

Piperine Alkaloid Induces Anti-Cancer Effects in Human Breast Carcinoma Cells by Targeting Phosphatidylinositol 3-Kinase/Protein Kinase B Signaling Pathway

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Zhang *et al.*: Effects of Piperine Alkaloid against Human Breast Carcinoma

Piperine is a dietary alkaloid with huge pharmacological importance and was first isolated from pepper extract. It has been reported with anti-cancer, anti-amoebic, anti-mycobacterial, anti-mutagenic, anti-convulsant, anti-asthmatic, immunomodulatory, anti-inflammatory and anti-oxidant activity. Therefore, the current investigation was designed to unveil the anticancer effects of piperine molecule against human breast carcinoma. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was executed for determination of effects of piperine on breast cancer cell viability. Clonogenic assay was implemented for checking the effect of piperine on clonogenic potency of breast cancer cells. To monitor morphology of cancerous breast cells, phase contrast microscopy was performed after piperine exposure. Apoptosis was checked by acridine orange/ethidium bromine staining assay and by execution of Western blotting analysis. Cell migration and invasion was monitored by transwell chambers assay. Muse flow cytometric analysis was performed for assessing of different cell cycle phases. Western blotting assay was implemented to check the levels of phosphatidylinositol 3-kinase and protein kinase B. Results revealed that piperine induced dose reliant cytotoxicity in MCF-7 human breast cancer cells. Further, application of piperine on cancer cell colonies remarkably reduced the number of cell colonies to minimum. Phase contrast microscopy evidenced some specific morphological modifications indicating apoptosis allied cytotoxicity of piperine like apoptotic bodies, condensed nucleus and membrane blabbing. Acridine orange/ethidium bromine staining assay revealed that piperine induced apoptosis in MCF-7 cells which was further investigated by Western blotting. This revealed increased expression of B-cell lymphoma 2 associated X and reduced expression of B-cell lymphoma 2, indicating apoptosis induction by piperine. Cell migration as well as cell invasive ability of MCF-7 cells was reduced to minimum by the application of piperine in dose dependent-manner. Flow cytometric analysis evidenced that piperine arrested the cell cycle at growth 2/mitosis phase, hence suppressed the breast cancer progression. Finally, Western blotting assay predicted constant expression of phosphatidylinositol 3-kinase and protein kinase B and reduced expression of phosphorylated phosphatidylinositol 3-kinase. Hence, evidenced the blocking of phosphatidylinositol 3-kinase/protein kinase B signaling pathway. In conclusion, the current investigation regarding anticancer effects of piperine against breast cancer revealed remarkable suppression of cancer cells. It induced apoptosis, suppressed cell migration and invasion, blocked cell cycle and phosphatidylinositol 3-kinase/protein kinase B signaling pathway.

Key words: Breast cancer, alkaloids, piperine, apoptosis, cell migration, cell invasion

Breast Cancer (BC) is a dangerous and most frequent malignancy prevailing in women globally. The incidences of developing BC have gone up with nearly 3.1 % increase annually over last 30 y. Alone in the year of 2010, nearly 1.6 million cases of BC were registered^[1]. BC comprises of variant subtypes and those subtypes arise from basal progenitor or luminal progenitor cells undergoing different genetic mutations^[2]. BC is both histopathologically as well as genetically heterogeneous, thus underlying mechanisms

of developing BC still remains uncertain^[3], which cause hurdles in BC diagnosis as well as treatment, owing to this conventional treatment for BC like surgery, radiation and chemotherapy does not hold good

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efficiency. Currently, subtypes of BC are categorized on the basis of activity of Hormone Receptors (HR) and Human Epidermal Growth Factor Receptor 2 (HER2)^[4]. These subtypes show difference in metastasis, biology, treatment as well as prognosis. It has been reported in various studies that the reproductive pattern (breastfeeding and pregnancy) has a direct relationship with developing BC^[5-7]. Before the introduction of modern medicine for treatment of human ailments, medicinal plants were extensively used and were effective too^[8,9]. Afterwards, the knowledge of medical properties of medicinal plants were explored, which lead to the formation of variant therapies and traditional medicine systems including Traditional Chinese Medicine (TCM), indigenous medicine, Ayurvedic medicine, aromatherapy and naturopathy^[10-12]. Apart from conventional medicine, modern medicine comprises of numerous plant derived chemical entities including anti-cancer drugs like vinblastine and taxol^[13,14]. Naturally occurring alkaloids have long been serving as a pool of drug discovery and few of these have already been permitted by United States Food and Drug Administration (US FDA) like camptothecin. Piperine, an alkaloid is a key dietary phytochemical due to its pharmacological importance and prevalence in spicy foods. It acts as anti-cancer, antimetastatic, larvicidal, anti-inflammatory, immunosuppressive, leishmanicidal and antiparasitic^[15-22]. Orsted first isolated piperine from the extract of pepper^[23]. The main purpose of the current study was to investigate anticancer and apoptotic effects of piperine alkaloid against human BC cells which was found to be mediated *via* cell migration and cell invasion inhibition, Growth 2 (G2)/Mitosis (M) phase cell cycle arrest and targeting Phosphatidylinositol 3-Kinase/Protein Kinase B (PI3K/AKT) signaling Pathway.

