar chart represents the mean  $\pm$  SD of 2 independent experiments with significant differences indicated as \* $p < 0.05$ ; \*\* $p < 0.01$ ; and \*\*\* $p < 0.001$ , compared to the controls. One-way ANOVA and Dunnett's multiple comparisons test were used for the statistical analysis.