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SYNTHESIS, CHARACTERIZATION AND ANTICANCER ACTIVITY OF PIPERAZINE AMIDE DERIVATIVE

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ABSTRACT: Piperazine is a nitrogen-containing heterocyclic compound and has fascinating applications in drug discovery and development. In the present study, novel 4-(benzo[1,3]dioxol-5-ylmethyl) piperazine amide derivative was prepared by acid amine coupling of 1-piperonyl piperazine derivative and benzoic acid. Synthesized compound was characterized by IR, NMR and mass spectral studies. Further, *in-vitro* cytotoxic effects were carried out in MTT in various human cell lines HeLa, MCF7, MDA-MB-231, HCT116, and HT29. Out of these diverse cell lines, compound 3 showed promising cytotoxic effects on MDA-MB-231 with the IC₅₀ estimation of 11.3 μM. The surface morphology by Colonogenic assay and *in-vitro* cell migration assay measures better movement on MDA-MB-231 in compound 3. Further, apoptotic-related examination under AO/Eb staining, commet assay, and cell cycle investigation exhibited that compound 3 demonstrated significant activities on MDA-MB-231 by activates apoptosis and blocking cell cycle moment in the G₀/G₁ stage. All the more altogether, staining the cells in the meantime with Annexin V-FITC/PI. The synthesized compound 3 indicated tremendous repressing capacities in cell cycle and activates apoptosis because of the structural morphology and heteroatom present in the molecule.

INTRODUCTION: Nitrogen-containing heterocycles constitute an important scaffold in biological and medicinal chemistry, which has fascinating applications in drug discovery and development ¹⁻².

Piperazine is the class of organic compound which consists of six-member rings contains the two nitrogen atom in the opposite positions. Piperazine is the extensive structural unit and a wide variety of success experienced in drug design. Piperazine unit found in the core moiety found in the wide variety of the natural active substance across the different therapeutic drugs ³. 1-(3, 4-Methylene-dioxybenzyl) piperazine or 1-piperonyl-piperazine derivatives have α-adrenergic antagonist properties ⁴ and peripheral vasodilator properties ⁵.

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Piperazines are among the most important building blocks in today's drug discovery and are found in biologically active compounds across a number of different therapeutic areas ⁶⁻⁷. Benzothiazole-

piperazine derivatives are active against HUH-7, MCF-7 and HCT-116 cancer cell lines and these compounds also showed causes apoptosis by cell cycle arrest at subG1 phase ⁸.