MATERIALS AND METHODS

Cellular viability measurement:

Viability of human breast carcinoma cells (MCF-7) was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) assay after piperine exposure. In brief, MCF-7 cells were cultured onto 96-well plate at 2×10^4 cells/well of density for 24 h at 37°. After culturing of the cells, treatment with varying piperine doses (0, 10, 20, 50 and 100 μ M) was initiated for 24 h and 48 h. Afterwards, MCF-7 cells were incubated at 37° in a Carbon dioxide (CO₂) incubator (5 %). Viability of piperine treated cells was then observed

by performing MTT assay as designated by Mosmann *et al.*^[24]. For Optical Density (OD) measurements absorbance was recorded with micro plate Enzyme Linked Immunosorbent Assay (ELISA) reader (Tecan, United States) at 570 nm.

Assessment of clonogenic potency:

Human MCF-7 BC cells were plated onto 6-well plate (3500 cells each well) containing Fetal Bovine Serum (FBS) 10 %. Seeding of MCF-7 cells was followed by piperine exposure at changing doses *viz.* 0, 20, 50 and 100 μ M for 10 d. Piperine treated cells were then stained with crystal violet after fixation in paraformaldehyde (Beyotime, China). Thereafter, cell colonies were studied under a digital camera (Olympus, Japan) followed by further investigations.

Cell morphology determination by phase contrast microscopy:

Morphological changes induced by piperine in MCF-7 BC cells were recorded by performing phase contrast microscopy. Briefly, MCF-7 cells were cultured in 6 mm cultural dishes containing 2×10^3 cells each. After culturing, cells were treated with piperine at changing doses *viz.* 0, 20, 50 and 100 μ M for 24 h each dish. Piperine treatment was followed by removal of cultural media and washing of treated MCF-7 cells with Phosphate Buffered Saline (PBS). Finally, MCF-7 cells were observed and studied with a phase contrast inverted microscope (Leica, Germany) at 200x magnification.

Apoptosis assessment by Acridine Orange/Ethidium Bromine (AO/EB) staining:

Ariffin *et al.* method of AO/EB staining was employed to assess the effect of piperine on apoptotic cell morphology^[25]. MCF-7 BC cells were harvested at 80 % confluence of growth and subjected to piperine exposure at varying doses *viz.* 0, 20, 50 and 100 μ M. Thereafter, AO/EB staining was performed followed by visualization. Visualization was performed with a confocal laser-scanning microscope (Olympus Fluoview FV1000) under 40x of magnification using 1.4 Numerical Aperture (NA) as an objective.

Transwell chambers assay for cell migration and cell invasion:

Transfection of MCF-7 BC cells was executed with piperine drug at changing concentrations of 0, 20, 50 and 100 μ M. The lower transwell chambers were filed with Roswell Park Memorial Institute (RPMI)-1640

cultural medium and FBS (10 %) (Corning Incorporated, Corning, New York, United States). Transfected cells were filled in upper chambers of transwell bearing cultural medium at a density of 1×10^5 cells/well. These transwell chambers were incubated for 12 h and then incubated for 10 min at 4° . The cells which didn't migrate were cleared with a cotton swab followed by staining with crystal violet with 5 min of incubation. Under a light microscope (TS100; Nikon Corporation, Tokyo, Japan) with 200x of magnification different sections were pictured. The cell invasion potency of MCF-7 BC cells was determined by following similar procedure except transwell chambers coated with Matrigel was used.

