RESEARCH ARTICLE

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Evaluating the Cytotoxic Potential of 3-(2-(3,4 dimethoxyphenyl)-2-oxoethylidene) indolin-2-one) (RAJI) on Triple Negative Breast Cancer Cells

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Abstract

Background: Triple-negative breast cancer (TNBC) is an aggressive and treatment-resistant subtype of breast cancer (BC) that is a leading global malignancy. A novel drug candidate, 3-(2-(3,4-dimethoxyphenyl)-2-oxoethylidene) indolin-2-one (RAJI), was synthesized using piperidine, isatin, and 3,4-dimethoxy acetophenones. Although these components have established roles in various drug syntheses and malaria treatment, their anti-cancer potential remains underexplored. Hence, the RAJI was designed to bridge this gap. **Methods:** The cytotoxic effects of RAJI on TNBC cell lines (MDA-MB-231 and MDA-MB-468) were evaluated using MTT assay, cell migration assay, apoptosis analysis (Annexin V), mitochondrial membrane potential tests, qRT-PCR, and tumor-induced mouse model evaluation. **Results:** RAJI exhibited cytotoxicity against TNBC cells, with IC_{50} values of 20 and 25 µg/mL for MDA-MB-231 and MDA-MB-468 cells, respectively. It reduced cell migration and induced apoptosis, as evident from the cell populations in the early and late apoptotic stages. Mitochondrial membrane potential assays revealed mitochondrial depolarization and cellular stress. Gene expression analysis via RT-PCR revealed that RAJI significantly downregulated Akt, PTEN, mTOR (*AKT/PI3K* signaling), Cyclin D1, indicating the induction of apoptosis in MDA-MB-231 cells via modulation of apoptotic genes such as Bax and Bcl-2. In the in In-vivo analysis, RAJI significantly reduced tumor volume in BALB/c athymic nude mice implanted with MDA-MB-231 cells over four weeks, with no notable toxicity. **Conclusion:** RAJI demonstrated potent anticancer activity, induced apoptosis, and reduced TNBC tumor progression by altering the Akt/PI3K pathway, making it a promising therapeutic candidate for breast cancer treatment.

Keywords: Breast cancer- Triple Negative Breast Cancer- Cytotoxicity- Cell migration- Apoptosis

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Introduction

According to the World Health Organization (WHO), breast cancer is the most prevalent form of cancer in 2020, with over 2.3 million recorded cases. In 2020, the main causes of cancer-related deaths were reported as - lung cancer accounting for 1.80 million fatalities, colon and rectum cancer resulting in 916 thousand deaths, liver cancer leading to 830 thousand deaths, and breast cancer causing 685 thousand deaths. Several countries have a high prevalence of breast cancer [1]. Recent updates in 2022 from the World Health Organization (WHO) identified 2.3 million cases of breast cancer in women worldwide, resulting in 6,70,000 deaths. In nations characterized by a high Human Development Index (HDI), the incidence of breast cancer is 1 in 12 women, with a corresponding mortality rate of 1 in 71 women. Based on an empirical study, it is projected that the worldwide prevalence of breast cancer will surpass 2 million cases by the year 2030. According to the WHO data, breast cancer has a prevalence of 99% in women and 0.5-1% among the male population. Between 1965 and 1985, the number of breast cancer cases in India increased by 50%, and in 2016, India reported more than 1.18,000 occurrences of breast cancer, with females accounting for 98.1% of the overall population [2].

The presence or absence of progesterone receptor (PR), estrogen receptor (ER), and Human Epidermal growth factor receptor 2 (*HER2*) determines various subtypes of breast cancer. TNBC is a type of breast cancer in which *PR, ER*, and *HER2* expression is absent. TNBC is aggressive in nature and frequently results in poor diagnosis, which in turn leads to a lack of targeted therapies [3]. Several experimental data have revealed that

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TNBC has a poor prognosis owing to its tendency towards early metastasis [4]. TNBC accounts for approximately 10% of all breast cancers [5], and the survival rate of individuals with TNBC is lower than that of individuals with other subtypes of BC. TNBC is more prevalent among women under the age of 40 years, who pose a two-fold increased risk of TNBC when compared with women over other age groups. According to a recent report, TNBC is more prevalent in India and has a higher incidence rate than in the Western population [6].