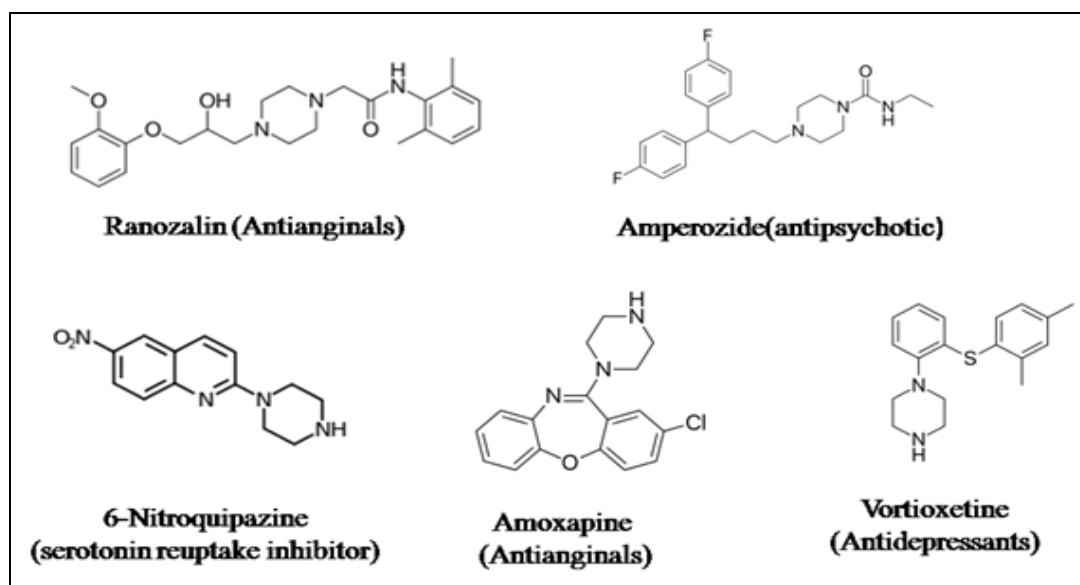


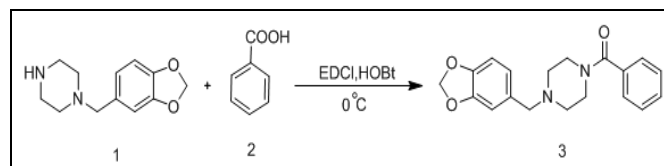
FIG. 1: PIPERAZINE CONJUGATED DRUG MOLECULES

Benzothiazole-Piperazine-1, 2, 3-Triazole hybrids also showed the antiproliferative inhibition potency against four selected human cancer cell lines (MCF7, T47D, HCT116, and Caco2) ⁹. The hybrid molecular designing is an effective method in modern medicinal chemistry to get highly active molecules. This hybridization may result in complementary pharmacophoric functions or emerge as a new mechanism of action ¹⁰⁻¹¹. Benzoyl-benzofuran derivatives possessing piperazine linker shows the anticancer activity against a panel of human tumor cell lines and apoptosis in A549 cell ¹². Based on the above facts, the present work involves the synthesis of a new novel molecule of benzodioxal piperazine derivatives and screened for their anti-cancer study.

EXPERIMENTAL:

Synthesis of 1-(benzo[1,3]dioxol-5-ylmethyl)piperazine amide (Compound 3): The title compound was synthesized as shown in scheme 1. Benzoic acid (111 mg, 0.91mmol) was dissolved in minimum amount of DMF and constantly stirred and placed in an ice bath. Triethylamine (0.4 mL, 2.73 mmol) is added to the reaction mixture and stirred again for 15 min. After that, EDC HCl (209 mg, 1.092 mmol) and of HOBt (123 mg, 0.91 mmol) was added. After 15 min of stirring the solution of 1-piperonyl piperazine (200mg, 0.91

mmol) was added to the reaction mixture and stirred for overnight. Reaction progress is checked by Thin Layer Chromatography.



SCHEME 1: SYNTHESIS OF 4-(BENZO[1,3]DIOXOL-5-YLMETHYL)PIPERAZINE AMIDE DERIVATIVES

After the completion of reaction, 5mL distilled water was added to the reaction mixture and the layers were separated. The organic layer was washed with 5 drops of 10 % HCl solution, 10% aq. NaHCO₃ solution (10 ml), brine (10 ml), dried over anhydrous Na₂SO₄, filtered and concentrated under vacuum to obtain the crude product.

Synthesis of (4-(benzo[d][1,3]dioxol-5-ylmethyl)piperazin-1-yl)(phenyl)methanone (3): (C₁₉ H₂₀ N₂O₃): White solid, mp 363.15K-367.15K, FT-IR (KBr, ν /cm⁻¹): 1627.82 cm⁻¹ (C=O amide), 1034.87 cm⁻¹ (C-N aromatic), 1499.76 cm⁻¹ (C=C aromatic), 1253.41 cm⁻¹ (-C-O-C- acetal), 712.22 cm⁻¹ (mono-substituted benzene), ¹H NMR (CDCl₃, ppm, 400MHz): δ 7.38(s, 5H), 6.85 (s, 1H), 6.73 (s, 2H), 5.93-5.92(d, 2H), 3.79 (s, 2H), 3.45 (s, 4H), 2.52-2.38 (d, 4H).

MTT Cell Viability Assay: The cytotoxic impact of compound 3 was assessed by utilizing 3(4, 5 dimethyl-2-yl) - 2, 5 diphenyl tetrazolium bromide (MTT) (1). In brief, 1×10^4 cells were seeded to 96-well plate, and cells were presented to compound 3 with an alternate focus (0– 200 mg). The cytotoxic impact of the compound 3 test was estimated by including 20 ml of (5 mg/ml) MTT for each well, incubate at 37 °C for 4 h, and absorbing the plate at 570 nm¹³.

