https://doi.org/10.2478/acph-2022-0034

Naturally occurring bergapten exhibits potent anticancer effects in cisplatin-resistant breast cancer cells: Role of PI3K/AKT signaling pathway

XILIANG ZHANG
YUXING ZHANG
ZHEN CAO
ZHANWEI ZHAO
HUIBIN ZHAO
XIAODONG YANG*

Department of General Surgery, The sixth Medical Center of PLA General Hospital, Beijing, 100048, China

*Correspondence, e-mail: xiaodongyang2001@gmail.com

ABSTRACT

The main objective of the current study was to examine the anticancer effects of naturally occurring bergapten on human breast cancer cells (MDA-MB-231) and investigate its impact on apoptosis, cell cycle, cell migration and invasion and PI3K/AKT signaling pathway. MTT assay and clonogenic assay were used to study effects on cell viability and cancer colony formation. Fluorescence microscopy and flow cytometry were used to study apoptotic effects induced by bergapten. In vitro, transwell migration assay and transwell chambers assay were used to study the impact on cell migration and invasion, respectively. Western blotting was used to study the impact of bergapten on the PI3K/AKT signaling pathway. Results indicated that bergapten led to potent cell proliferation inhibition and reduced cancer colony formation in a dose-dependent manner. Bergapten induced apoptotic effects as shown by acridine orange/ethidium bromide staining assay and annexin-V FITC assay. The percentage of both early and late apoptotic cells increased significantly with increasing doses of bergapten. Bergapten also led to the dose-dependent G2/M phase cell cycle arrest. Bergapten also caused

inhibition of cell migration and cell invasion along with causing suppression of PI3K/AKT signaling pathway. In conclusion, bergapten exhibits strong anticancer effects in human breast cancer cells. These effects are mediated *via* apoptosis, cell migration and invasion inhibition and suppression of the PI3K/AKT signaling pathway.

Keywords: breast cancer, coumarins, bergapten, apoptosis, cell cycle

Accepted March 3, 2022
Published online March 8, 2022

Natural products have impressed and captured the interest of researchers because of their enormous structural varieties and rich biological activity profile (1). Since times immemorial, plant-based natural products have aided human beings in different livelihood purposes like food, shelter and medicine. Phytochemicals are widespread as secondary metabolites in plants and are involved in plant defensive mechanisms against pathogens and other plants (2). Colossal natural products have been isolated from plants, yet their hidden therapeutic potential and chemical content have not been fully explored. Coumarins are plant isolated natural products with tremendous structural diversities (3). They show outstanding bioactivity profiles like antiviral (*e.g.* anti-HIV, anti-influenza), antimicrobial (*e.g.* antituberculosis), anti-inflammatory, anti-tumor, antinociceptive, antioxidant, antidepressant, antiasthmatic, anti-Alzheimer, and antihyperlipidemic (4–6). It has been reported that anticancer effects of coumarins and their derivatives mediate *via* different mechanisms like inhibition of protein kinase, telomerase enzyme and oncogene expression, stimulation of caspase-dependent apoptosis, and targeting cell cycle at G2/M-phase and G0/G1-phase (7, 8). Bergapten or 5-methoxypsoralen is a member of coumarins isolated from many plant species including, *Cnidium monnieri* (L.) Cusson (Chinese medicinal plant) (9). Bergapten is among active coumarins with remarkable biological activity and rich pharmacological potential. It has been reported to induce anticancer effects in different human cancer cell lines, including breast cancer ZR-75 and MCF7 cells (10). Anticancer potency of bergapten ~~drug~~ follows different mechanisms, including the stimulation of autophagy (PTEN upregulation, targeting of p-AKT/p-mTOR and Beclin 1, and transforming LC3-I to LC3-II) and apoptosis (p38 MAPK and ERK1/2 activation) (11). Breast cancer is a leading and lethal neoplasm prevailing in women globally (12–14). Distant disease spread enhances the disastrous effects and lethality of breast cancer. Besides, potential advancements have been achieved in breast cancer, the

overall survival rate remains very low. Therefore, the current research was carried out to demonstrate the effects of the bergapten ~~drug~~ on breast cancer cells and assesses its impact upon cellular apoptosis, migration and invasion, PI3K/AKT signaling pathway and cell cycle. Breast cancer prevalence remains very high among women of the Western world (15). Recent years have also seen an alarming increase in breast cancer cases and fatalities in some Asian countries including, China, Pakistan and India (16). Several risk factors contribute to the emergence of breast cancer, including late diagnosis, inaccessibility of appropriate treatment, age, less awareness, breastfeeding, poor lifestyle and socioeconomic barriers subsidizing to higher mortality rates (17). The current research was designed to explore the possible anti-breast cancer potency of the bergapten ~~drug~~ and its possible mechanism.

EXPERIMENTAL

Chemicals and antibodies

Bergapten was purchased from Sigma Aldrich (69664-25MG, purity = ≥99.0 %). Unless otherwise stated, all other chemicals used were acquired from Sigma-Aldrich (USA). Anti-p-PI3K, anti-PI3K, anti-p-AKT, anti-AKT, anti-β-actin and HRP-labelled secondary antibodies were purchased from Cell Signalling Technology (USA).

Cell culture and conditions

The MDA-MB-231 cell line was procured from American Type Culture Collection (USA) and was maintained in DMEM (Dulbecco's modified Eagle's medium (Sigma-Aldrich). DMEM was supplemented with fetal bovine serum (ThermoFisher Scientific, Inc., USA) of 10 % and suitable antibiotic (streptomycin (100 µg mL^{-1}) and penicillin (100 U mL^{-1}) obtained from ThermoFisher Scientific, Inc. Cell cultures were maintained in a humid environment bearing 95 % air and 5 % CO_2 at 37 °C.