Examination of different cell cycle phases:

For cell cycle analysis, MCF-7 cells were first treated with varying piperine doses (0, 20, 50 and 100 μ M) for 24 h. Then these cells were subjected to washing, once with PBS followed by 5 min of centrifugation. After centrifugation, cells were fixed in 70 % ethanol and repeatedly washed twice with PBS. Cell suspensions were supplemented with Ribonucleic Acid (RNA) 50 μ l and Propidium Iodide (PI) 25 μ l and then incubated for 15 min. Finally, Deoxyribonucleic Acid (DNA) content was determined through Muse flow cytometry (Millipore, Billerica, Massachusetts, United States).

Western blotting analysis:

Human MCF-7 BC cells were collected at 80 % confluence of growth followed by lysing with Radioimmunoprecipitation Assay (RIPA) buffer (Sigma-Aldrich). The protein content within each lysate was determined with Bicinchoninic Acid (BCA) protein assay kit (Beyotime, China). Thereafter, 10 % Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used for uniformly arranging protein amounts (40 μ g) followed by loading these over Polyvinylidene Difluoride (PVDF) membranes (Millipore, Billerica, Massachusetts, United States). PVDF membranes were then subjected to primary antibodies treatment (antibodies against B-Cell Lymphoma Protein-2 (BCL-2), Bcl-2 Associated X (BAX), PI3K and AKT) (1:1000; Epitomics, Burlingame, California, United States). Primary antibody exposure was followed by secondary antibody exposure (anti-rabbit Immunoglobulin G (IgG) conjugated to Human Resource Planning (HRP)) with incubation overnight at 4° . Finally, protein bands were envisaged through Enhanced Chemiluminescence (ECL) detection system (Pierce, Rockford, IL, United

States).

Statistical analysis:

Significant difference was evaluated by execution of Student's t-test using Statistical Package for Social Sciences (SPSS) for Windows software package (release 11.0.0, SPSS Inc., United States). Variance was considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Cellular viability of MCF-7 BC cells was testified by MTT assay after piperine (fig. 1A) drug exposure at varying doses (0, 10, 20, 50 and 100 μ M). Cellular viability was found to be retarding with increasing drug doses as well as with the extent of drug exposure. The cell viability percentage decreased from nearly 95 % to 15 % after 24 h of exposure and reduced from 90 % to 10 % after 48 h of drug exposure (0-100 μ M) (fig. 1B). Hence, evidencing the cytotoxicity effects of piperine in MCF-7 BC cells.

Clonogenic potency of MCF-7 BC cells was determined by execution of clonogenic assay. Along with cell viability inhibition the MCF-7 cell colonies were also inhibited in a dose-dependent manner. The number of cell colonies pictured under a digital camera at varying drug doses (0, 20, 50 and 100 μ M) of MCF-cell colonies can be seen reduced to minimum (fig. 2A).

To assess the morphological changes in MCF-7 cells after exposure with piperine, phase contrast microscopy was executed. The results evaluated that the piperine induced cell shrinkage, apoptotic bodies, bubbling, condensed nuclei, membrane blabbing and echinoid spikes (fig. 2B). These morphological modifications evidenced induction of apoptosis mediated cytotoxicity by piperine in MCF-7 cells.

AO/EB staining was performed to monitor the apoptotic cells after piperine exposure. Results indicated regular tumor cells, late and early apoptotic cells, and necrotic cells were inspected *via* fluorescent microscopy, granular yellow-green or crescent-shaped AO nuclear staining marked early-stage apoptotic cells. Unevenly localized and concentrated orange nuclear ethidium bromide staining represents late-stage apoptotic cells. Uneven orange-red fluorescence represents necrotic cells with increased volume. Cells seemed to be in the progression of disintegration (fig. 3A). Further, Western blotting assay unveiled that the activity of BAX increased significantly with increasing piperine doses and activity of BCL-2 reduced at higher drug doses (fig. 3B).

Therefore, AO/EB staining and Western blotting assay represent that the anti-proliferative effects of piperine drug are mediated *via* induction of apoptosis in MCF-7 BC cells.

Transwell chambers were used to monitor cell migration as well as cell invasive tendency of piperine treated MCF-7 cells. After 48 h of exposure in transwell chambers migrated cells were examined. It was established from the results that number of migrated cells reduced from maximum at controls to minimum after piperine drug exposure (fig. 4A). The number of invasive cells also reduced dose dependently from high to low after piperine exposure (fig. 4B). Thus transwell chamber assays unveiled that along with inducing cytotoxicity in MCF-7 cells, piperine significantly retarded the cell migratory and cell invasive potency of MCF-7 cells.

