Lantana camara leaf extract-mediated suppression of DNA methyltransferase 1 promotes G0/G1 cell cycle arrest and apoptosis, and impedes migration in MDA-MB-231 triple-negative breast cancer cells

Sourav Sanyal [a], Arundhaty Pal [a], and Tapas K Sengupta [a, *]

[a] Department of Biological Sciences, Indian Institute of Science Education and Research Kolkata, Mohanpur, Nadia - 741246, West Bengal, India

[*] Correspondence: senguptk@iiserkol.ac.in

Abstract

Background

Triple-negative breast cancer is an aggressive subtype of breast cancer with a poor prognosis and limited therapeutic options. DNA methyltransferase 1 maintains aberrant silencing of tumor suppressor genes, contributing to cancer progression. Our previous work showed that *Lantana camara* leaf ethanolic extract induces G0/G1 arrest, apoptosis, and inhibits migration in MDA-MB-231 triple-negative breast cancer cells.

Purpose

This study investigated the role of DNA methyltransferase 1 in mediating the anticancer effects of *Lantana camara* extract.

Study Design

An *in vitro* experimental study was performed using MDA-MB-231 triple-negative breast cancer cells.

Methods

Cells were treated with the extract, and the expression of DNA methyltransferase 1 was analyzed at mRNA and protein levels. Tumor suppressor gene expression was assessed at the mRNA level, and functional assays were conducted following DNMT1 overexpression to examine its impact on extract-induced effects. Expression of methyl-CpG binding domain protein 2, histone deacetylase 1, and histone deacetylase 2 was also evaluated at the mRNA level.

Results

The extract significantly downregulated DNA methyltransferase 1, resulting in the reactivation of multiple tumor suppressor genes. Overexpression of DNA methyltransferase 1 reduced extract-induced G0/G1 arrest, attenuated apoptosis, and restored migration, while suppressing tumor suppressor gene expression. Additionally, the extract downregulated methyl-CpG binding domain protein 2, histone deacetylase 1, and histone deacetylase 2, suggesting broader modulation of epigenetic regulators.

Conclusion

Suppression of DNMT1 is a central mechanism underlying the anticancer activity of *Lantana camara* extract. These findings highlight its potential as a natural epigenetic modulator and a promising therapeutic candidate for TNBC.

Keywords

Triple-negative breast cancer; *Lantana camara*; DNA methyltransferase 1; G0/G1 arrest; Apoptosis; Migration; Epigenetics.

1. Introduction

Breast cancer continues to be one of the foremost causes of cancer-related mortality and morbidity among women globally. Despite significant advances in diagnosis and treatment, the disease continues to present a major clinical burden, with approximately 2.3 million new cases and 665,700 deaths reported in 2022 (Bray et al., 2024). Breast cancer is a heterogeneous disease comprising several molecular subtypes with distinct therapeutic responses and prognoses. Among these, triple-negative breast cancer (TNBC), characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), represents approximately 15–20% of all breast cancers (Lee, 2023). TNBC is clinically aggressive, often diagnosed at advanced stages, and associated with higher rates of recurrence and metastasis compared to other subtypes (Liang et al., 2020). Current treatment for TNBC largely relies on conventional chemotherapy, as the lack of defined molecular targets restricts the use of hormonal or HER2-targeted therapies (Yin et al., 2020). However, chemoresistance and systemic toxicity limit therapeutic efficacy, underscoring the urgent need for novel, more effective treatment strategies.

Epigenetic alterations have emerged as critical drivers of tumorigenesis, metastasis, and therapy resistance in breast cancer. Unlike genetic mutations, epigenetic modifications such as DNA methylation, histone modifications, and non-coding RNAs are reversible, making them attractive therapeutic targets (Lu et al., 2020). Aberrant DNA methylation is a characteristic of cancer, leading to the silencing of tumor suppressor genes and promoting oncogenic pathways (Lu et al., 2020). DNA methyltransferases (DNMTs) catalyze the addition of methyl groups to cytosine residues in CpG dinucleotides, maintaining epigenetic gene silencing (Lu et al., 2020). Among them, DNA methyltransferase 1 (DNMT1) is primarily responsible for preserving methylation patterns during DNA replication and is frequently overexpressed in a variety of malignancies, including TNBC (Z. Li et al., 2022; Wong, 2021). Elevated DNMT1 levels have been associated with enhanced proliferation, evasion of apoptosis, and increased migratory and invasive capacity in TNBC cells (Z. Li et al., 2022). Consequently, DNMT1 has gained attention as a promising target for epigenetic therapy in cancer.

Several synthetic DNMT inhibitors, such as 5-azacytidine and decitabine, have been approved for clinical use in hematological malignancies (Lu et al., 2020). However, their application in solid tumors, including breast cancer, has been limited by instability, off-target effects, and systemic toxicity. This has spurred growing interest in natural products as alternative epigenetic modulators. Plant-derived compounds have historically played a central role in anticancer drug development, with notable examples including paclitaxel, vinblastine, and camptothecin (Soumya et al., 2021). Beyond their cytotoxic effects, a growing body of evidence suggests that phytochemicals can regulate epigenetic mechanisms, including inhibition of DNMT activity, reversal of aberrant DNA methylation, and reactivation of silenced tumor suppressor genes. Natural compounds such as epigallocatechin gallate (EGCG), curcumin, and genistein have been shown to exert DNMT inhibitory activity, thereby restoring normal gene expression patterns and suppressing tumor progression (Saldívar-González et al., 2018). These findings highlight the potential of plant-derived agents as epigenetic therapies for aggressive cancers such as TNBC.

Extracts from different parts of medicinal plants are rich sources of bioactive phytochemicals, including flavonoids, alkaloids, terpenoids, and polyphenols, many of which exhibit anticancer

activities (Gahtori et al., 2023). Several studies have demonstrated that plant extracts can suppress proliferation, induce apoptosis, and inhibit migration of breast cancer cells through diverse molecular pathways (Gahtori et al., 2023). It was reported previously from our laboratory that *Lantana camara* leaf ethanolic extract (LCLEE) is cytotoxic to MDA-MB-231 cells, induces G0/G1 cell cycle arrest and apoptosis, and also inhibits the migratory potential (Pal et al., 2024). However, the ability of LCLEE to target DNMT1-mediated epigenetic regulation in MDA-MB-231 cells remains unexplored. Linking DNMT1 suppression by natural extracts with functional anticancer outcomes, such as cell cycle arrest, apoptosis induction, and inhibition of migration, would provide novel insights into the therapeutic potential of phytochemicals in managing TNBC.

In this study, we investigated the effects of LCLEE on TNBC cells, focusing on its role in regulating DNMT1 expression. Using the MDA-MB-231 cell line as a representative model of TNBC, we examined whether LCLEE-mediated suppression of DNMT1 was associated with alterations in key cellular processes, including cell cycle progression, apoptosis, and migration. We demonstrate that LCLEE treatment promotes G0/G1 cell cycle arrest, induces apoptosis, and inhibits the migratory activity of TNBC cells, highlighting DNMT1 suppression as a critical mechanistic link. Our findings provide mechanistic evidence for the epigenetic activity of LCLEE and suggest its potential as a source of novel therapeutic candidates for triple-negative breast cancer.

2. Materials and methods

2.1. Cell culture

MDA-MB-231 cell line was procured from the National Centre for Cell Science (NCCS), Pune, India and was maintained in Dulbecco's Modified Eagle Medium (DMEM) with High Glucose (HiMedia, India) and enriched with 10% (v/v) Fetal Bovine Serum (FBS) (HiMedia, India), Penicillin (10000 U/mL), and Streptomycin antibiotic solution (10 mg/mL) (HiMedia, India). Cells were maintained in a humidified incubator with 5% CO_2 at 37ºC. For cell harvesting and sub-culturing, 1X Trypsin-EDTA solution (HiMedia, India) was used.

2.2. *Lantana camara* leaf ethanolic extract (LCLEE) preparation

Healthy leaves of *Lantana camara* were collected from the campus and surroundings of the Indian Institute of Science Education and Research Kolkata. *Lantana camara* leaf ethanolic extract was prepared from the collected leaves following the protocol discussed previously (Pal et al., 2024). Finally, the resultant extract residues were dissolved in molecular-grade ethanol to prepare a 50 mg/mL stock solution and were stored at 4°C for future use.

To treat the cells with different concentrations of the extract, keeping the final concentration of ethanol as the vehicle at 0.8%, the stock solution was diluted in ethanol to make working stocks for respective treatments, and a fixed volume of the respective working stocks was added to the cell culture media for all treatments.