Analysis of cell viability

To estimate the toxicity of bergapten ~~drug~~ against MDA-MB-231 cells, MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was implemented. Cell lines were subjected to culturing for 24 h before bergapten exposure. Afterwards, MDA-MB-231 cells were loaded to 96-well plates with a concentration of 2.5×10^4 cells each well followed by supplementation of bergapten ~~drug~~ at variant doses viz 0, 12.5, 25, 50 and 100 µmol L^{-1}, for 24 and 48 h. After incubation, ~~bergapten~~ bergapten-treated MDA-MB-231 cells were

washed in phosphate buffer saline (PBS) and stained using MTT dye with 4 h of further incubation. In living cells, NADPH-dependent cellular oxidoreductase enzymes reduce MTT dye (yellow) into insoluble formazan crystals (blue). These crystals are then dissolved with dimethyl sulphoxide for colorimetric analysis. Optical density was calculated from absorbance measurements at 490 nm by using a spectrophotometer (PerkinElmer, Inc., USA).

Colony generation assay

To estimate the colony generation capability of MDA-MB-231 cells after exposure to bergapten ~~drug exposure~~, a clonogenic assay was performed. 1,000 MDA-MB-231 cells were plated onto 6-well plates and grown for 12 h. Then, each well plate was supplied with bergapten ~~drug at variant doses~~at various concentrations viz 0, 12.5, 50 and 100 µmol L^{-1} and ~~left on incubation~~incubated without any disturbance for 48 h. After completion of the exposure period, cells were collected, fixed in alcohol and stained with crystal violet (1 %) dye. Cell colonies were finally estimated under an inverted microscope (Leica Microsystems GmbH, Germany) and only those colonies were considered significant for counting bearing ≥ 50 cells.

Apoptosis analysis

To estimate apoptosis in MDA-MB-231 cells ~~by~~ after bergapten exposure ~~drug~~, acridine orange (AO)/ethidium bromide (EB) and annexin V/FITC (fluorescein isothiocyanate) staining assays were executed. The MDA-MB-231 cells were cultured onto chamber slides (Corning, USA) with a density of 1.5×10^3 cells/slide. Afterwards, treatment with bergapten ~~drug~~ was instigated at ~~variant~~ various concentrations viz 0, 12.5, 50 and 100 µmol L^{-1} for 24 h. Later, detached cells were washed within ice-cold ~~phosphate~~ phosphate-buffered saline followed by fixation of 15 min with 95 % ethanol. After that, fixed cells were dehydrated and stained using 5 µL AO/EB staining solution of 50 µg mL^{-1} concentration. Finally, Leica DM 14000B microscope with a digital camera (Leica Microsystems GmbH) was used to analyse fluorescence, and each concentration was analysed thrice. For quantification of apoptosis in MDA-MB-231 cells after bergapten exposure, the annexin V/FITC assay was implemented. For annexin V FITC assay, Annexin V/ Propidium Iodide (PI) apoptosis detection kit (Santa Cruz, Texas, United States) was used by strictly obeying the manufacturer's instructions followed by cytometric analysis with Partec Cyflow® Cube 6 Flow Cytometer.

Flow cytometry

Flow cytometric studies were carried out to examine different cell cycle phases in bergapten-treated MDA-MB-231 cells. In brief, tumorous breast MDA-MB-231 cells were seeded overnight onto sterile cultural plates at a density of 1.5×10^3 cells/plate, followed by bergapten exposure at variant doses viz 0, 12.5, 50 and 100 µmol L^{-1}. After 48 h of bergapten treatment, MDA-MB-231 cells were washed with phosphate-buffered saline (PBS), fixed in PFA (4 %) and stained using staining solution bearing RNase (100 µg mL^{-1}), PI (50 µg mL^{-1}), sodium citrate (0.1 %), and triton×100 (0.1 %) for half an hour. Finally, different cell cycle phases were recorded with flow cytometry using FACS Calibur™.

Transwell assay

Transwell migration and invasion assays were performed to monitor migration rate and invasion of MDA-MB-231 cells after bergapten exposure. In brief, upper chambers of the 24-well trans-well chambers were fitted with membranes (Corning, USA) bearing 8 mm of pore size and were supplied with DMEM containing different bergapten ~~drug~~ doses viz 0, 12.5, 50 and 100 µmol L^{-1}. Lower chambers were supplied with fetal bovine serum (10 %) and DMEM followed by incubation of all the upper and lower transwell chambers for 48 h. Unmigrated cells were cleaned off using a cotton swab, and migrated cells were stained using 0.1 % crystal violet (Sigma). Finally, migrated number of cells were counted and photographed under an inverted microscope (Olympus, Japan). ~~Similar~~ A similar procedure was followed for invasion assessment of MDA-MB-231 cells, except transwell chambers were coated with Matrigel.

Western blotting

~~Western~~ The western blotting assay was executed to monitor the activity levels of PI3K/AKT signaling pathway allied proteins in bergapten treated MDA-MB-231 cells. In brief, cells were harvested at ≥ 90 % of confluence followed by treatment with different bergapten doses viz 0, 12.5, 50 and 100 µmol L^{-1} for 24 h. Later on, MDA-MB-231 cells were lysed using RIPA lysis buffer (Sigma). The protein content within each lysate was quantified by BCA assay, and equal portions of proteins (40 µg) from each lysate were separated on SDS-PAGE. Separation of the proteins was followed by electrophoretic transference of proteins to PVDF membranes. After that, PVDF membranes were blocked by using skimmed milk (5 %) followed by incubation with suitable primary ~~antibody~~ antibodies (anti-PI3K and anti-AKT) for 12 h at 4 °C in the dark. After washing, membranes were incubated with HRP-labelled secondary antibodies for 4 h at 25 °C. Finally, blots were developed using HRP substrate.

Statistical analysis

Analyses of variance (ANOVA) followed by Dunnett's test were used to analyse all the experimental data with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 4.0, respectively. Considering $p < 0.05$ as statistically significant, all the data was expressed as mean ± SD.