Muse flow cytometry was executed to investigate DNA content for determination of different cell cycle phases. After exposure with varying piperine drug doses (0, 20, 50 and 100 μ M) for 24 h different cell cycle phases were determined. The results revealed that the number of G0/G1 and S-phase cells reduced significantly and dose-reliantly to minimum after piperine exposure. In contrast with this the number of G2/M phase cells accumulated and reached to higher concentrations at higher drug doses (fig. 5). This indicated that piperine induced cell cycle arrest at G2/M phase of cell cycle. Hence, evidencing that piperine allied cytotoxicity in MCF-7 cells is also mediated *via* induction of cell cycle arrest.

The effect of piperine over the PI3K/AKT signaling pathway was investigated *via* Western blotting assay. This pathway plays a vital role in cancer cell development and progression. Hence, hampering this pathway leads to suppression of cancer. After, piperine treatment cellular protein was quantified and subjected to Western blotting. Results indicated that the activity of phosphorylated PI3K and AKT reduced remarkably with increased piperine doses. In contrast with this PI3K and AKT activity remained unchanged (fig. 6). Thus indicated blocking of PI3K/AKT signaling pathway in MCF-7 cells by piperine.

Different chemotherapeutics induce anti-cancer effects *via* induction of apoptosis in cancer cells. Apoptosis is accomplished *via* two different pathways; extrinsic (death receptor allied) and intrinsic (mitochondria allied)^[26]. Piperine has a tendency to alter the expressions of apoptosis engaged proteins thus has a capability of inducing both intrinsic as well extrinsic apoptosis. Piperine has been reported with anti-tumor activity against mouse BC 4T1 model and also suppressed tumor metastasis^[27]. The anti-cancer effects of piperine in 4T1 cells were due to induction of caspase-3-reliant intrinsic apoptosis and cell cycle arrest at G2/M phase *via* reduction of cyclin B1 activity. Piperine was also reported with blocking of Sterol Regulatory Element-Binding Protein 1 (SREBP-1), Extracellular Signal-Regulated Kinases (ERK) 1/2 and Fas Cell Surface Death Receptor (FAS) expression in over expressive HER-2 BC^[28]. Piperine inhibits Epidermal Growth Factor (EGF) allied Matrix Metalloproteinase 9 (MMP-

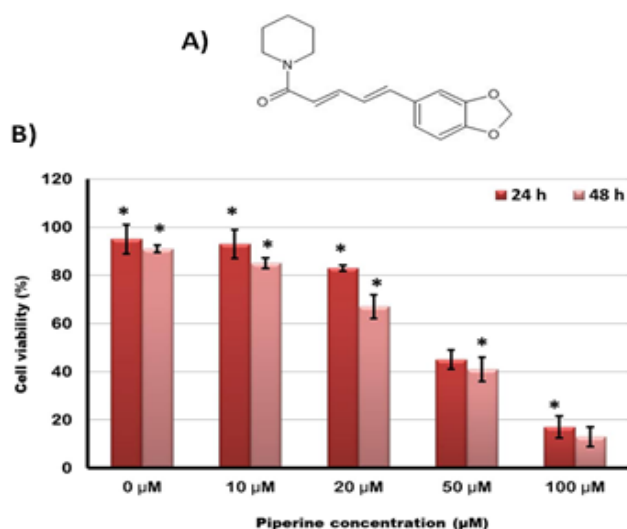


Fig. 1: (A): Chemical structure of piperine molecule and (B): Results representing cellular viability of MCF-7 cells after piperine treatment. MCF-7 cells were treated with piperine for 24 h and 48 h at changing doses of 0, 10, 20, 50 and 100 μ M

Note: All the experimental data are revealed as mean \pm standard deviation. Experiments were executed in triplicates with p<0.05, (■): 24 h and (■): 48 h