2.3. RNA isolation, cDNA synthesis, and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

MDA-MB-231 cells were cultured in 60 mm dishes (0.4 x 10^6 cells/dish) to 70-80% confluency. The cells were then treated with vehicle (0.8% ethanol) and doses of LCLEE at 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL for 24 hours. Total RNA was isolated from

vehicle-treated and LCLEE-treated MDA-MB-231 cells using RNAiso Plus (Takara, Japan) according to the manufacturer's instructions.

The RNA samples were further used for the synthesis of cDNA using PrimeScript 1st strand cDNA synthesis kit (Takara, Japan) according to the protocol mentioned by the manufacturer. The detailed protocol has been mentioned previously (Pal et al., 2024).

Real-time PCR was performed using TB Green Premix Ex Taq (Tli RNaseH Plus) kit (Takara, Japan) following the instructions mentioned by the manufacturer. The detailed protocol has been described previously (Pal et al., 2024). To check and justify the obtained results, dissociation curves were built after the PCR run. Primers of 18S, DNMT1, p21, PTEN, FOXO3a, Claudin-1, MBD2, HDAC1, and HDAC2 were used for a gene expression study (Supplementary Table S1). The gene expression was normalized against the 18S rRNA housekeeping gene, and the data were analysed using the $2^{-\Delta\Delta Ct}$ method and plotted as fold change versus vehicle control (Livak and Schmittgen, 2001).

2.4. Western Blotting

MDA-MB-231 cells were grown in 60 mm dishes (0.4 x 10^6 cells/dish) till 70-80% confluency, followed by treatment with vehicle (0.8% ethanol) and doses of LCLEE at 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL for 24 hours, along with untreated control cells. The cells were harvested by trypsinization, and washed using phosphate-buffered saline (PBS). Lysis of the cells was done using RIPA buffer (HiMedia India) supplemented with protease inhibitor cocktail (Sigma-Aldrich, USA) with frequent agitation for 30 minutes on ice. A clear protein lysate was then obtained as supernatant by centrifugation at 12,000 rpm for 20 minutes at 4 °C. Bradford assay was then performed to measure the protein concentration in the cell lysate. An equal amount of total protein (60 µg) from all the treatment conditions was separated by electrophoresis on 8% polyacrylamide gel and then electro-transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% (w/v) bovine serum albumin (BSA) fraction V (Amresco, USA) in TBST. The membrane was incubated overnight with the respective primary antibody at 4°C, followed by horseradish peroxidase (HRP)-conjugated secondary antibody. Finally, the protein bands were detected using a chemiluminescent (West Pico ECL Substrate, Thermo Fisher Scientific, USA) on the G: BOX Chemi XRQ imaging system (Syngene). The NIH-ImageJ 1.53k software was used to quantify the intensity of the protein bands, which were then normalized using the GAPDH (loading control). The details of the antibodies used in the experiment are listed in Supplementary Table S2.

2.5. Generation of stable cell lines

To generate stable MDA-MB-231 cell lines, cells were transfected with either pcDNA3.1(+) empty vector encoding a neomycin resistance gene (neoR) or pcDNA3.1(+)-DNMT1 construct encoding both the transgene (DNMT1) and a neomycin resistance gene (neoR). Details of the plasmid and cloning procedure are provided in the supplementary section (Supplementary Figure S1). MDA-MB-231 cells were seeded at a density that allowed them to reach ~80% confluence at the time of transfection. Transfection was performed using Lipofectamine™ 2000 Transfection Reagent (ThermoFisher Scientific, USA) by following the instructions given by the manufacturer. 48 hours post-transfection, the culture medium was then replaced with fresh complete medium supplemented with G-418 sulphate (Geneticin) (SRL, India).

Before selection, a kill curve was established to determine the minimum concentration of G-418 sulphate required to eliminate non-transfected cells. Briefly, non-transfected parental MDA-MB-231 cells were seeded into a 96-well plate and treated with increasing concentrations of G-418 sulphate (ranging from 25 µg/mL to 2000 µg/mL). Media was replaced every 3 days, and cell viability was monitored after 7 days. The concentration at which > 90% of the cells died within 7 days was selected as the optimal G-418 sulphate concentration for selection.

For selection, transfected cells were cultured in complete medium containing 700 µg/mL G-418 sulphate (predetermined concentration). Selection was continued for 14 days, with media changes every 3 days, until non-resistant cells were eliminated and resistant colonies emerged. Surviving cells were expanded in the presence of G-418 to generate a polyclonal stable cell population.

Stable integration and expression of the transgene (DNMT1) were verified by qRT-PCR. Here, the polyclonal stable cell line containing the empty vector is termed MDA-MB-231/pcDNA3.1(+), whereas the polyclonal stable cell line containing the pcDNA3.1(+)-DNMT1 construct is termed MDA-MB-231/pcDNA3.1(+)-DNMT1. For long-term maintenance, cells were cultured in G-418 sulphate-containing medium at a maintenance concentration (200 µg/mL) and also cryopreserved in DMEM medium containing 30% FBS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

2.6. Cell cycle phase distribution assay

MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells were cultured in 35 mm cell culture dishes (0.2 x 10^6 cells/dish) till 70-80% confluency. Cells were then treated with vehicle (0.8% ethanol) and doses of LCLEE at 60 µg/mL, 80 µg/mL, 100 µg/mL, 120 µg/mL, 150 µg/mL, and 180 µg/mL for 24 hours, along with untreated control cells. Cells were then harvested by trypsinization along with the floating cells derived from the consumed media. Cell pellets were washed with ice-cold 1X PBS, and the cells were fixed with 70% ethanol and incubated at 4°C overnight. Thereafter, the cell cycle phase distribution assay was performed following the protocol discussed previously (Pal et al., 2024), and the samples were analysed using a BD LSR Fortessa flow cytometer (BD Biosciences, USA). The distribution of the cells in different phases (Sub-G1, G0/G1, S, and G2/M) was determined.

2.7. Annexin V-FITC/PI double staining

The detection of apoptosis post treatment with the extract was done by the Annexin V-FITC/PI double staining using the Annexin V-FITC Apoptosis detection kit (BD Biosciences, USA) following the protocol mentioned by manufacturer. Briefly, MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells were cultured in 35 mm cell culture dishes (0.2 x 10^6 cells/dish) till 70-80% confluency. Cells were then treated with vehicle (0.8% ethanol) and doses of LCLEE at 60 µg/mL, 120 µg/mL, 180 µg/mL, 240 µg/mL, and 300 µg/mL for 24 hours, along with untreated control cells. Cells were then harvested by trypsinization along with the floating cells obtained from the consumed media. The cell pellets were further washed once with ice-cold 1X PBS. Thereafter, the Annexin V-FITC/PI double staining was performed following the protocol discussed previously (Pal et al., 2024), and the samples were analysed using a BD LSR Fortessa flow cytometer (BD Biosciences, USA). Data was represented as scatter plots showing the distribution of cells after Annexin V-FITC/ PI double staining. The

cell populations which were negative for both Annexin V-FITC and PI were considered as live cells (lower left quadrant). Whereas, only Annexin V-FITC-positive (lower right quadrant), both Annexin V-FITC and PI-positive (upper right quadrant), and only PI-positive (upper left quadrant) cell populations were considered as early apoptotic, late apoptotic, and non-apoptotic, respectively.

2.8. Wound healing assay

Wound healing assay was performed following the protocol discussed previously (Pal et al., 2024). Briefly, MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells were cultured in 35 mm dishes (0.3 x 10^6 cells/dish) and grown till the formation of a uniform monolayer. A wound was made in each dish using a sterile 0.2-10 µL micro pipette tip once the monolayer was formed. The consumed media was discarded from the culture dish. Then, the monolayer of cells was washed once with 1X PBS. Cells were then treated with 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL, and 120 µg/mL doses of LCLEE, along with vehicle-treated and untreated control cells. 0.5 µg/mL cycloheximide was added in every treatment condition to inhibit cell proliferation. Images of the cells were captured at 0 hours and 24 hours post-treatment using an inverted light microscope (Olympus, Japan) at 10X magnification. The images were analysed, and the area of the wounds was calculated by ImageJ 1.53k (ImageJ software, NIH, USA) using the following formula:

$$\text{Wound closure \%} = \frac{\text{Area of wound at 0 hours - Area of wound at 24 hours}}{\text{Area of wound at 0 hours}} \text{ X } 100\ \%$$

9. Statistical analysis

All the experiments were conducted independently at least three times. Data were plotted using GraphPad Prism 8.0.1 (GraphPad software, CA, USA) and represented as mean ± SEM (Standard Error of Mean). Statistical analysis was done by performing a parametric Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. *Lantana camara* leaf ethanolic extract downregulated the mRNA and protein expression of DNMT1

In triple-negative breast cancer, DNMT1 plays a significant role in promoting tumor development and aggressive behavior. DNMT1 is overexpressed in TNBC, and its increased expression is associated with poorer survival outcomes (Z. Li et al., 2022; Wong, 2021). It contributes to tumorigenesis by silencing tumor suppressor genes through hypermethylation of their promoter regions (Wong, 2021; Yu et al., 2019). The mRNA expression of DNMT1 decreased dose-dependently when MDA-MB-231 cells were treated with different doses of LCLEE compared to vehicle control. DNMT1 mRNA expression (Figure 1A) was found to be significantly downregulated to 0.55 ± 0.02, 0.36 ± 0.01, 0.30 ± 0.03, and 0.26 ± 0.04-fold when MDA-MB-231 cells were treated with 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of LCLEE, respectively. When the protein expression of DNMT1 was checked under the same treatment conditions, it was found that the protein level of DNMT1 (Figure 1B and 1C) was decreased to 0.40 ± 0.10, 0.53 ± 0.21, 0.03 ± 0.02, and 0.01 ± 0.00-fold when the cells were treated with 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of LCLEE, respectively.