TNBC exhibits heterogeneity, and despite advancements in cancer treatment, it remains an unresolved medical challenge with few therapeutic options beyond the usual approach [7]. Basic treatment options for breast cancer include surgery, radiation, and chemotherapy. Lumpectomy is a surgical procedure that involves removal of the tumor and some adjacent normal tissues, while mastectomy is the complete removal of the afflicted breasts [8]. Following a lumpectomy, patients may undergo radiation treatment, which entails exposing the affected areas of the breasts and tumors to high-energy irradiation to eliminate any remnant cancer cells [9]. However, radiation therapy is effective in patients with non-metastatic tumors and is administered either as a standalone treatment or in conjunction with surgical intervention and chemotherapy. Chemotherapy involves the use of specific formulations [10,11]. However, these cytotoxic drugs have adverse effects that negatively affect the patients. Some negative effects of chemotherapy include nausea, pain, stress, sleep disturbances, anxiety, and gastrointestinal disorders. Therefore, it is crucial to identify specific hallmarks and drug candidates that may be utilized to increase the effectiveness of chemotherapy in the treatment of TNBC.

Deregulation of the phosphoinositide 3-kinase (*PI3K)/AKT* signalling pathway is a characteristic feature of several cancers, including TNBC. This pathway is essential for cellular activities such as proliferation, survival, and metabolism. Several recent studies have provided insight into the role of the *PI3K/Akt* pathway in facilitating the growth of TNBC. In MDA-MB-231 cells, dysregulation of *PTEN*, a tumor suppressor that antagonizes *PI3K*, leads to persistent activation of *Akt*, which promotes the growth of tumors via downstream effectors such as *mTOR*, *Cyclin D1*, and *VEGF* [12]. Additionally, this pathway interacts with other oncogenic regulators, such as p53 and c-Myc, which further enhance the advancement of the cell cycle and inhibit apoptosis [13]. Thus, targeting *PI3K/Akt* signalling has emerged as a viable therapeutic approach for the treatment of TNBC. This is especially true in situations in which conventional hormonal or *HER2*-directed treatments are unsuccessful. Hence, targeting this signalling pathway provides a viable option for the development of successful treatment options, given the prominent role of the *PI3K/Akt* pathway in the pathogenesis of TNBC and its impact on therapeutic resistance.

This study involved the formulation of a novel drug called RAJI, which encompasses three main components: piperidine, isatin, and 3,4-dimethoxy acetophenones in a one-pot protocol. It has shown significant potential in the field of cancer research owing to its wide range of pharmacological characteristics and potential for precise intervention. Piperidine is a heterocyclic molecule with a variety of properties. It possesses a promising framework for the development of drugs, enabling precise regulation of biological targets associated with the advancement of breast cancer. Isatin has distinctive pharmacological and biological attributes. It is utilized as a precursor for drug synthesis because of its critical class of heterocyclic compounds [14]. In contrast, 3,4-dimethoxy acetophenones possess two methoxy groups connected to their three and four locations. Multiple studies have demonstrated their antimalarial properties. However, its potential for cancer research has not been extensively investigated [15]. This limited understanding in this domain prompted us to investigate its potential as an anti-cancer agent against malignant cells.

Materials and Methods

Maintenance of Cell Lines

TNBC cell lines - MDA-MB-231 and MDA-MB-468 purchased from NCCS, Pune, India, and normal a non-malignant breast epithelial cell line (MCF-10A) procured from ATCC, USA were maintained in DMEM (high glucose media) supplemented with 10 % FBS and 1% antibiotic-antimycotic solution in an 5% CO2 atmosphere at 37^{o}C temperature.