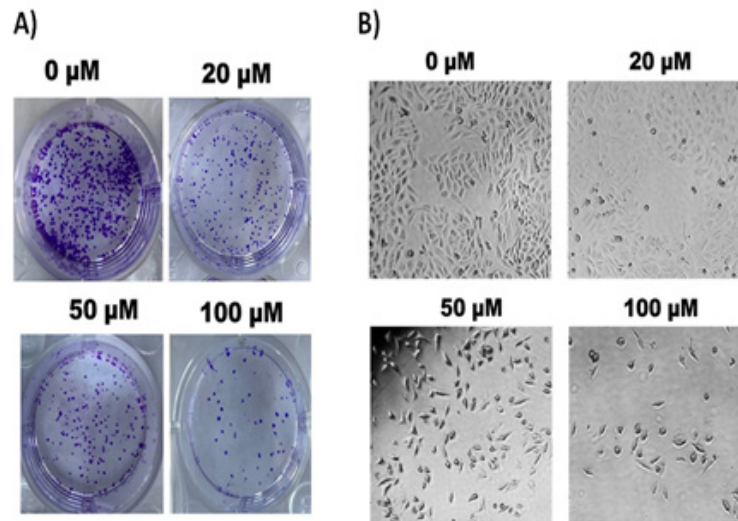


Fig. 2: (A): Pictures representing MCF-7 cell colonies after 10 d of piperine exposure. Experiments were executed in triplicates and (B): Phase contrast microscopy indicating morphological changes at indicating piperine drug doses
 Note: Experiments were executed in triplicates

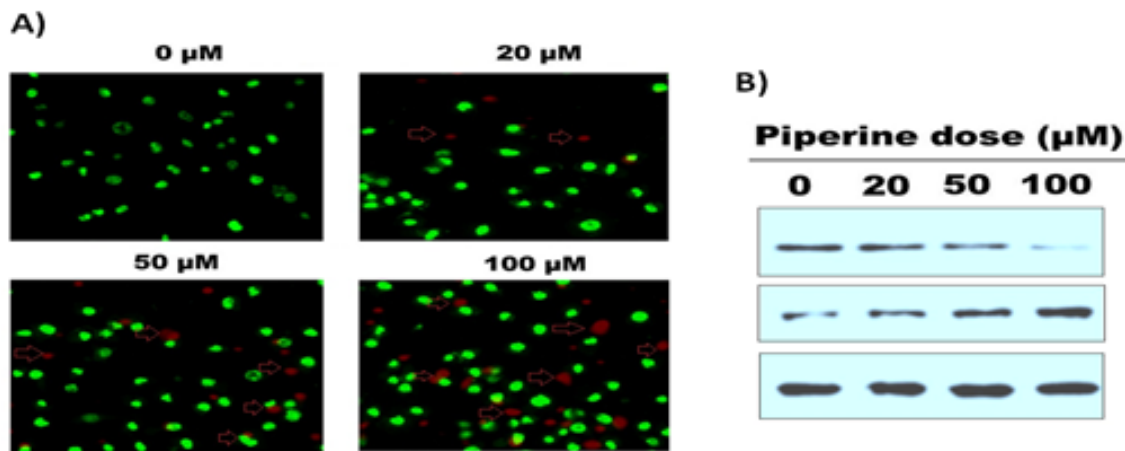


Fig. 3: (A): Assessment of apoptosis *via* AO/EB staining revealing apoptotic cells at different stages including normal, early, late apoptotic and necrotic cells, and (B): Western blotting assay results representing expressions of BAX and BCL-2 after piperine exposure of MCF-7 cells
 Note: Experiments were performed in triplicates

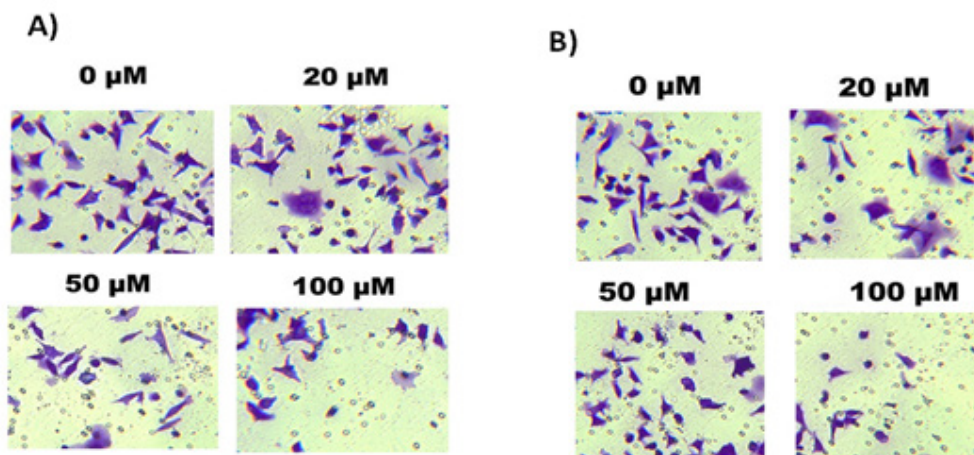


Fig. 4: (A): Transwell chamber assay representing population of migrated cells after drug exposure of MCF-7 cells. Results revealed dose dependent suppression of MCF-7 cell migration and (B): Transwell chamber assay representing population of invaded cells after drug exposure of MCF-7 cells. Results revealed dose dependent suppression of MCF-7 cell invasion tendency
 Note: Experiments were performed in triplicates