3.2. *Lantana camara* leaf ethanolic extract upregulated the mRNA expression of tumour suppressor genes that are hypermethylated due to overexpression of DNMT1 in MDA-MB-231 cells

Several studies have reported that overexpression of DNMT1 in MDA-MB-231 cells silences tumour suppressor genes that are involved in cell proliferation, apoptosis, and migration, such as p21 (Al-Kharashi et al., 2017; Mirza et al., 2013), PTEN (Liu et al., 2021; Stefanska et al., 2012), FOXO3a (Liu et al., 2019), and Claudin-1 (Chiang et al., 2019) through hypermethylation of their promoter regions. p21 is a protein that inhibits Cyclin D-CDK4/6 complex and induces G0/G1 cell cycle arrest (Xia et al., 2023). PTEN is an important tumour suppressor protein that blocks PI3K signaling by inhibiting PIP3-mediated membrane recruitment and activation of AKT, thereby impeding cell proliferation, growth, and survival (Miricescu et al., 2020). FOXO3a is a transcription factor belonging to the forkhead box O (FOXO) family and a tumour suppressor protein that suppresses cell cycle progression and promotes cell death (Liu et al., 2019). Claudin-1 is a tight junction protein that increases cell-cell adhesion and inhibits cell migration in TNBC cell lines (Geoffroy et al., 2020). As it was observed that LCLEE downregulated the mRNA and protein expression of DNMT1, the mRNA expression of p21, PTEN, FOXO3a, and Claudin-1 was examined after treating MDA-MB-231 cells with different doses of LCLEE, along with a vehicle control. Compared to vehicle control, the transcript level of p21 cell cycle inhibitor (Figure 2A) increased to 3.56 ± 0.31, 4.14 ± 0.21, 7.87 ± 2.08, and 9.09 ± 2.00-fold when treated with 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of LCLEE, respectively. PTEN mRNA expression (Figure 2B) was found to be significantly upregulated to 1.32 ± 0.09, 1.46 ± 0.10, 1.64 ± 0.26, and 1.65 ± 0.15-fold when MDA-MB-231 cells were treated with LCLEE at 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL, respectively. Similarly, FOXO3a mRNA expression (Figure 2C) was found to be significantly upregulated to 1.27 ± 0.10, 1.51 ± 0.21, and 2.23 ± 0.12-fold when MDA-MB-231 cells were treated with 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of LCLEE, respectively; however, no significant change in the mRNA expression was observed in the case of 40 µg/mL dose of LCLEE. The transcript level of Claudin-1 (Figure 2D) was found to be significantly upregulated to 2.93 ± 0.68, 5.06 ± 1.10, and 6.42 ± 1.79-fold when treated with 40 µg/mL, 60 µg/mL, and 80 µg/mL doses of LCLEE, respectively, compared to vehicle control.

3.3. Ectopic overexpression of DNMT1 attenuated *Lantana camara* leaf extract-mediated G0/G1 cell cycle arrest

Previously, it was reported that LCLEE induced G0/G1 cell cycle arrest in MDA-MB-231 cells (Pal et al., 2024). To further delineate the mechanism of action of the extract, and to be precise, to check whether the downregulation of DNMT1 has any role behind the anti-proliferative effect of the extract, cell-cycle phase distribution was analysed in MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells to compare the effect of the extract and to evaluate whether the overexpression of DNMT1 can attenuate the effect of the extract. Figure 3A and 3C are representative images of the distribution of MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells in distinct cell cycle phases when treated with a range of doses of the extract from 60 µg/mL to 180 µg/mL for 24 hours, along with vehicle-treated and untreated controls. Analysis of the results (Figure 3B) revealed that G0/G1 population of MDA-MB-231/pcDNA3.1(+) cells increased from 60.967 ± 0.878% (vehicle control) to 65.917 ± 1.049%, 68.833 ± 1.160%, 72.467 ± 0.495%, 74.517 ±

316 0.588%, 72.817 ± 1.131%, and 68.700 ± 1.479% when treated with 60 µg/mL, 80 µg/mL, 100
317 µg/mL, 120 µg/mL, 150 µg/mL, and 180 µg/mL doses of LCLEE, respectively. This
318 observation suggested that treatment with the extract induced G0/G1 cell cycle arrest of MDA-
319 MB-231/pcDNA3.1(+) cells. The increase in the G0/G1 population was also accompanied by
320 significant decrease in S and G2/M phase populations in the extract-treated cells as compared
321 to the vehicle control. In the sub-G1 population, a slight increase in the percentage of the cell
322 population was also observed. However, the differences were statistically insignificant.
323 Whereas, Figure 3D revealed that the G0/G1 population of MDA-MB-231/pcDNA3.1(+)-
324 DNMT1 cells increased from 66.917 ± 1.097% (vehicle control) to 69.133 ± 0.304%, 71.083
325 ± 0.776%, 72.617 ± 0.516%, 73.400 ± 0.842%, 73.133 ± 0.638%, and 69.167 ± 0.429% when
326 treated with 60 µg/mL, 80 µg/mL, 100 µg/mL, 120 µg/mL, 150 µg/mL, and 180 µg/mL doses
327 of LCLEE, respectively. This finding indicated that treatment with the extract induced G0/G1
328 arrest in MDA-MB-231/pcDNA3.1(+)-DNMT1 cells, too. However, the increase in G0/G1 cell
329 population at the 60 µg/mL dose of the extract is non-significant in the case of MDA-MB-
330 231/pcDNA3.1(+)-DNMT1 cells compared to the significant increase in MDA-MB-
331 231/pcDNA3.1(+) cells. It is also noteworthy that the extent to which the G0/G1 cell population
332 increased is less in MDA-MB-231/pcDNA3.1(+)-DNMT1 cells compared to MDA-MB-
333 231/pcDNA3.1(+) cells. The observed increase in the G0/G1 population in MDA-MB-
334 231/pcDNA3.1(+)-DNMT1 was also associated with a significant decrease in the S-phase
335 population. However, no significant change was observed in the G2/M cell population in
336 MDA-MB-231/pcDNA3.1(+)-DNMT1 cells compared to the MDA-MB-231/pcDNA3.1(+)
337 cells, where G2/M cell population decreased significantly from 60 µg/mL to 150 µg/mL doses
338 of LCLEE. A comparative table of cell cycle phase distribution of MDA-MB-
339 231/pcDNA3.1(+) cells and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells at different treatment
340 conditions has been provided in Supplementary Table S4. Taken together, the results
341 established that the ectopic overexpression of DNMT1 can attenuate the induction of G0/G1
342 cell cycle arrest by LCLEE.

343 3.4. Ectopic overexpression of DNMT1 impeded *Lantana camara* leaf extract-mediated 344 apoptotic cell death

345 Previously, it was reported that LCLEE induced apoptotic cell death in MDA-MB-231 cells
346 (Pal et al., 2024). To further delineate the mechanism of action of the extract, and to be specific,
347 to check whether the downregulation of DNMT1 is responsible behind the induction of
348 apoptosis by the extract, Annexin V-FITC/PI double staining was performed in MDA-MB-
349 231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells to compare the effect of
350 the extract and to evaluate whether the overexpression of DNMT1 can attenuate the effect of
351 the extract. Figure 4A and 4C are representative images of the scatter plots showing percentage
352 viable or live (Q3), early apoptotic (Q4), late apoptotic (Q2), and non-apoptotic (Q1)
353 populations of MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells
354 when treated for 24 hours with 60 µg/mL, 120 µg/mL, 180 µg/mL, 240 µg/mL, and 300 µg/mL
355 doses of LCLEE, along with vehicle-treated and untreated controls. Analysis of the results
356 (Figure 4B) revealed that late apoptotic cell population of MDA-MB-231/pcDNA3.1(+) cells
357 increased from 6.275 ± 0.557% (vehicle control) to 10.70 ± 1.901%, 12.975 ± 2.478%, 17.85
358 ± 4.777%, 18.175 ± 4.826%, and 28.275 ± 7.393%; non-apoptotic cell population increased
359 from 1.90 ± 0.319% (vehicle control) to 4.075 ± 0.68%, 4.475 ± 0.829%, 7.275 ± 1.736%,
360 18.025 ± 5.693%, and 37.400 ± 12.906% when treated with 60 µg/mL, 120 µg/mL, 180 µg/mL,