RESULTS AND DISCUSSION

Bergapten suppresses viability of MDA-MB-231cells

Bergapten (Fig. 1) ~~molecule~~ has been previously reported to exhibit ~~anti-viability~~cytotoxic ~~effects~~ effects against different human cancer cell lines. ~~On~~ After applying bergapten ~~drug~~ against MDA-MB-231 breast cancer cells, remarkable cytotoxicity was induced. It couldn't only inhibit the proliferation rate but also retarded the cancer colony formation by MDA-MB-231 cells in a dose reliant-manner.

MTT assay was executed to assess the effects of bergapten on the cellular viability of MDA-MB-231 cells. Results showed significant retardation of viability in target cancer cells by bergapten ~~drug with~~in a concentration-reliant manner. It was observed that viability percentage reduced from 100 % in the case of controls to almost 10 % at 100 µmol L^{-1} after 24 h of treatment. After 48 h of treatment using the same compound~~drug~~ doses, the viability was reduced to almost 5 % (Fig. 2). Therefore, it was concluded that the bergapten ~~drug~~ could remarkably inhibit the normal proliferation of MDA-MB-231 cells. Further, the efficacy of these cells to generate colonies was monitored by clonogenic assay. The outcomes of which designated that bergapten suppressed the potential of colony generation (Fig. 3a). In the case of the control group, 400 colonies and in the treated group only about 20 colonies were generated after 24 h of bergapten treatment (Fig. 3b).

Bergapten induced apoptosis in MDA-MB-231 cells

~~Maximum~~ A huge number of chemotherapeutic drugs target apoptosis to stop carcinogenesis (18). Bergapten has been previously reported to stimulate apoptosis in SKBR-3 and MCF7 breast cancer cells (19). Herein, the bergapten ~~drug~~ against MDA-MB-231 cells produced remarkable proapoptotic effects and enhanced the number of apoptotic cells in a concentration-reliant manner.

The anti-proliferative effects of bergapten against MDA-MB-231 cells were probed *via* AO/EB and Annexin V-FITC staining assays for apoptosis mediation. Bergapten ~~drug~~ has already been reported of remarkable proapoptotic potential. AO/EB staining revealed that the control group showed normal cells with green fluorescence, and the treated group showed multi-colour fluorescence, denoting different stages of apoptosis. The yellow-green fluorescence represents early-stage apoptosis, orange-red fluorescence represents late-stage apoptosis and red represents necrotic cells (Fig. 4). On treatment with bergapten, the green fluorescence was observed to decline in contrast to the others. Therefore, ~~the~~ bergapten ~~drug~~ could enhance the number of apoptotic MDA-MB-231 cells in concentration reliant-manner. Further, apoptosis was quantified using the Annexin V-FITC assay. Results indicated that apoptosis percentage in the control group was almost negligible while ~~as,~~ in the treated group, apoptosis percentage almost reached 90 % at 100 µmol L^{-1} of bergapten concentration (Fig. 5). Hence, it may be concluded that the ~~anti-viability~~cytotoxic effects of bergapten ~~drug~~ are mediated *via* apoptosis stimulation.

Bergapten causes cell cycle arrest in MDA-MB-231 cells

Many drugs inhibit the progression of the cell cycle at different of its check-points like G2/M, G0/G1 and S. Bergapten ~~drug~~ has been reported of substantial potency to target cell cycle and previously it has been shown with targeting of the cell cycle in different lung cancer cells including NCI-H460 and A549 NSCLC cell lines (20). Herein, ~~the~~ bergapten ~~drug~~ targeted the cell cycle progression in MDA-MB-231 cells at the G2/M-phase of the cell cycle and hence reduced tumor development.

The effect of ~~the~~ bergapten ~~drug~~ over the cell division potency of MDA-MB-231 cells was evaluated *via* flow cytometry. Results indicated that the peak of other cell cycle checkpoints (S and G0/G1) reduced except G2/M-phase, which amplified on bergapten (0–100 µmol L^{-1}) exposure (Fig. 6). The percentage of G2/M-phase cells in the control group was detected as 9.7 %, and in the treated group, the percentage was enhanced up to 46.4 %. Therefore, it may be concluded that bergapten induces ~~anti-viability~~cytotoxic effects against MDA-MB-231 cells *via* blocking the cell cycle progression at the G2/M phase.

Bergapten suppresses MDA-MB-231 cell migration and invasion

Cell migration and invasion are the two lethal features of metastatic disease and need to be addressed by chemotherapeutic drugs. Migration and invasion of cancer cells to distant places augment the dangerousness of the disease and hinder its diagnosis. In the recent past,

bergapten-containing drugs have been shown with remarkable modulatory effects on IDOL/LDLR and LXR/PI3K/Akt pathways, which show active participation in cell migration and invasion (21). Herein, the bergapten drug-induced substantial anti-migratory and anti-invasive effects against MDA-MB-231 cells.

The transwell chambers assay was used to monitor the Bbergapten drug's effect on migration and invasion of MDA-MB-231 cells. After exposure to different drug concentrations (0–100 μM), both the migration (Fig. 7) as well as an invasion (Fig. 8) was detected to reduce significantly. Therefore, these results suggested that bergapten could inhibit metastasis of MDA-MB-231 cells.

Bergapten targets PI3K/AKT signaling pathway in MDA-MB-231 cells

The PI3K/AKT signaling pathway is vital in for cancer cell survival and differentiation (22). Therefore, this pathway serves as a leading target for chemotherapeutics in blocking the normal passage of cellular processes. Western The western blotting assay was used to monitor the effects of the bergapten drug on the PI3K/AKT signaling pathway. Diminished The diminished expression level of p-PI3K and p-AKT proteins was were found in the bergapten-treated group compared to the control (Fig. 9). These results indicate that PI3K/AKT signaling pathway could be retarded by the bergapten drug and contribute to its antiproliferative property against MDA-MB-231 cells.

CONCLUSIONS

In conclusion, the current research results demonstrated substantial anti-breast cancer activity of the bergapten molecule. The anticancer effects of bergapten were attributed to its cellular apoptotic induction, G2/M phase cell cycle arrest, inhibition of cell migration and invasion and targeting of PI3K/AKT signaling pathway.