Clonogenic Assay: The clonogenic assay was performed to clear up the possible differentiations in the whole deal effects of compound 3 on the MDA-MB-231 cell line with minor alterations¹⁴. Cells were refined in a 6-well plate (400 cells/all around), exhibited to compound, and incubated at 37 °C for 24 h. Survived regions were fixed with methanol and acidic acid and stained in 0.4% crystal violet. Colonies were checked using an amplifying instrument, and culture plates were shot, using the Sony modernized camera.

In-vitro Cell Migration Assay: The measure elucidates the effects of compound 3 on the updating of the cell attachment affiliations both with the adjoining cells and with the extracellular cross-section, cell-cell communication of the harmful development cells in the midst of its duplication¹⁵. Rapidly, the correct number of MDA-MB-231 cells (1×10^6 cells/ml) were refined in the specified media in 12-well plates to shape a monolayer (Up to 80% transformation), and 1-mm measure wounds were made by scratching with a microtip. New media was incorporated, and the cells were treated with compound 3 for 48 h. The "recuperating" of this gap by cell development and improvement towards the point of convergence of the opening is watched and shot at an undefined region, using a turned around amplifying instrument (Carl Zeiss Axio Vert.A1). The dimension of cell development/recovering of the gap was dictated by differentiating the last gap width with the basic gap width.

Acridine Orange/Ethidium Bromide Staining: Nuclear staining was performed by using AO/EB dual staining technique¹⁶. The HT29 cells were assembled from both the control and compound 3 treated gatherings. The cells were spread on clean glass slides and fixed in a fixative course of action

[methanol: acidic destructive (3:1)]. The slides were hydrated with PBS and stained with Acridine orange/Ethidium bromide (1:1). The cells were immediately washed with PBS, saw under a fluorescent amplifying instrument with the wavelength 400nm– 500nm and caught.

Assessment of DNA Damage by Single-Cell Gel Electrophoresis (Comet Assay): The oxidative DNA damage of cells, single-cell gel electrophoresis was performed¹⁷. For single cell gel electrophoresis measure, the cells were washed with PBS and reaped by centrifugation. The harvested cells were exposed to compound 3 for 48 h and washed with PBS. The cell suspension (100 µL) was mixed with 10 mL 0.5% Low Melting Agarose (LMA) and spread on a minute slide precoated with 1% ordinary dissolving agarose. The slide is then drenched in lysis arrangement (2.5 M NaCl, 10 mM Na-EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4 °C. Gel electrophoresis was performed with 300 mM NaOH and 10 mM Na EDTA at pH 13 to permit the unwinding up of DNA. An electric field of 3000 mA and 25 V was connected for 20 min to permit the relocation of adversely charged DNA towards the anode. After electrophoresis, the tiny slides were incubated with neutralizing buffer (0.4 M Tris, pH 7.5) trailed by staining with 75 µL (40 µg/mL) PI and pictured under fluorescence magnifying instrument. The pictures got were dissected utilizing CASP programming to know the level of DNA damage.

Cell Cycle Investigation: HT29 cells were plated into 60-mm culture dishes (1×10^5 cells for every dish) and hatched for 24 h in 10% serum-containing media. Cells were dealt with or not treated with compound 3 for 24 h in 10% serum-containing media after incubation for 24 h in 1% serum medium. Cells were gathered by trypsinization and washed with phosphate-buffered saline (PBS) and, after that, settled in 1 ml of 70% cold ethanol.

After rehydration, cells were processed with RNase (100 µg/ml) and stained with propidium iodide (PI, 20 µg/ml). PI staining was practiced adhering to the item directions (Clontech, Palo Alto, CA). The cells were analyzed by Flow cytometry.

Annexin V Apoptosis Examine: HT29 cells were plated into 12 well culture dishes (1×10^5 cells for every well) and hatched for 24 h in a medium containing 10% FBS. Cells were treated with compound 3 for 72 h in 10% serum-containing media. Cells were gathered by trypsinization and washed with phosphate buffered saline (PBS) and after that stained with Annexin V (Bio Legend, San Diego, CA, USA) and PI and afterward, apoptosis was investigated by flow cytometry.