240 μg/mL, and 300 μg/mL doses of LCLEE, respectively. This finding implied that treatment with LCLEE increased late and non-apoptotic populations of MDA-MB-231/pcDNA3.1(+) cells, and the observed increase was accompanied by a significant concomitant decrease in live or viable cell population, but no significant change was observed in the early apoptotic cell population compared to the vehicle control. Whereas, analysis of the results (Figure 4D) revealed that the late apoptotic cell population of MDA-MB-231/pcDNA3.1(+)-DNMT1 cells increased significantly from 6.20 ± 0.484% (vehicle control) to 10.083 ± 0.712 and 21.867 ± 4.708% when treated with 240 μg/mL and 300 μg/mL doses of the extract, respectively. Non-apoptotic cell population increased significantly from 3.983 ± 0.631% (vehicle control) to 44.117 ± 8.818% only when treated with 300 μg/mL dose of the extract. Viable or live cell population decreased significantly from 86.483 ± 1.311% (vehicle control) to 31.500 ± 4.906% only when treated with 300 μg/mL dose of the extract. However, no significant change was observed in the early apoptotic cell population compared to the vehicle control. Taken together, it was observed that the late apoptotic population increased significantly in all the doses from 120 μg/mL to 300 μg/mL of the extract, the non-apoptotic population increased significantly in all the doses from 60 μg/mL to 300 μg/mL of the extract, and the live cell population decreased significantly in all the doses from 120 μg/mL to 300 μg/mL of the extract in the case of MDA-MB-231/pcDNA3.1(+) cells. On the other hand, the late apoptotic population increased significantly only in the 240 μg/mL and 300 μg/mL doses of LCLEE, the non-apoptotic population increased significantly only in the 300 μg/mL dose of LCLEE, and the live cell population decreased significantly only in the 300 μg/mL dose of the extract in the case of MDA-MB-231/pcDNA3.1(+)-DNMT1 cells. A comparative table of live and dead cell populations of MDA-MB-231/pcDNA3.1(+) cells and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells at different treatment conditions has been provided in Supplementary Table S5. The above findings culminated in the fact that the ectopic overexpression of DNMT1 can attenuate the induction of apoptosis by LCLEE.

3.5. Ectopic overexpression of DNMT1 promoted the migratory potential

It was reported previously that LCLEE impeded the migration of MDA-MB-231 cells (Pal et al., 2024). To further delineate the mechanism of action of the extract, and to be precise, to check whether the downregulation of DNMT1 is responsible behind the inhibition of migration by the extract, wound healing assay was performed in MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells to compare the effect of the extract and to evaluate whether the overexpression of DNMT1 can attenuate the effect of the extract. To delineate the effect of the extract on the migratory potential, MDA-MB-231/pcDNA3.1(+) and MDA-MB-231/pcDNA3.1(+)-DNMT1 cells were treated with lower doses of LCLEE along with vehicle-treated and untreated controls, and a wound healing assay was performed. Compared to the untreated control and vehicle control, a smaller wound area was healed by the LCLEE-treated cells in the case of MDA-MB-231/pcDNA3.1(+) cells (Figure 5A). Whereas, in comparison to the untreated control and vehicle control, a non-significant wound area was healed by the LCLEE-treated cells in the case of MDA-MB-231/pcDNA3.1(+)-DNMT1 cells (Figure 5B). The graphical representation of the wound closure percentage (Figure 5C) indicated a significant dose-dependent reduction of migratory activity post 24 hours of treatment in the case of MDA-MB-231/pcDNA3.1(+) cells. On the other hand, the graphical representation of the wound closure percentage (Figure 5C) indicated a non-significant reduction in the migratory activity post 24 hours of treatment in the case of MDA-MB-

231/pcDNA3.1(+)-DNMT1 cells. The wound closure percentage in MDA-MB-231/pcDNA3.1(+) cells was found to be 61.76 ± 4.96% for untreated control and 61.06 ± 7.82% for vehicle control, whereas the wound closure percentage reduced dose-dependently to 55.10 ± 6.04%, 48.72 ± 6.82%, 43.09 ± 1.40%, 33.51 ± 0.91%, and 30.83 ± 1.47% at 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL, and 120 µg/mL, respectively. The wound closure percentage in MDA-MB-231/pcDNA3.1(+)-DNMT1 cells was found to be 91.44 ± 2.34% for untreated control and 89.17 ± 4.09% for vehicle control, whereas the wound closure percentage became 88.32 ± 4.01%, 85.60 ± 4.54%, 82.56 ± 3.95%, 73.88 ± 1.66%, and 77.61 ± 1.45% at 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL, and 120 µg/mL, respectively. Not only that, but when considering only the untreated cells, it was observed that the migratory potential of MDA-MB-231/pcDNA3.1(+)-DNMT1 cells is higher compared to that of MDA-MB-231/pcDNA3.1(+) cells. Taken together, the results indicated that the ectopic overexpression of DNMT1 promoted the migratory potential of the cells and attenuated the inhibition of migration by LCLEE.

3.6. Ectopic overexpression of DNMT1 downregulated the mRNA expression of p21, PTEN, FOXO3a, and Claudin-1 tumour suppressor genes

Previously, it was observed that LCLEE upregulated the mRNA expression of p21, Claudin-1, PTEN, and FOXO3a tumour suppressor genes (section 3.2) that are hypermethylated and silenced due to DNMT1 in MDA-MB-231 cells. To further check whether the ectopic overexpression of DNMT1 can change the mRNA expression of these essential genes involved in proliferation, survival, and migration, RT-qPCR was performed, and the mRNA expression of these genes was measured in MDA-MB-231/pcDNA3.1(+)-DNMT1 cells compared to MDA-MB-231/pcDNA3.1(+) cells. It was found that the transcript level of DNMT1 increased to 2.643 ± 0.400-fold in MDA-MB-231/pcDNA3.1(+)-DNMT1 cells compared to MDA-MB-231/pcDNA3.1(+) cells, whereas the mRNA expression of p21, PTEN, FOXO3a, and Claudin-1 was decreased to 0.369 ± 0.082, 0.387 ± 0.084, 0.375 ± 0.087, and 0.379 ± 0.075-fold respectively (Figure 6) in MDA-MB-231/pcDNA3.1(+)-DNMT1 cells compared to MDA-MB-231/pcDNA3.1(+) cells.

3.7. *Lantana camara* leaf ethanolic extract downregulated the mRNA expression of Methyl-CpG binding domain protein 2 (MBD2) and Histone Deacetylases 1 and 2 (HDAC1 and HDAC2) in MDA-MB-231 cells

The observed downregulation of DNMT1 by LCLEE suggests a potential mechanism of epigenetic reprogramming through reduced maintenance of promoter hypermethylation. As a result, genes silenced via DNMT1-mediated hypermethylation are upregulated, indicating functional reversal of transcriptional repression. However, DNA methylation does not operate in isolation; it is tightly integrated with methyl-CpG binding proteins and histone-modifying enzymes that collectively establish and stabilize repressive chromatin states. MBD2 is a critical methyl-CpG "reader" that recognizes methylated promoters and recruits co-repressor complexes. Importantly, MBD2 interacts with histone deacetylases, particularly HDAC1 and HDAC2, to enforce gene silencing. This DNMT1–MBD2–HDAC axis constitutes a multi-layered epigenetic machinery, whereby DNA methylation and histone deacetylation cooperate to compact chromatin and block transcription (Lekesiz et al., 2025). Thus, even if DNMT1 levels decline and partial promoter hypomethylation occurs, persistent expression or activity of MBD2 and HDACs may sustain transcriptional repression. Assessing the expression of MBD2, HDAC1, and HDAC2 is therefore essential to delineate the broader epigenetic impact

of the extract. MBD2 mRNA expression (7A) was found to be significantly downregulated to 0.56 ± 0.11, 0.76 ± 0.05, 0.25 ± 0.12, and 0.2 ± 0.02-fold when MDA-MB-231 cells were treated with 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of LCLEE, respectively. The transcript level of HDAC1 (Figure 7B) was significantly decreased to 0.49 ± 0.06, 0.47 ± 0.01, 0.37 ± 0.04, and 0.29 ± 0.05-fold when MDA-MB-231 cells were treated with 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of LCLEE, respectively. Similarly, HDAC2 mRNA expression (Figure 7C) was found to be significantly downregulated to 0.53 ± 0.13, 0.63 ± 0.07, 0.22 ± 0.07, and 0.28 ± 0.01-fold when MDA-MB-231 cells were treated with 40 µg/mL, 60 µg/mL, 120 µg/mL, and 180 µg/mL doses of the extract,F respectively.