REFERENCES

1. X. Liu, Z. Wang, X. Feng, E. Bai, Y. Xiong, X. Zhu, B. Shen, Y. Duan and Y. Huang. Platensimycin-Encapsulated Poly(lactic-co-glycolic acid) and Poly(amidoamine) Dendrimers Nanoparticles with Enhanced Anti-Staphylococcal Activity in Vivo,

Bioconjugate chemistry. **31** (2020) 1425–1437; https://doi.org/10.1021/acs.bioconjchem.0c00121

2. M. Ekor, The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety, *Frontiers in pharmacology.* **4** (2014) 177; https://doi.org/10.3389/fphar.2013.00177
3. G.B. Bubols, R. Vianna Dda, A. Medina-Remon, G. von Poser, R.M. Lamuela-Raventos, V.L. Eifler-Lima, and S.C. Garcia, The antioxidant activity of coumarins and flavonoids, *Mini reviews in medicinal chemistry.* **13** (2013) 318–334; https://doi.org/10.2174/138955713804999775
4. E. Jameel, T. Umar, J. Kumar, and N. Hoda, Coumarin: A Privileged Scaffold for the Design and Development of Antineurodegenerative Agents, *Chemical biology & drug design.* **87** (2016) 21–38; https://doi.org/10.1111/cbdd.12629
5. I. Najmanova, M. Dosedel, R. Hrdina, P. Anzenbacher, T. Filipsky, M. Riha, and P. Mladenka, Cardiovascular effects of coumarins besides their antioxidant activity, *Current topics in medicinal chemistry.* **15** (2015) 830–849; https://doi.org/10.2174/1568026615666150220112437
6. H. Li, Y. Yao, and L. Li, Coumarins as potential antidiabetic agents, *The Journal of pharmacy and pharmacology.* **69** (2017) 1253–1264; https://doi.org/10.1111/jphp.12774
7. J.S. Lopez-Gonzalez, H. Prado-Garcia, D. Aguilar-Cazares, J.A. Molina-Guarneros, J. Morales-Fuentes, and J.J. Mandoki, Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines, *Lung cancer.* **43** (2004) 275–283; https://doi.org/10.1016/j.lungcan.2003.09.005
8. J.Y. Chuang, Y.F. Huang, H.F. Lu, H.C. Ho, J.S. Yang, T.M. Li, N.W. Chang, and J.G. Chung, Coumarin induces cell cycle arrest and apoptosis in human cervical cancer HeLa cells through a mitochondria- and caspase-3 dependent mechanism and NF-kappaB down-regulation, *In vivo.* **21** (2007) 1003–1009
9. H.B. Lia and F. Chen, Preparative isolation and purification of bergapten and imperatorin from the medicinal plant Cnidium monnieri using high-speed counter-current chromatography by stepwise increasing the flow-rate of the mobile phase, *Journal of chromatography A.* **1061** (2004) 51–54; https://doi.org/10.1016/j.chroma.2004.10.091
10. F. De Amicis, S. Aquila, C. Morelli, C. Guido, M. Santoro, I. Perrotta, L. Mauro, F. Giordano, A. Nigro, S. Ando, and M.L. Panno, Bergapten drives autophagy through the

up-regulation of PTEN expression in breast cancer cells, *Molecular cancer*. **14** (2015) 130; https://doi.org/10.1186/s12943-015-0403-4

11. M.L. Panno, F. Giordano, M.G. Palma, V. Bartella, V. Rago, M. Maggiolini, D. Sisci, M. Lanzino, F. De Amicis, and S. Ando, Evidence that bergapten, independently of its photoactivation, enhances p53 gene expression and induces apoptosis in human breast cancer cells, *Current cancer drug targets.* **9** (2009) 469–481; https://doi.org/10.1016/j.febslet.2010.04.001
12. I.L. Wapnir, K.N. Price, S.J. Anderson, A. Robidoux, M. Martin, J.W.R. Nortier, A.H.G. Paterson, M.F. Rimawi, I. Lang, J.M. Baena-Canada, B. Thurlimann, E.P. Mamounas, C.E. Geyer, Jr., S. Gelber, A.S. Coates, R.D. Gelber, P. Rastogi, M.M. Regan, N. Wolmark, and S. Aebi, Efficacy of Chemotherapy for ER-Negative and ER-Positive Isolated Locoregional Recurrence of Breast Cancer: Final Analysis of the CALOR Trial, *Journal of clinical oncology*. **36** (2018) 1073–1079; https://doi.org/10.1200/JCO.2017.76.5719
13. S.K. Singh, S. Singh, J.W. Lillard, Jr., and R. Singh, Drug delivery approaches for breast cancer, *International journal of nanomedicine.* **12** (2017) 6205–6218; https://doi.org/10.2147/IJN.S140325
14. H. Sung, P.S. Rosenberg, W.Q. Chen, M. Hartman, W.Y. Lim, K.S. Chia, O. Wai-Kong Mang, C.J. Chiang, D. Kang, R.K. Ngan, L.A. Tse, W.F. Anderson, and X.R. Yang, Female breast cancer incidence among Asian and Western populations: more similar than expected, *Journal of the National Cancer Institute.* **107** (2015); https://doi.org/10.1093/jnci/djv107
15. C.E. DeSantis, J. Ma, M.M. Gaudet, L.A. Newman, K.D. Miller, A. Goding Sauer, A. Jemal, and R.L. Siegel, Breast cancer statistics, 2019. *CA: a cancer journal for clinicians.* **69** (2019) 438-451; https://doi.org/10.3322/caac.21583
16. S. Mubarik, S.S. Malik, Z. Wang, C. Li, M. Fawad, and C. Yu, Recent insights into breast cancer incidence trends among four Asian countries using age-period-cohort model, *Cancer management and research.* **11** (2019) 8145-8155; https://doi.org/10.2147/CMAR.S208323
17. A. Lee, N. Mavaddat, A.N. Wilcox, A.P. Cunningham, T. Carver, S. Hartley, C. Babb de Villiers, A. Izquierdo, J. Simard, M.K. Schmidt, F.M. Walter, N. Chatterjee, M. Garcia-Closas, M. Tischkowitz, P. Pharoah, D.F. Easton, and A.C. Antoniou, BOADICEA: a comprehensive breast cancer risk prediction model incorporating

genetic and nongenetic risk factors, *Genetics in medicine.* **21** (2019) 1708-1718; https://doi.org/10.1038/s41436-018-0406-9