RESULTS AND DISCUSSION: Compound 3 was screened for the cytotoxic movement for different human disease cell lines MDA-MB-231, MCF-7 (breast adenocarcinoma), HeLa (cervical malignant growth), HT-29 and HCT 116 (Colorectal Adenocarcinoma) by MTT measure. The MTT is tetrazolium salt which gets decreased to formazan precipitates. The force of formazan formed is an immediate measure of the feasibility of the cells. Thus the compound 3 was assessed for the cytotoxic impacts alongside standard medication cisplatin. Compound 3 shows strong anticancer action with IC_{50} estimation of $11.3 \mu M$ Fig. 2A.

In perspective on the *in-vitro* investigations, we were further fascinated to look at the morphological examinations through Colony improvement measures in MDA-MB-231 cell lines. For each well, 400 cells/well were incorporated, after 24 h of incubation, specified compound 3 was treated with $11.3 \mu M$ nearby the standard medication cisplatin. At that point, the cells were fixed with methanol and acidic corrosive later, cells were stained by using 0.5% crystal violet.

After 24 h of incubation, compound 3 demonstrates significant effects on MDA-MB-231 when compare to control. The number of the colonies was watched and captured by using Sony digital camera. Fig. 2B A while later, *in-vitro* cell migration analyze was performed in 1×10^6 MDA-MB-231 cells were plated in a 6-well plate and medium-term to confluency in serum-containing media to shape a uniform monolayer (up to 80-85%). A while later, 1 mm wounds were made to make the lines in the cells by using a smaller scale tip. New media was again included, and cells were treated with an intense compound 3 for 48 h of incubation.