4. Discussion

In the present study, we demonstrate that treatment with *Lantana camara* leaf ethanolic extract suppresses the expression of DNMT1 at both mRNA and protein levels in MDA-MB-231 cells. This suppression was accompanied by the re-expression of tumor suppressor genes commonly silenced through DNMT1-mediated promoter hypermethylation. Importantly, ectopic overexpression of DNMT1 impeded G0/G1 cell cycle arrest and apoptosis, and promoted migration of MDA-MB-231 cells, underlining the pivotal role of DNMT1 downregulation in mediating the anticancer activity of the extract. These findings not only support our previous report that the extract induces cytotoxicity, cell cycle blockade, apoptosis, and antimigratory effects in MDA-MB-231 cells but also provide novel mechanistic insights into the molecular pathways involved.

Aberrant DNA methylation, primarily mediated by DNMT1 overexpression, has been recognized as a key driver of tumor progression in breast cancer, particularly triple-negative breast cancer, which lacks hormone receptors (ER and PR) and HER2-directed therapeutic targets. Elevated DNMT1 expression has been associated with poor prognosis, tumor aggressiveness, and resistance to therapy (R., 2025; Wong, 2021). Our observations that suppression of DNMT1 restores the expression of hypermethylated tumor suppressor genes and induces G0/G1 cell cycle arrest and apoptosis, along with the inhibition of migration, consistent with reduced malignancy, are in line with previous reports implicating DNMT1 as a crucial regulator of TNBC (H. Li et al., 2022; Z. Li et al., 2022; Pan et al., 2022).

Treatment with *Lantana camara* leaf ethanolic extract upregulated the mRNA expression of the genes often silenced by DNMT1-driven hypermethylation. Restoration of these genes likely contributed to the observed G0/G1 arrest, apoptosis, and impaired migration. These results parallel the effects of synthetic DNMT inhibitors, such as 5-azacytidine and decitabine, which are clinically approved for hematological malignancies (R., 2025). However, unlike these nucleoside analogues, which are often associated with systemic toxicity and off-target effects, plant-derived agents may offer the advantages of multi-target modulation and potentially improved safety profiles (Aanniz et al., 2024; Mirza et al., 2013). This highlights the translational significance of identifying natural compounds capable of epigenetic reprogramming in TNBC.

A major highlight of the current work lies in the functional validation of DNMT1 suppression. Ectopic overexpression of DNMT1 attenuated the extract-mediated induction of apoptosis, G0/G1 arrest, and inhibition of migration, while also repressing the expression of tumor suppressor genes reactivated by the extract. This finding provides strong causal evidence that

DNMT1 downregulation is not merely an associated event but is mechanistically central to the anticancer activity of the extract.

In addition to DNMT1, the extract also downregulated the mRNA expression of MBD2, HDAC1, and HDAC2. These molecules are well-known epigenetic corepressors that cooperate to form transcriptional silencing complexes, maintaining stable repression of tumor suppressor genes. Previous studies have demonstrated that disruption of the DNMT1–MBD2–HDAC complex results in the derepression of silenced genes and suppression of tumorigenesis (Lekesiz et al., 2025). Therefore, the simultaneous downregulation of DNMT1, MBD2, HDAC1, and HDAC2 by the extract may exert a synergistic effect, leading to the reactivation of tumor suppressor genes and thereby enhancing the anticancer efficacy of the extract.

Collectively, our findings suggest that *Lantana camara* leaf ethanolic extract exerts its anticancer effects in TNBC cells through epigenetic reprogramming, specifically by suppressing DNMT1 and associated repressors. By reactivating tumor suppressor genes and impairing proliferation, survival, and migration, the extract addresses three critical hallmarks of cancer. Given the lack of effective targeted therapies for TNBC, these results highlight the potential of plant-derived compounds as novel epigenetic modulators. The dual ability of the extract to inhibit both tumor growth and metastatic potential strengthens its relevance as a candidate for further preclinical development.

Despite these promising results, the present study also has several limitations. Our findings are based on *in vitro* analyses, and further *in vivo* studies are required to establish the therapeutic efficacy and safety profile of the extract. Additionally, the specific phytochemical constituents responsible for DNMT1 suppression remain to be identified. Future research should focus on bioactivity-guided fractionation to isolate active compounds, genome-wide methylation profiling to elucidate global epigenetic changes, and evaluation of the extract in animal models of TNBC. Furthermore, combinatorial approaches integrating the extract with conventional chemotherapeutic agents may enhance its therapeutic potential and provide a rational strategy for managing TNBC.

5. Conclusion

In summary, we report that *Lantana camara* leaf ethanolic extract downregulates DNMT1 and associated epigenetic repressors, leading to re-expression of silenced genes, promotion of G0/G1 cell cycle arrest and apoptosis, and suppression of migration in MDA-MB-231 triple-negative breast cancer cells. The mechanistic link between DNMT1 suppression and these observed changes was confirmed by ectopic overexpression studies. These findings not only provide mechanistic insights into the anticancer activity of the extract but also highlight its promise as a natural epigenetic therapeutic candidate for TNBC.

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Authors' contributions

Sourav Sanyal: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Arundhaty Pal:** Methodology, Investigation, Formal analysis, Writing – original draft. **Tapas Kumar Sengupta:** Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition.

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Data Availability

All data generated or analysed during this study are included in this present article and supplementary section.

Declaration

Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval

Not applicable.

Consent to participate

All authors have given consent to participate in the study.

Consent for publication

All authors have given consent for publication.

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652

653 **Figure 1.**

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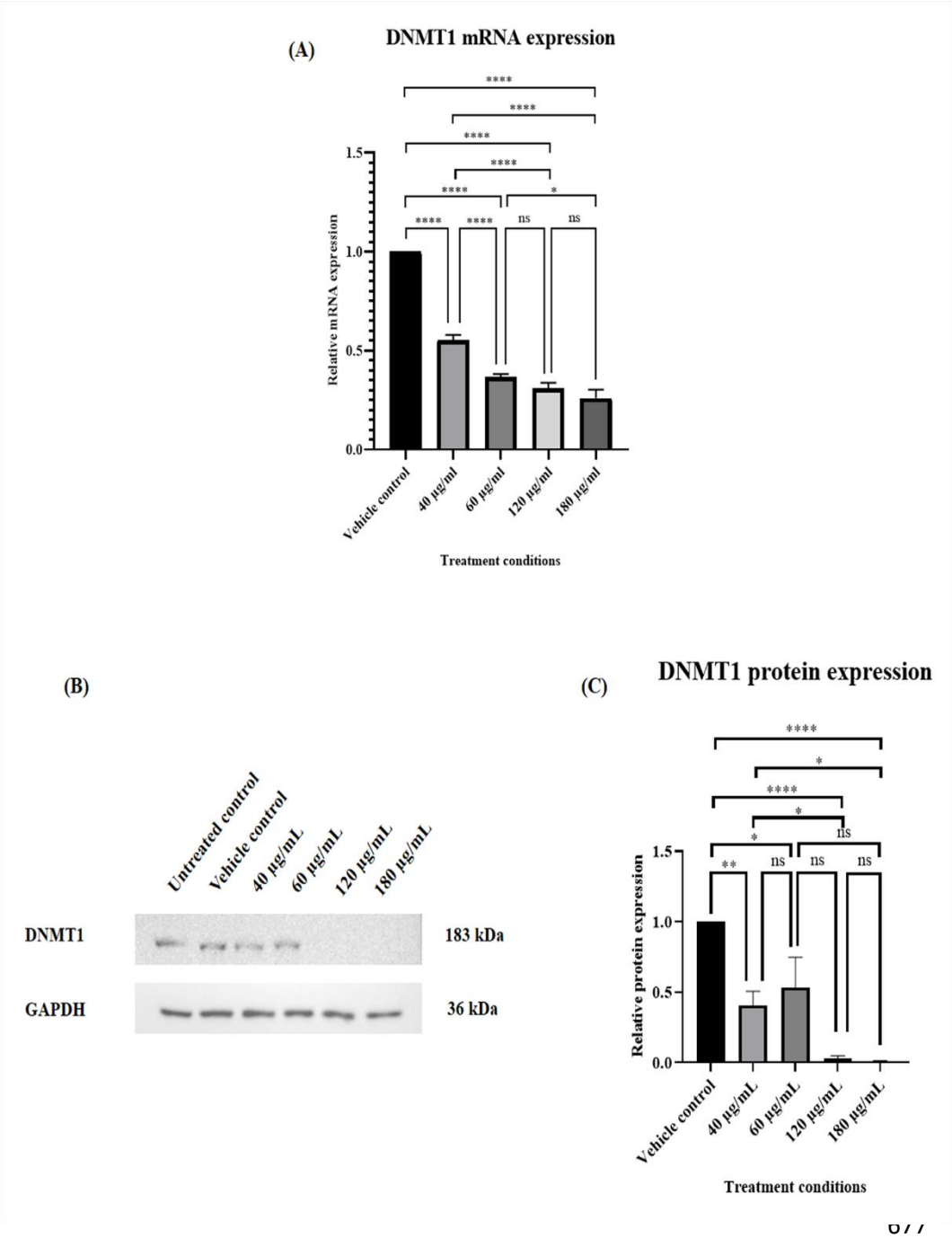