Assessment of Cell Viability Using MTT Assay

To evaluate the cytotoxic effect of RAJI on TNBC cell lines, MDA-MB-231, MDA-MB-468, and MCF-10A cells, 96-well plates were inoculated with 1×10^{4} cells, followed by a 24-hour incubation period. The cells were then exposed to various concentrations of RAJI for 24 and 48 h. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. Cell viability was determined using the MTT reagent (Sigma-Aldrich) in accordance with the specifications provided by the manufacturer, and the IC_{50} values in each cell line were determined [16].

Live/Dead Staining using Acridine Orange and Ethidium Bromide

TNBC cell lines – both MDA-MB-231 and MDA-MB-468 were cultured under preset conditions followed by treatment with RAJI in three different concentrations - (10, 20, 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells) in the respective cell lines. After 24 h of treatment, Acridine Orange (AO) and Ethidium Bromide (EtBr) were added to the cells and incubated for 1 h. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. Images were obtained using a fluorescence microscope.

Cell Migration Assay

TNBC cell lines, both MDA-MB-231 and MDA-MB-468, were cultured under preset conditions in six well plates and after the cells attained 100% confluency, wounds were created by scraping the cell monolayer with

a sterile tip of a micropipette. Phosphate buffered saline (PBS) was used to wash the wells to remove floating cells that were scraped, followed by treatment with RAJI at three different concentrations in the respective cell lines (10, 20, and 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells). Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. A light microscope was used to view and photograph the scratch at the same spot both before and after scrap, at 0, 24, and 48 h. The gap distance between each wound was measured for each cell line.

Cell Cycle Analysis by Flow Cytometry

TNBC cell lines – both MDA-MB-231 and MDA-MB-468 were cultured under preset conditions in six well plates for 24 hrs post which they were treated with RAJI in three different concentrations in the respective cell lines - (10, 20, 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells). Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. After RAJI treatment, the cells were washed with PBS and incubated with trypsin-EDTA incubation for 3-4 minutes at 37°C, after which additional medium was added and centrifuged at 300 × g at 25°C. The pellets were washed with PBS, fixed in 70% ethanol and incubated at -20˚C for 30 mins followed by another round of centrifugation and PBS wash post which the pellet was treated with Propidium Iodide (PI)/RNase staining buffer, incubation in the dark for 15 to 20 minutes at room temperature and subjected through flow cytometry in PI/RNase solution for analysing the specific phase in the cell cycle where the cell division is arrested in each cell lines [16].

Apoptosis Assessment using Annexin-V-FITC Staining

TNBC cell lines – both MDA-MB-231 and MDA-MB-468 were cultured under preset conditions in six well plates for 24 hrs post which they were treated with RAJI in three different concentrations in the respective cell lines - (10, 20, 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells). Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. After RAJI treatment, the cells were washed with PBS and incubated with trypsin-EDTA incubation for 3-4 minutes at 37°C, after which additional medium was added and centrifuged at 300 × g at 25°C. The pellets were washed with PBS, treated with Annexin-V, and vortexed for 15 min at RT (25°C) in the dark, followed by the addition of propidium iodide (PI) and Annexin V binding buffer. The cell mixture was then subjected to flow cytometry to analyze the percentage of cells in the Early' and 'Late apoptotic phases in each cell lines [16].

Determination of Intracellular ROS Levels

TNBC cell lines – both MDA-MB-231 and MDA-MB-468 were cultured under preset conditions in six well plates for 24 hrs post which they were treated with RAJI in three different concentrations in the respective cell lines - (10, 20, 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells). Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. After RAJI treatment, the cells were washed with PBS and incubated with trypsin-EDTA incubation for 3-4 minutes at 37°C, after which additional medium was added and centrifuged at 300 × g at 25°C. The pellets were washed with PBS, treated with D2DCFDA, and centrifuged at 150 g for 5 min. The supernatant was discarded and the pellets were resuspended in pre-warmed DPBS, followed by subjection to flow cytometry to analyze the ROS levels in each cell line according to the manufacturer's protocol.