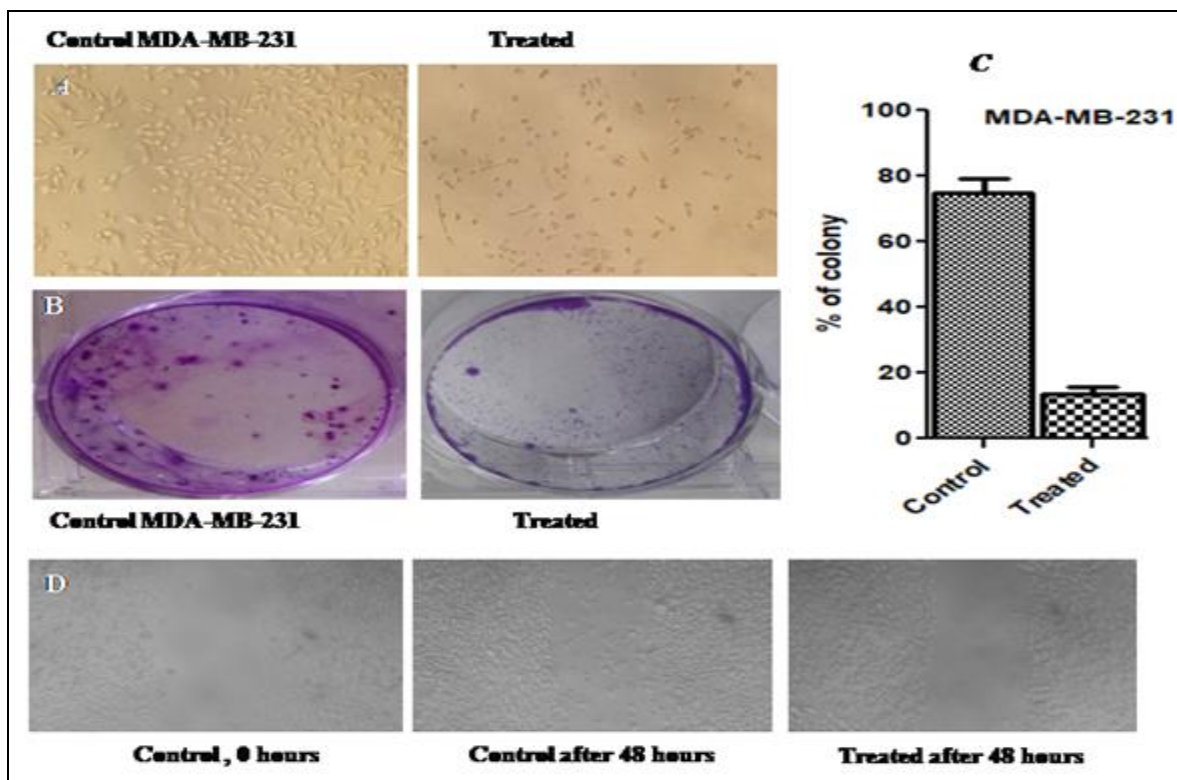


FIG. 2: A) IMAGES OF INVERTED MICROSCOPY. CELLS WERE TREATED WITH $11.3 \mu M$ COMPOUND 3 FOR 24 h. CONTROLS INCLUDED UNTREATED CELLS. B) COLONY DEVELOPMENT EXAMINE WAS PERFORMED FOR SPECIFIC CELL LINES MDA-MB-231 OF COMPOUND 3 $11.3 \mu M$. C) *IN-VITRO* CELL MOVEMENT EXAMINE WAS PERFORMED FOR PARTICULAR CELL LINES MDA-MB-231 FOR 0 h to 48 h WITH $1.7 \mu M$ CONCENTRATION OF COMPOUND 3 (RED LINES SHOWED RELOCATION LEVELS IN MDA-MB-231)

The recuperation of the cells made by the gap was analyzed and shot under the rearranged magnifying instrument (Carl Zeiss Axio vert.A1). The healing rate was determined by contrasting the beginning gap of control MDA-MB-231 against treated compound 3. Following 48 h of incubation, compound 3 restrains the relocation of the cells, starting with one spot of the gap then onto the next **Fig. 2C**.

Acridine orange is an essential color and stains both live and dead cells. After treated with compound 3 the morphology of MDA-MB-231 cells have red in shading while ordinary cells have sound morphology with flawless atomic design, and they were green in shading. Cell debasement, cell contracting, blebbing are one of the signs of cell pulverization, and their morphology has appeared in **Fig. 3A**.