677

678 **Figure 1.** mRNA and protein expression of DNA methyltransferase 1 were altered in MDA-
679 MB-231 cells compared to vehicle control when treated with various doses of *Lantana camara*
680 leaf ethanolic extract for 24 hours. **(A)** relative mRNA expression of DNMT1, **(B)**
681 Representative western blot images, and **(C)** normalized graph of DNMT1 protein expression.
682 Data is represented as mean ± SEM of three independent biological replicates, where * is
683 $P \leq 0.05$, ** is $P \leq 0.01$, **** is $P \leq 0.0001$, and ns signifies non-significant.

684

Figure 2.

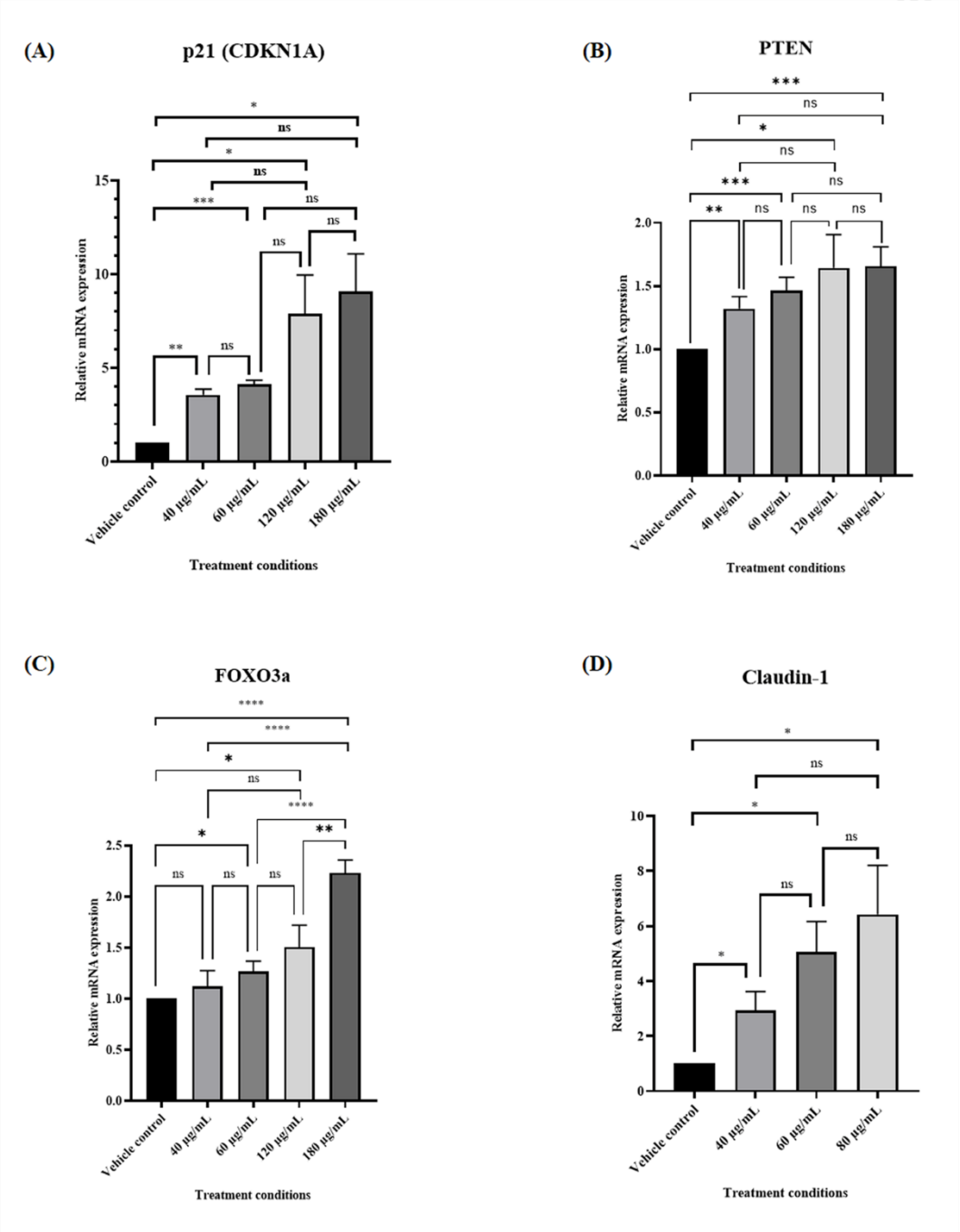


Figure 2. mRNA expression levels of tumour suppressor genes were upregulated in MDA-MB-231 cells compared to vehicle control when treated with various doses of *Lantana camara* leaf ethanolic extract for 24 hours. Relative expression of **(A)** p21, **(B)** PTEN, **(C)** FOXO3a, and **(D)** Claudin-1. Data is represented as mean ± SEM of three independent biological replicates, where * is P≤0.05, ** is P≤0.01, *** is P≤0.001, **** is P≤0.0001, and ns signifies non-significant.

Figure 3.