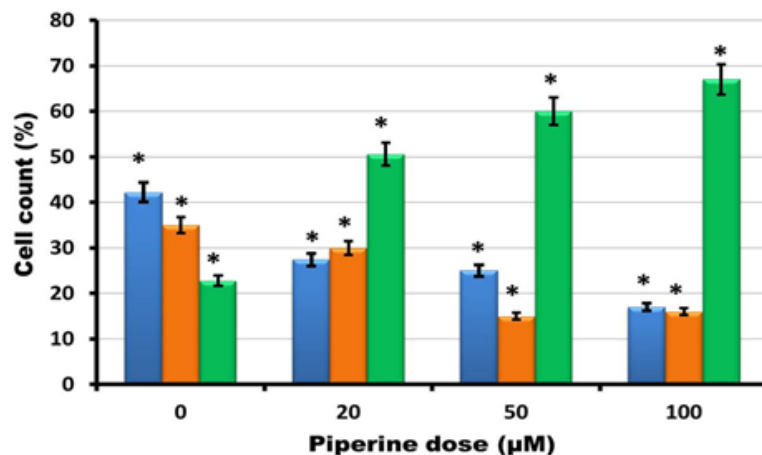


Fig. 5: Flow cytometric analysis of piperine treated MCF-7 cells at indicated doses. Results indicated amplified G2/M-phase cells evidencing cell cycle arrest at given phase of cell cycle

Note: Experiments were performed in triplicates, (■): G0/G1; (■): S and (■): G2/M

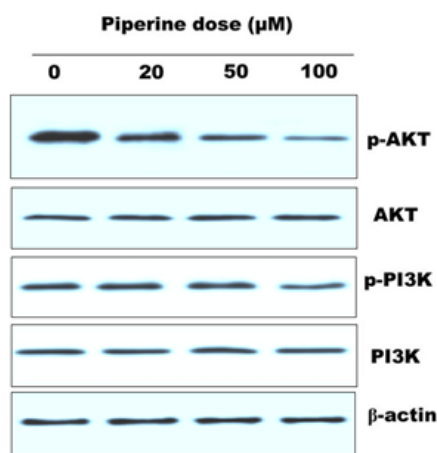


Fig. 6: Western blotting assay results representing expressions of phosphorylated and non-phosphorylated PI3K and AKT protein levels at indicated doses. Results represented blocking of PI3K/AKT signaling pathway

Note: Experiments were repeated in triplicates

9) activity and acts *via* suppression of Nuclear Factor Kappa B (NF-κB) and Activator Protein 1 (AP-1) activation and by intervening with p38 Mitogen-Activated Protein Kinase (MAPK), AKT and ERK1/2 signaling pathways which leads to suppression of cellular migration^[29]. In the current investigation, piperine alkaloid was testified for induction of anticancer and apoptotic effects in human BC cells, along with investigating its effects on cell migration and cell invasion, cell cycle and PI3K/AKT signaling pathway. It was observed that piperine induced cytotoxicity in MCF-7 cells in a dose dependent manner along with suppression of cancer cell colonies. Further phase contrast microscopy predicted specific morphological modifications that pointed induction of cytotoxicity by piperine may be mediated *via* induction of apoptosis. It was evidenced by performing AO/EB staining that piperine induced apoptotic cell death in MCF-7 cells and further supported by Western blotting (showed

increased BAX and decreased BCL-2 levels). Cell migration as well as cell invasion ability was reduced to minimum by application of piperine on MCF-7 cells. Next, piperine arrested the cell cycle at G2/M phase depicted after execution of flow cytometric analysis. Finally, Western blotting assay revealed blocking of PI3K/AKT signaling pathway.

In conclusion, the current investigation evidenced that piperine alkaloid induces anticancer and apoptotic effects in human BC cells. The effects of BC suppression were found to be mediated *via* suppression of cell migration and cell invasion, G2/M phase cell cycle arrest and targeting PI3K/AKT signaling pathway.

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Conflict of interests:

The authors declared no conflict of interest.

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