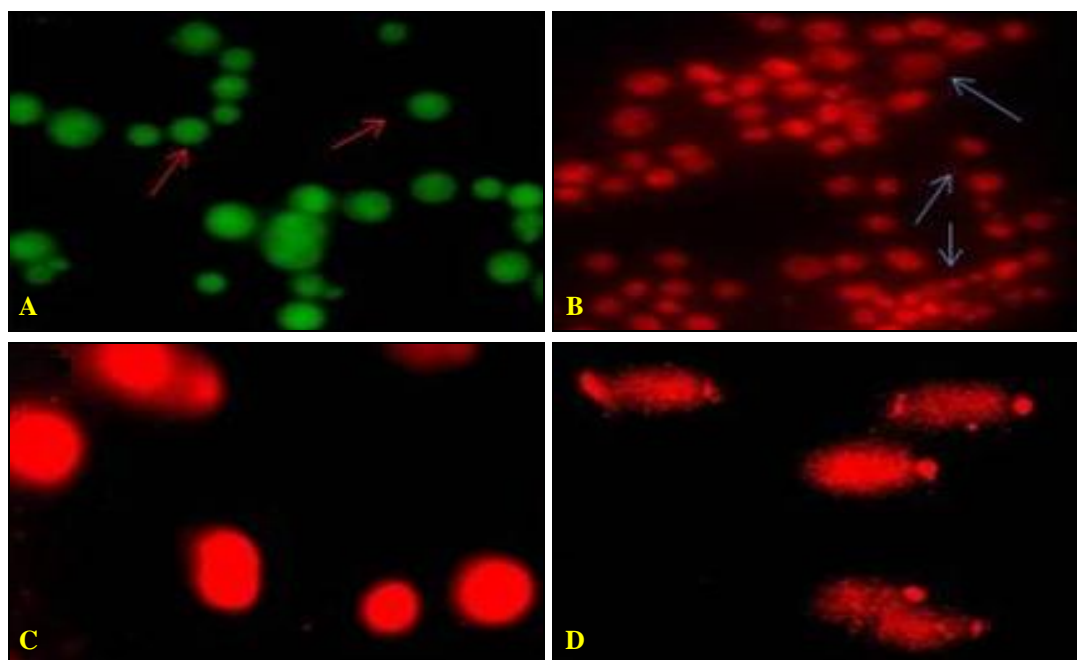


FIG. 3: A. APOPTOSIS INDUCING-ACTIVITY OF COMPOUND 3 BY AO/EB STAINING FOR SPECIFIC CELL LINE MDA-MB-231 IN COMPOUND 3 WITH 11.3 μ M CONCENTRATIONS. (BLUE COLORED ARROWS INDICATED CHANGES IN THEIR MORPHOLOGY WHEREAS RED INDICATES HEALTHY MORPHOLOGY). B. COMPOUND 3 DISPLAYED MORPHOLOGICAL DIFFERENCE IN MDA-MB-231 WHEN COMPARE WITH CONTROL

The consequences of comet test of the control cells and cells treated with compound 3 are outlined in **Fig. 3B**. As found in the fluorescence infinitesimal pictures, the nucleoids of control cells were consistently circular in shape; there was no any DNA damage. Be that as it may, total scoring of nucleoids in examinations reflected dimension of DNA single-strand breaks. Consequently, the normal comet tail DNA for control MDA-MB-231 **Fig. 3B**. Interestingly, compound 3 treated cells for 48 h. Apoptosis is a consecutive procedure in which the cell is coordinated towards implosion.

In malignancy, capacity of the cell to experience apoptosis is blocked as results a cell proceeds to proliferative aimlessly. By conjugating FITC to Annexin V it is possible to recognize and quantitate apoptotic cells on a lone cell premise by stream cytometry. Staining the cells in the meantime with FITC-Annexin V and the propidium iodide allows

the isolation of normal cells, early apoptotic and late apoptotic. Thus we performed cell cycle investigation to recognize whether cells experience apoptosis in various periods of the cell cycle. The compound 3 initiating apoptosis and blocking cell cycle development in the G0/G1 stage. The level of cells in different phases of cell cycle in mixes treated and un-treated populaces were resolved utilizing FACS Caliber (BD Biosciences, San Jose, CA) **Fig. 4A**.

To affirm whether the cells experience apoptosis or putrefaction, Annexin V-FITC/PI double staining techniques was researched. The predetermined cell lines MDA-MB-231 were treated with powerful compound 3 for 48 h. At that point, cells were stained with propidium iodide (PI) and investigated by stream cytometry. Propidium iodide (PI) can tie to DNA and discharge a fluorescent and the power of which is corresponding to the DNA content.

The cis-platin has initiated apoptosis where 10.30% of cells are in late apoptosis, and 56.00% of cells are necrotic, and 0.94% of cells are in early apoptosis. The MDA-MB-231 cells were treated with compound 3 at determined fixations at 11.3 μ M have appeared early apoptosis, 46.82% late

apoptosis. Henceforth, compound 3 has more prominent repressing capacities in MDA-MB-231 cell lines, even in a lesser focus when contrasted and that of the standard medication cisplatin as shown in Fig. 4C.