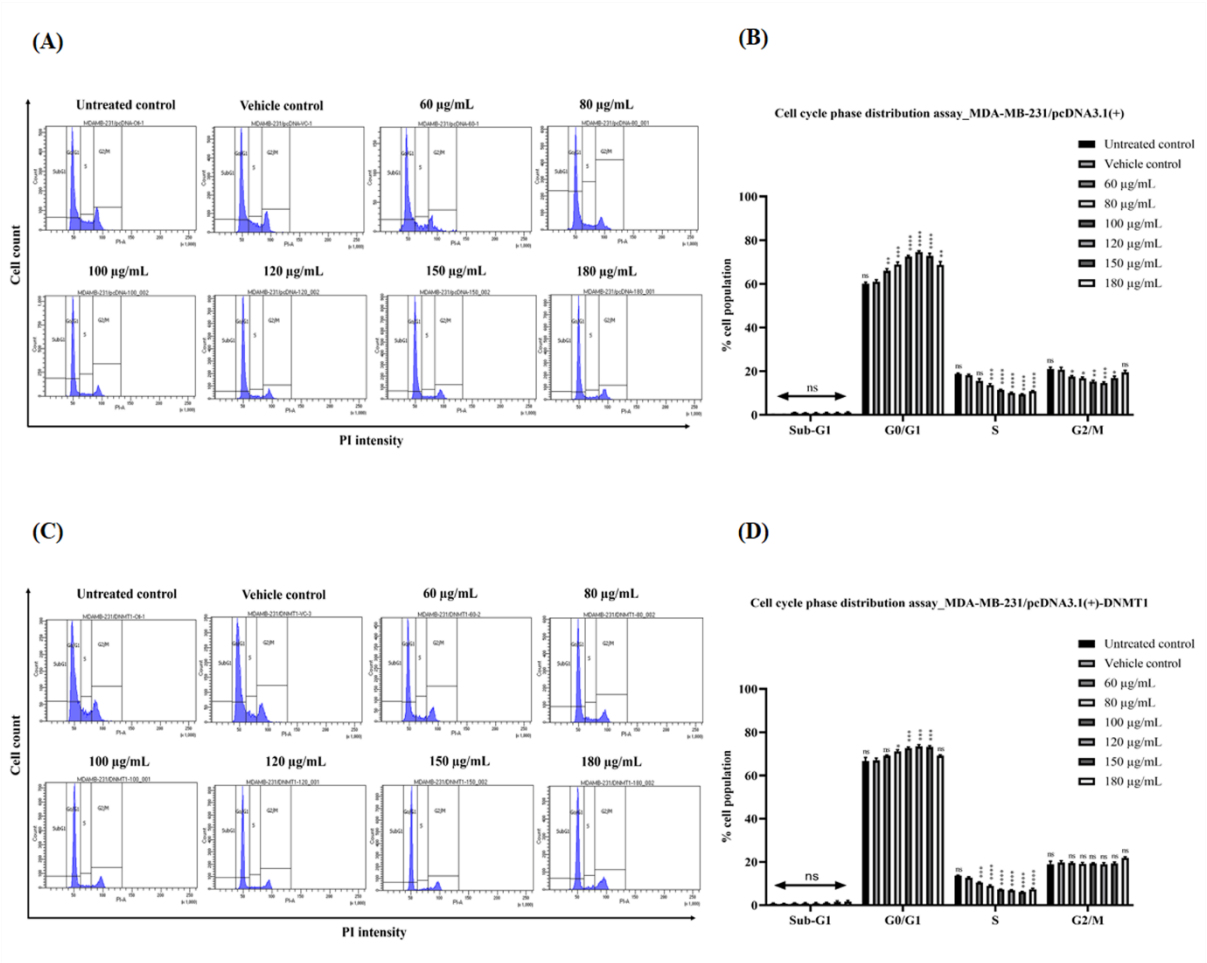


Figure 3. Representative images of cell cycle phase distribution of (A) MDA-MB-231/pcDNA3.1(+) cells and (C) MDA-MB-231/pcDNA3.1(+)-DNMT1 cells following 24 hours of treatment with the leaf extract at different doses. Bar diagram showing the percentage of cells in different cell cycle phases (G0/G1, S, G2/M, and sub-G1) of (B) MDA-MB-231/pcDNA3.1(+) cells and (D) MDA-MB-231/pcDNA3.1(+)-DNMT1 cells following 24 hours of treatment with the extract at different doses. Data are means of three independent experiments and presented as means ± SEM, where * is $P \leq 0.05$, ** is $P \leq 0.01$, *** is $P \leq 0.001$, **** is $P \leq 0.0001$, and ns signifies non-significant.

Figure 4.

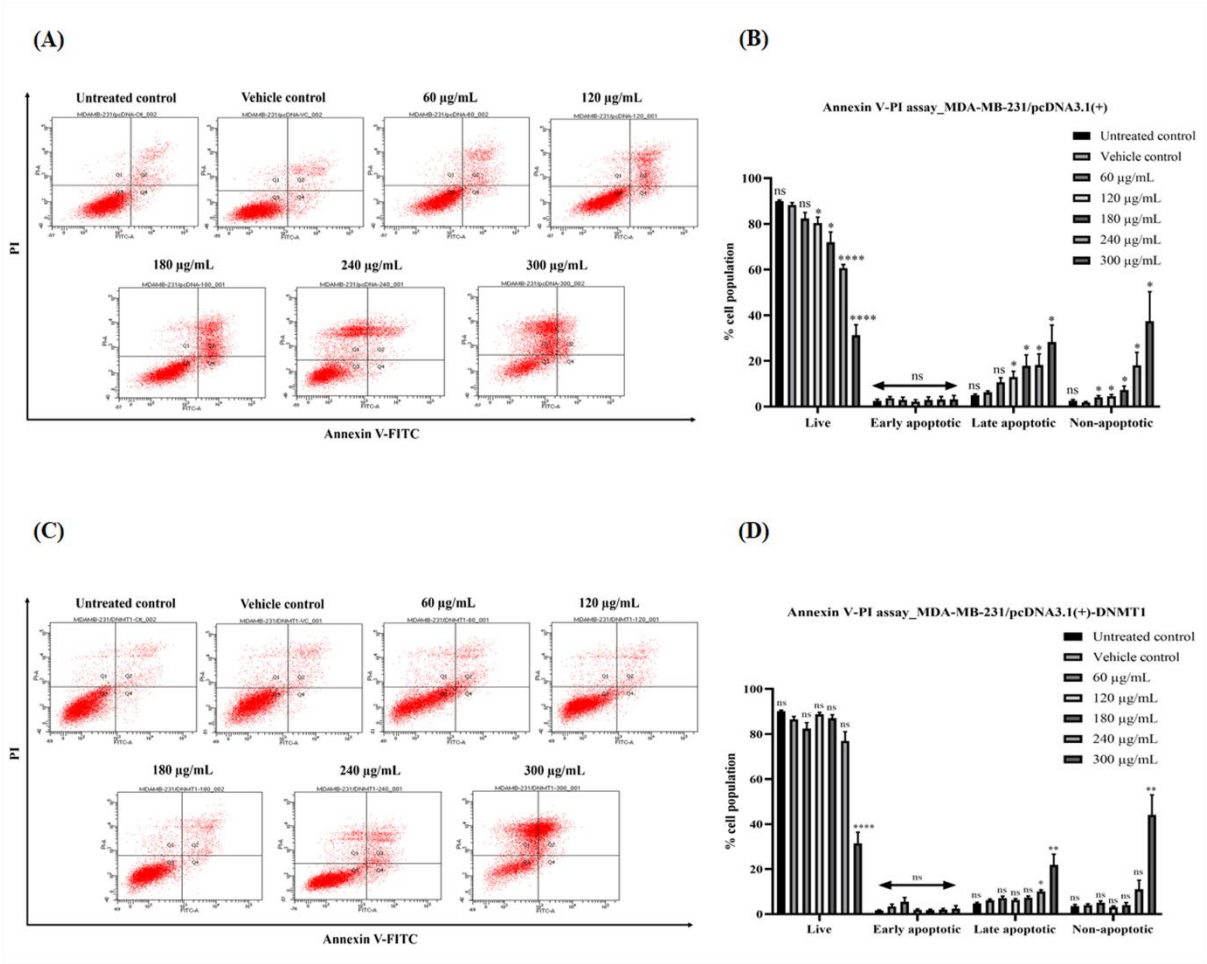
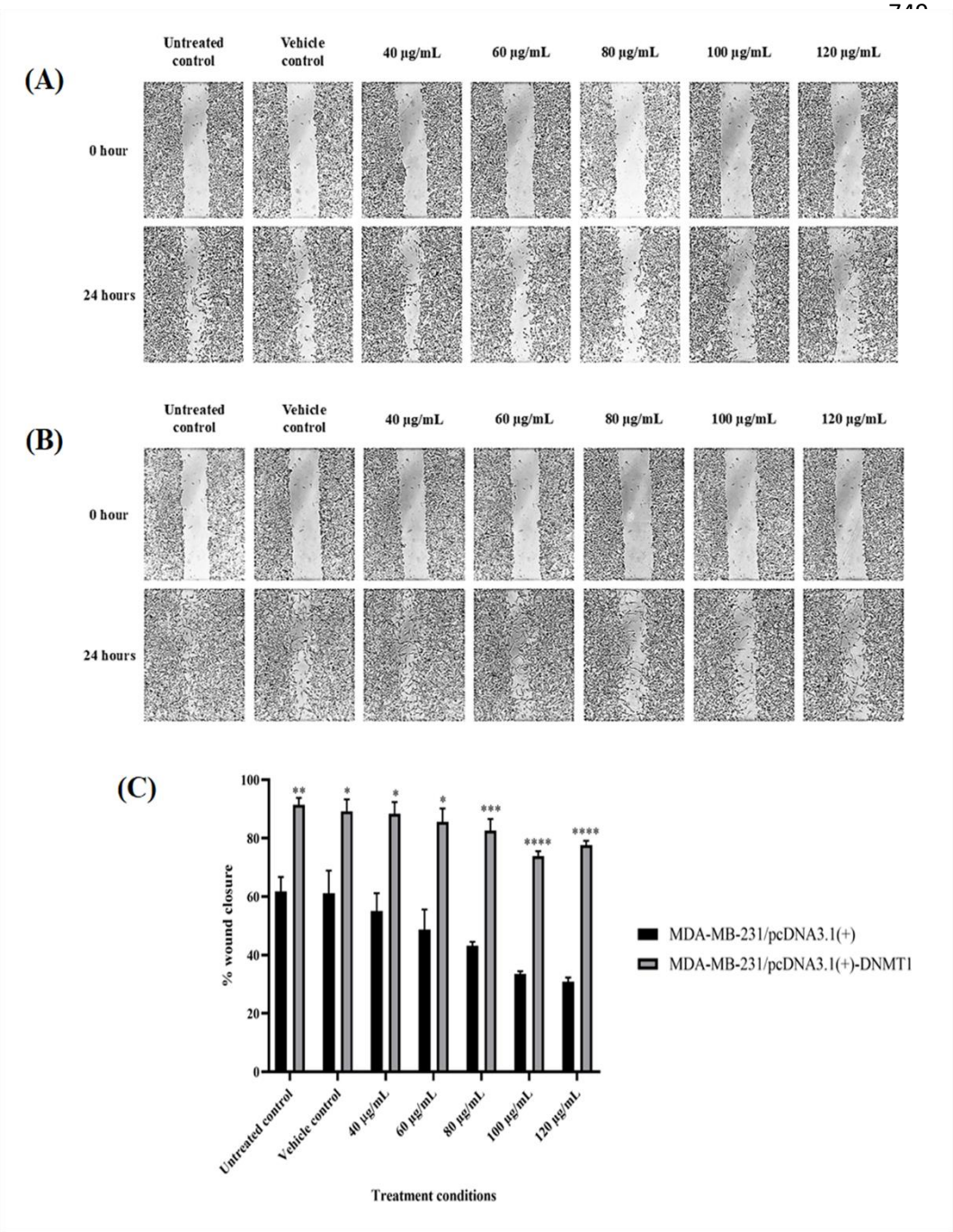


Figure 4. Representative scatter plots showing percentage viable or live (Q3), early apoptotic (Q4), late apoptotic (Q2), and non-apoptotic (Q1) (A) MDA-MB-231/pcDNA3.1(+) cells and (C) MDA-MB-231/pcDNA3.1(+)-DNMT1 cells following 24 hours of treatment with the leaf extract at different doses. Bar diagram showing the percentage of cells in different quadrants (live, early apoptotic, late apoptotic, and non-apoptotic) of (B) MDA-MB-231/pcDNA3.1(+) cells and (D) MDA-MB-231/pcDNA3.1(+)-DNMT1 cells following 24 hours of treatment with the extract at different doses. Data are means of three independent experiments and presented as means ± SEM, where * is $P \leq 0.05$, ** is $P \leq 0.01$, **** is $P \leq 0.0001$, and ns signifies non-significant.