Evaluating the Mitochondrial Membrane Potential

TNBC cell lines – both MDA-MB-231 and MDA-MB-468 were cultured under preset conditions followed by treatment with RAJI in three different concentrations in the respective cell lines - (10, 20, 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells). After 24 h of treatment, Hoechst and JC-1 were added to the cells and incubated for 30 min. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. Images were obtained using a fluorescence microscope according to the manufacturer's protocol.

Colorimetric Assessment of Caspase Levels

Caspase (3/7, 9) enzyme activity was measured by a colorimetric assay using a caspase assay kit (Promega). MDA-MB-468 cells were cultured under preset conditions in six well plates for 24 h, after which they were treated with RAJI at an IC_{50} of 25µg/ml. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard. After 24 h of treatment, caspase 3/7 and 9 expression was evaluated by UV absorbance at 410 nm, according to the manufacturer's protocol.

Quantitative Real-Time analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the relative expression levels of key genes associated with the PI3K/*Akt* signaling pathway, including *Akt, PTEN, mTOR, cyclin D1, Bax,* and *Bcl-2*. TNBC cells, specifically the MDA-MB-231 cell line, were cultured under standardized conditions in six-well plates for 24 h. Subsequently, the cells were treated with RAJI at its IC_{50} concentration (25 µg/mL). Untreated cells were used as the negative control, whereas cells treated with doxorubicin served as a positive control. After 24 h of treatment, total RNA was isolated using the RNA Isoplus Total RNA Extraction Kit (TaKaRa) according to the manufacturer's instructions. RNA purity and concentration were determined by measuring absorbance at 260/280 nm using a NanoDrop spectrophotometer. First-strand complementary DNA (cDNA) synthesis was performed using an iScript cDNA Synthesis Kit (TaKaRa). Quantitative PCR was conducted using TB Green® Premix Ex Taq™ II (TaKaRa) and the gene-specific primers listed in Table 1. Amplification was carried out using a QuantStudio 3 real-time PCR system under the following cycling conditions: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, and final denaturation at

Table 1. List of Genes and Their Respective Primer Sequences

Name of the Gene		Sequence	Length
AKT	FP	TGGACTACCTGCACTCGGAGAA	22
	RP	GTGCCGCAAAAGGTCTTCATGG	22
mTOR	FP	AGCATCGGATGCTTAGGAGTGG	22
	RP	CAGCCAGTCATCTTTGGAGACC	22
PTEN	FP	TGAGTTCCCTCAGCCGTTACCT	22
	RP	GAGGTTTCCTCTGGTCCTGGTA	22
Cyclin D1	FP	TCTACACCGACAACTCCATCCG	22
	RP	TCTGGCATTTTGGAGAGGAAGTG	23
Bax	FP	TCAGGATGCGTCCACCAAGAAG	22
	RP	TGTGTCCACGGCGGCAATCATC	23
Bcl-2	FP	ATCGCCCTGTGGATGACTGAGT	22
	RP	GCCAGGAGAAATCAAACAGAGGC	23
GAPDH	FP	GTCTCCTCTGACTTCAACAGCG	22
	RP	ACCACCCTGTTGCTGTAGCCAA	22

95 °C for 15 s. Relative gene expression was normalized to that of the internal reference gene *GAPDH* and calculated using the comparative Ct (ΔΔCt) method. All experiments were performed with three independent biological replicates, and each was analyzed in triplicate to ensure reproducibility and accuracy.