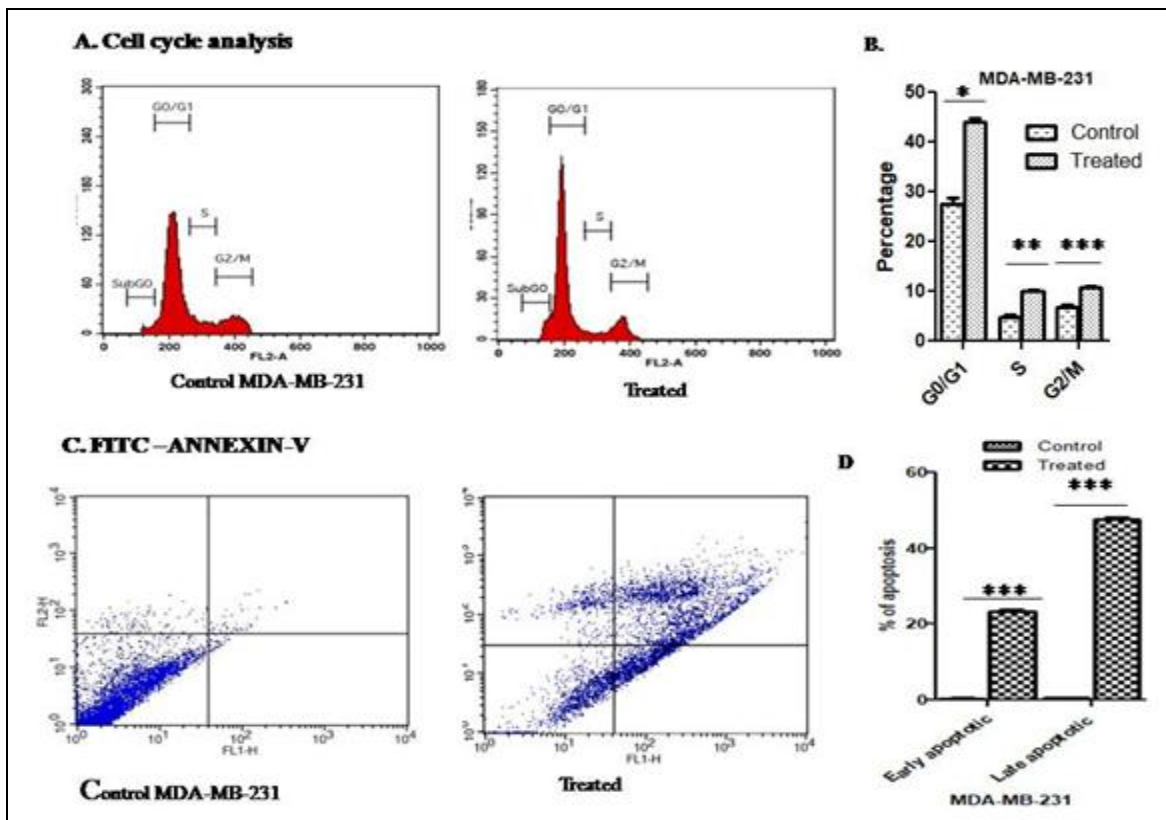


FIG. 4: A. THE COMPOUND 3 DISPLAYS CELL CYCLE ARREST AT G₀/G₁ STAGE. B. LEVEL OF G₀/G₁ IN CONTROL MDA-MB-231 TREATED. C. COMPOUND 3 INSTIGATED APOPTOSIS IN MDA-MB-231 WITH 1.7 μ M CONCENTRATION. IMPACTS OF 3 ON MDA-MB-231 CELLS WERE CONFIRMED BY ANNEXIN V-FITC/PI. D. LEVEL OF EARLY APOPTOSIS, LATE APOPTOSIS, AND NECROSIS IN CONTROL MDA-MB-231 TREATED AND STANDARD DRUG CISPLATIN. Statistical analysis was performed by Prism pad 5. Statistical significant values were expressed as (*P<0.05) (**P<0.001) and (**P<0.0001).

CONCLUSION: Compound 3 has the most restricting productivity through different non-covalent communications. The cytotoxic impacts were conveyed from a recently incorporated novel compound 3 against a board of human malignant growth cells, and compound 3 showed an intense anticancer movement with IC₅₀ assessed that 11.3 μ M. Further, *in-vitro* cell movement examine were additionally performed to research the restraining the dimension of staining of MDA-MB-231 cells from both treated and control cells. Apoptotic related examinations were likewise performed to contemplate the membrane blebbing, cell contracting, and layer corruptions have been affirmed with Acridine orange/Ethidium bromide staining. Further flow cytometry investigations uncovered that the novel compound 3 has

inhibiting capabilities of MDA-MB-231 cells by blocking cell cycle in G₀/G₁ period of the cell cycle and its demonstrates the activation of apoptosis. Consequently this novel compound 3 could be one of the conspicuous medications in the field of breast cancer.

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CONFLICTS OF INTEREST: The authors declare that they have no conflict of interest.

ETHICAL STANDARDS: Compliance with Ethical Standards

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