748 **Figure 5.**



772 **Figure 5.** Representative images of wound healing assay of untreated, vehicle-treated, and
773 LCLE-treated (A) MDA-MB-231/pcDNA3.1(+) cells and (B) MDA-MB-231/pcDNA3.1(+)-
774 DNMT1 cells. Cells were subjected to a scratch in a monolayer condition and then incubated
775 for another 24 hours. Wound closure was visualized with a phase contrast microscope, and
776 images were captured at 24-hour time intervals. (C) Bar diagram representing the wound
777 closure potential of the cells at 24-hour time intervals after being treated with various doses of
778 the extract. Data is represented as means ± SEM of three independent biological replicates,
779 where * is $P \leq 0.05$, ** is $P \leq 0.01$, *** is $P \leq 0.001$, and **** is $P \leq 0.0001$.

780

Figure 6.

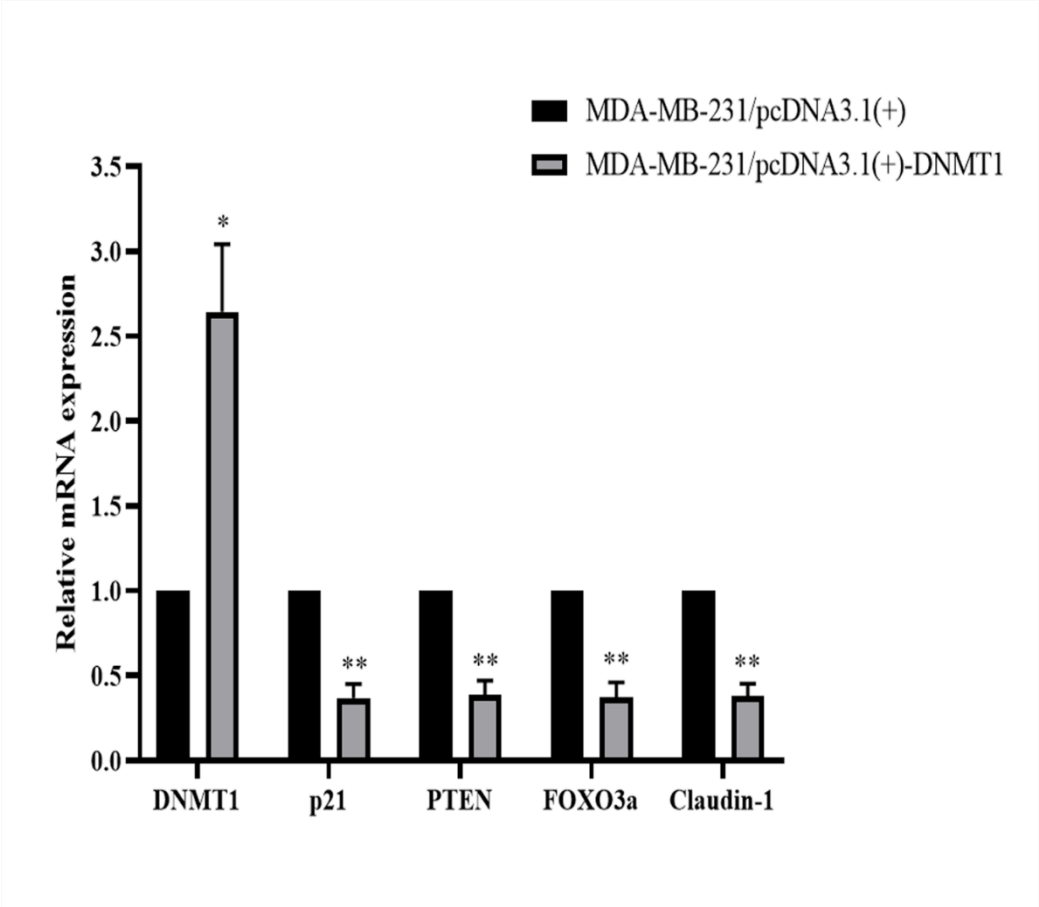
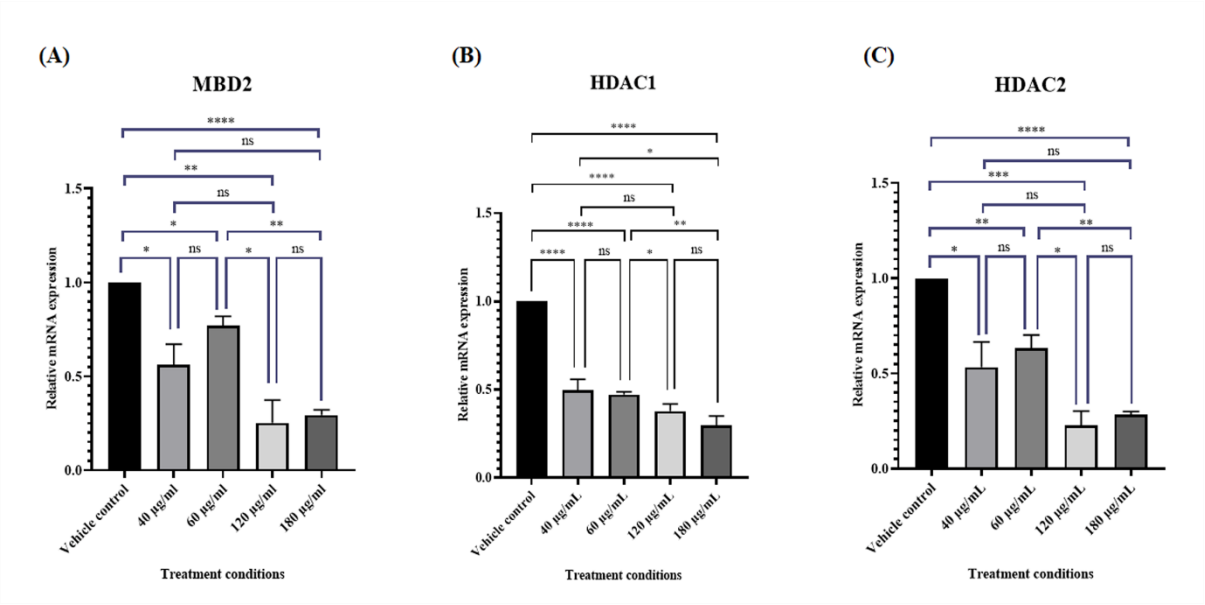


Figure 6. Relative mRNA expression of DNMT1, p21, PTEN, FOXO3a, and Claudin-1 in DNMT1 over-expressed MDA-MB-231/pcDNA3.1(+)-DNMT1 cells compared to empty vector containing MDA-MB-231/pcDNA3.1(+) cells. mRNA expression was measured by qRT-PCR using specific primer pairs. The relative expressions were calculated by the $2^{-\Delta\Delta Ct}$ method and normalized with 18S mRNA. Data are means of three independent experiments and presented as mean ± SEM, where * is $P \leq 0.05$ and ** is $P \leq 0.01$.

812 **Figure 7.**

813



814

815 **Figure 7.** mRNA expression levels of some key epigenetic regulators were altered in MDA-
816 MB-231 cells compared to vehicle control when treated with various doses of *Lantana camara*
817 leaf ethanolic extract for 24 hours. Relative expression of **(A)** MBD2, **(B)** HDAC1, and **(C)**
818 HDAC2. Data is represented as mean ± SEM of three independent biological replicates, where
819 * is $P \leq 0.05$, ** is $P \leq 0.01$, *** is $P \leq 0.001$, **** is $P \leq 0.0001$, and ns signifies non-significant.