18. X. Xu, Y. Lai, and Z.C. Hua, Apoptosis and apoptotic body: disease message and therapeutic target potentials, *Bioscience reports.* **39** (2019); https://doi.org/10.1042/BSR20180992
19. M.L. Panno, F. Giordano, F. Mastroianni, M.G. Palma, V. Bartella, A. Carpino, S. Aquila, and S. Ando, Breast cancer cell survival signal is affected by bergapten combined with an ultraviolet irradiation, *FEBS letters.* **584** (2010) 2321-2326; https://doi.org/10.1016/j.febslet.2010.04.001
20. S.R. Chiang, C.S. Lin, H.H. Lin, P.C. Shieh, and S.H. Kao, Bergapten induces G1 arrest of nonsmall cell lung cancer cells, associated with the p53mediated cascade, *Molecular medicine reports.* **19** (2019) 1972-1978; https://doi.org/10.3892/mmr.2019.9810
21. S.P. Pattanayak, P. Bose, P. Sunita, M.U.M. Siddique, and A. Lapenna, Bergapten inhibits liver carcinogenesis by modulating LXR/PI3K/Akt and IDOL/LDLR pathways, *Biomedicine & pharmacotherapy.* **108** (2018) 297-308; https://doi.org/10.1016/j.biopha.2018.08.145
22. C. Hu, K. Yang, M. Li, W. Huang, F. Zhang, and H. Wang, Lipocalin 2: a potential therapeutic target for breast cancer metastasis, *OncoTargets and therapy.* **11** (2018) 8099-8106; https://doi.org/10.2147/OTT.S181223

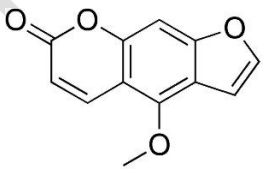

Fig. 1. Chemical structure of bergapten.

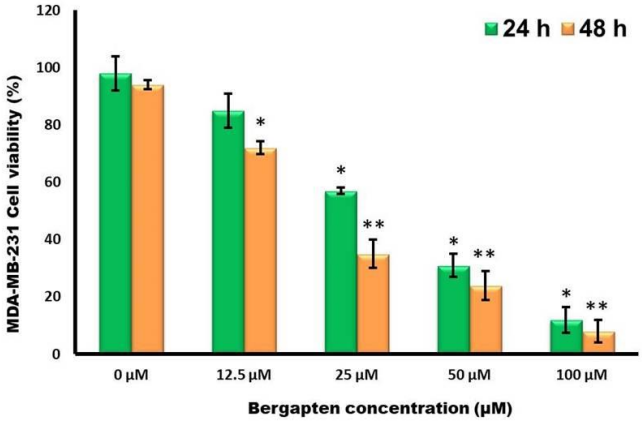

Fig. 2. MTT assay was used to study the effects of bergapten ~~drug~~ on the viability of breast cancer MDA-MB-231 cells. The figure represents inhibition of proliferation by the bergapten ~~drug~~ in a dose-dependent manner. All the independent experiments were performed in triplicates. Data ~~was~~ are expressed as mean ± SD. (*) and (**) represents $p < 0.05$ and $p < 0.01$ compared against control.

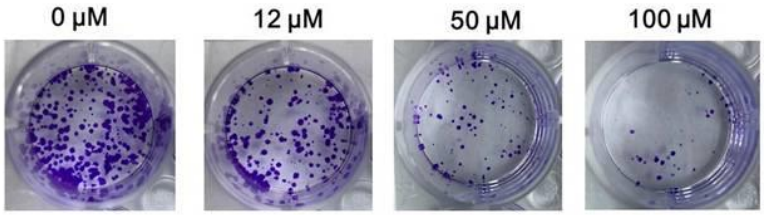

Figure 3A

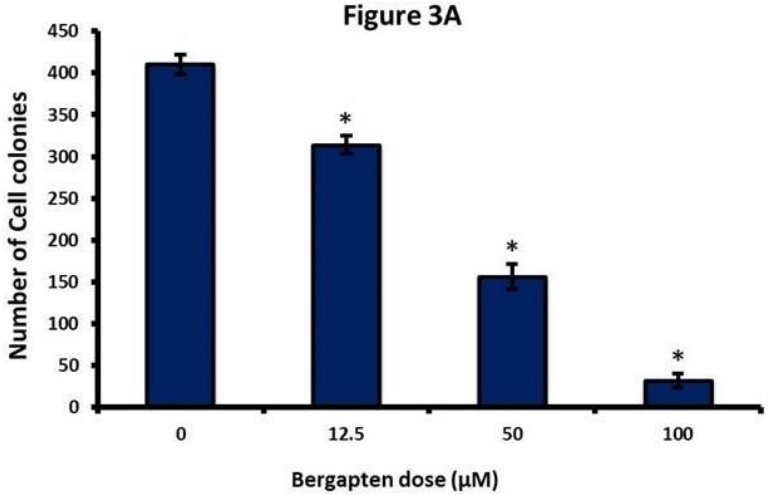

Figure 3B

Fig. 3. a) Clonogenic assay was implemented to examine the effects of bergapten ~~drug~~ of colony generation tendency of MDA-MB-231 cells. The results indicated decreasing MDA-MB-231 cell colonies after exposure to indicated doses of the bergapten ~~drug~~. b) Graphical representation of a number of MDA-MB-231 colonies generated after being exposed to indicated bergapten doses. All the independent experiments were performed in triplicates. Data ~~was~~ are expressed as mean ± SD. (*) represents $p < 0.05$ compared against control.

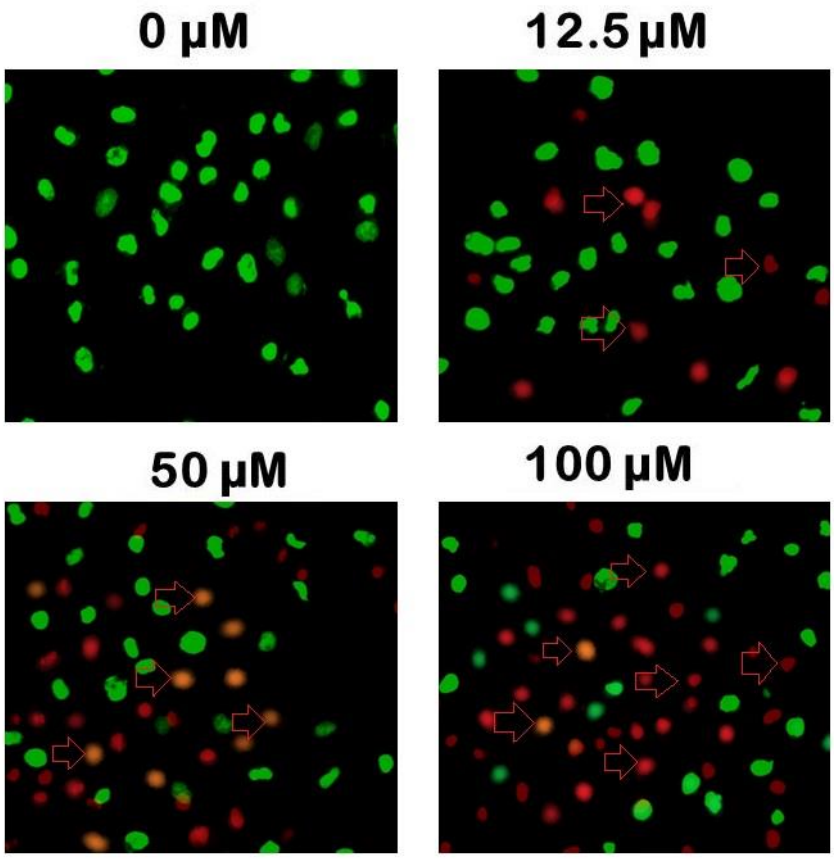

Fig. 4. AO/EB staining assay was used to study apoptosis in MDA-MB-231 cells after being exposed to various doses of the bergapten ~~drug~~. The figure represents early apoptotic, late apoptotic and necrotic cells. Individual experiments were performed three times. Early apoptotic cells show yellow-green fluorescence**,** late apoptotic cells show orange fluorescence, and necrotic cells show uneven orange-red fluorescence.

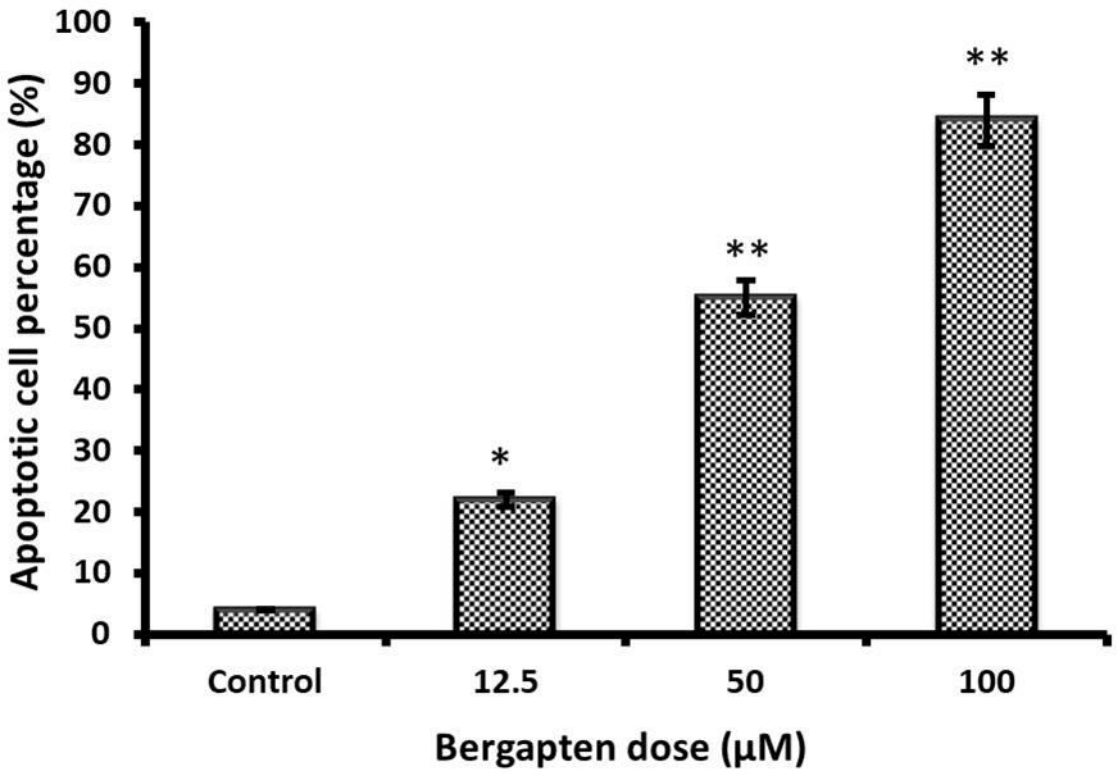

Fig. 5. Annexin V-FITC staining assay was implemented to quantify apoptosis in bergapten treated MDA-MB-231 cells. Results showed an enhanced percentage of apoptotic cells in the treated group compared to the control group. Individual experiments were performed in triplicates. Data ~~was~~ are expressed as mean ± SD. (*) and (**) represents $p < 0.05$ and $p < 0.01$ compared against control.

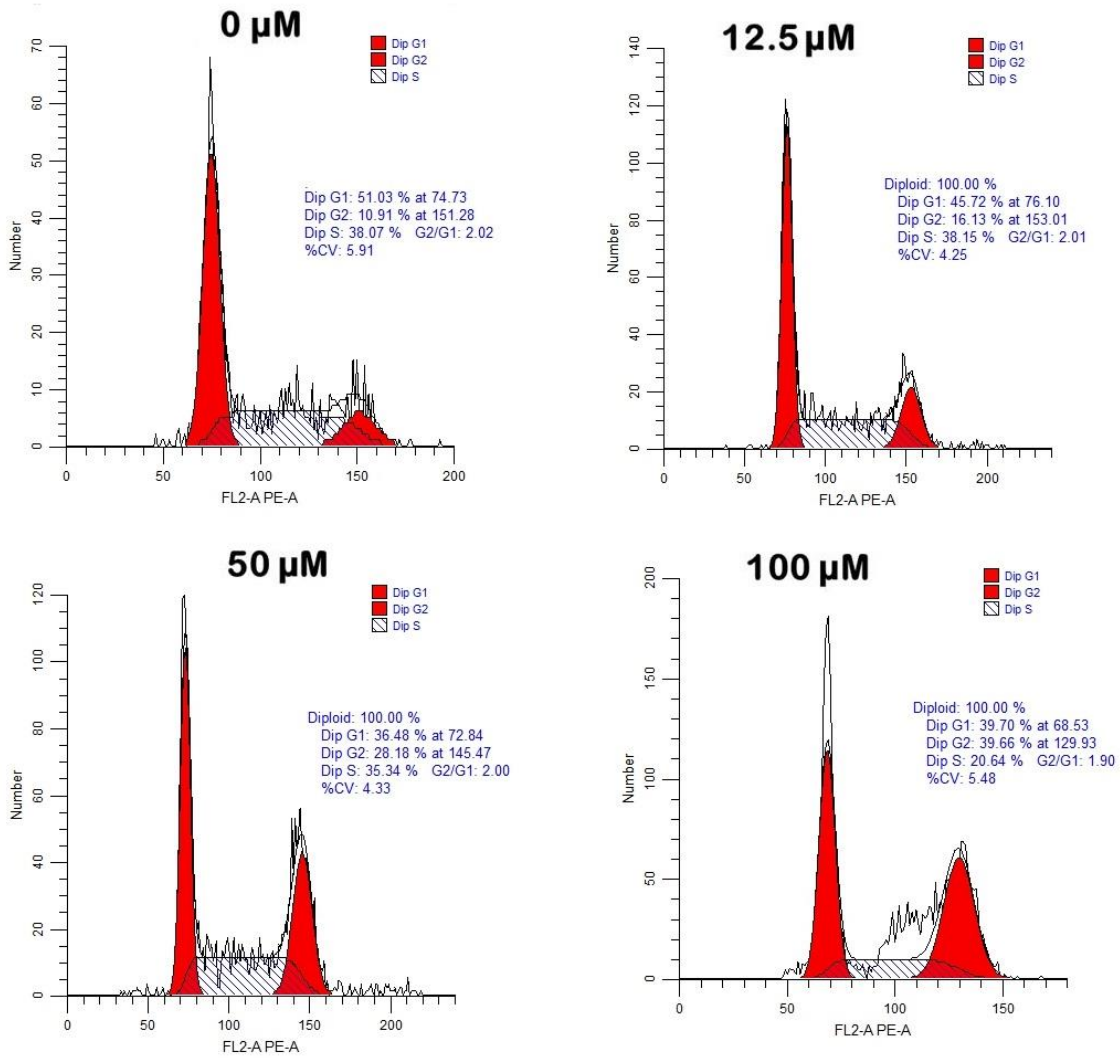

Fig. 6. Flow cytometric assessment of different phases of the cell cycle in bergapten treated MDA-MB-231 cells. All the independent experiments were performed in triplicates.

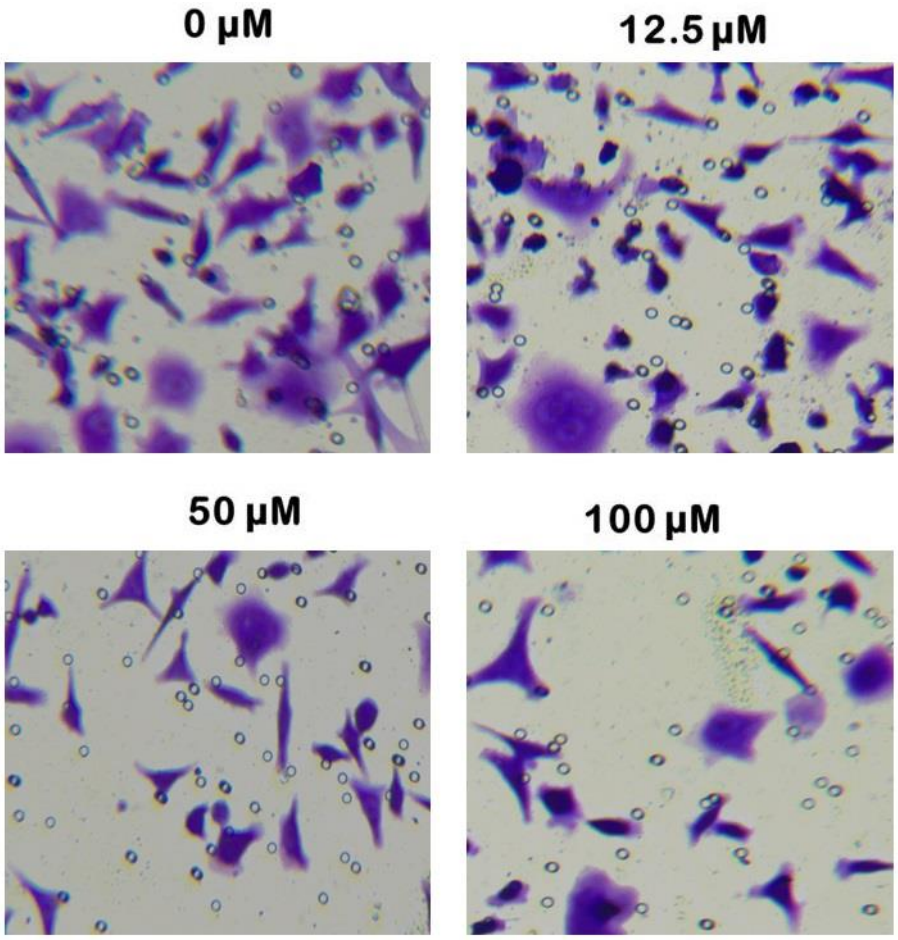

Fig. 7. Transwell chambers migration assessment to monitor MDA-MB-231 cells' ability to migrate after being exposed to different bergapten doses. Individual experiments were performed three times.

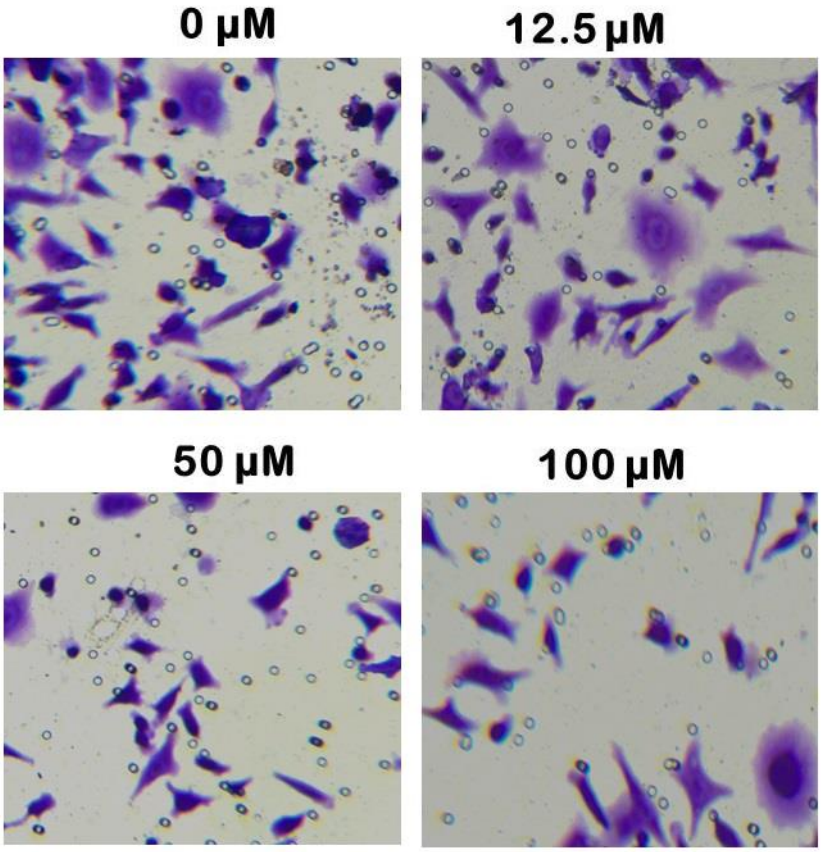

Fig. 8. Transwell chambers invasion assessment to monitor MDA-MB-231 cells' ability to migrate after being exposed to different bergapten doses. Individual experiments were performed three times.

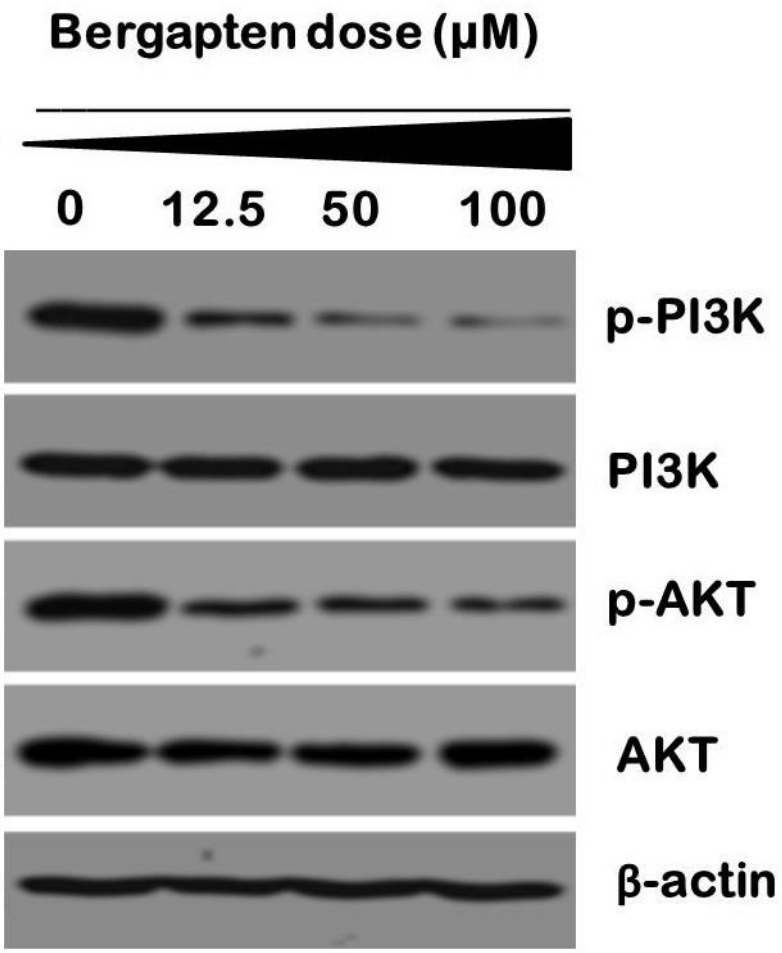

Fig. 9. ~~Western~~ The western blotting assay was performed to check the activity levels of PI3K/AKT signaling pathway allied proteins. Results showed a reduction in phosphorylation of PI3K and AKT in comparison to the overall expression of PI3K and AKT after being exposed to different bergapten doses. Individual experiments were performed three times.