Tumour Xenograft in Mice

Female nude athymic BALB/c mice with weighing approximately 25 gm and 4 weeks old were housed separately with a 2-week acclimatization period prior to start of the experiment and Good Animal Practice was followed as per Institutional Animal Ethics Committee (IAEC) with approval no SVCOP/IAEC/014/2021-22 in accordance with the Committee for the Purpose of Control and Supervision of Experiments (CPCSEA), India.

Induction of TNBC Cell Line

2 x 10^6 cells (MDA-MB-231) were suspended in 100 ml of Matrigel (Corning, USA) and serum-free DMEM and injected subcutaneously into the flanks of each animal using a tuberculin syringe with a needle of 21 gauge [16].

Tumour Progression and RAJI Treatment

To track the progression of breast tumors in the mice, the diameters of the tumors were measured both horizontally and vertically, and after the tumors attained an average size of approximately 200 and 300 mm^3, the mice were considered to be fit for the xenograft model study. All mice bearing tumors were categorized into four different groups with three mice in each group: group 1 or the control group with Phosphate Buffered Saline (PBS) administration, group 2 or RAJI-treated (25 mg/kg body weight in 1 × PBS), group 3 or doxorubicin-treated (1.5 mg/kg body weight in 1 × PBS), and group 4 or combination treatment of both RAJI and doxorubicin (25 mg/kg & 1.5 mg/kg body weight in 1 × PBS, respectively). The respective drugs were administered intraperitoneally every 2 days to the animals in the respective groups for a period of 30 days. Alterations in the tumor dimensions were recorded at periodic intervals, and tumor volumes were computed using the formula - ½ (Width × Length2). The study concluded 40 days post-tumor induction in MDA-MB-231cells by administering a lethal dose of ketaminexylazine. Tumors were collected for further studies [16].

Statistical Analysis

All experimental data were evaluated using the Student's T-Test and a P-value of < 0.05 was considered statistically significant.

Results

Assessment of Cell Viability Using MTT Assay

The cytotoxic potential of RAJI was evaluated in two TNBC cell lines, MDA-MB-231 and MDA-MB-468, and a normal epithelial breast cell line, MCF 10A, at different concentrations and at two different time periods: 24 and 48 h. The results suggest that the IC_{50} values for the two TNBC cell lines were 20µg and 25µg in the MDA-MB-231 and MDA-MB-468 cell lines and 40µg in the normal MCF 10A cell line. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Live/Dead Staining using Acridine Orange and Ethidium Bromide

Live/dead staining was performed on the two TNBC cell lines alone using the dyes acridine orange (AO) and ethidium bromide (EtBr) at three different RAJI concentrations (10, 20, and 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells). Live cells take up AO and emit a green hue, whereas dead cells take up EtBr and emit a red color. Hence, it can be concluded that with an increase in RAJI concentration, there is a significant decrease in the live cell population in both TNBC cell lines. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Cell Migration Assay

As TNBC cell lines are highly invasive in nature, the inhibitory efficiency of RAJI was evaluated in two TNBC cell lines, MDA-MB-231 and MDA-MB-468. The results clearly demonstrated that, with an increase in RAJI concentration, there was a distinct decrease in cell viability, which greatly influenced the gap/wound closure that was manually created during the course of the experiment (both 24 and 48 h). Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Cell Cycle Analysis by Flow Cytometry

To further understand the underlying mechanisms of RAJI-induced inhibition, cell cycle distribution patterns in MDA-MB-231 and MDA-MB-468 cells were examined using flow cytometry. These results clearly emphasize that RAJI significantly increased cell cycle arrest in the G0/G1 phase, thereby highlighting the fact that the anti-proliferative effects of RAJI may be due to cell cycle arrest, combined with other mechanisms when the concentration is increased. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Apoptosis Annexin-V Staining

Apoptosis is another significant factor that contributes to cell growth. Following labelling of TNBC cells with Annexin V/PI, flow cytometry was used to gauge the level of apoptosis in the TNBC cell lines MDA-MB-231 and MDA-MB-468. The results obtained clearly emphasize that with an increase in RAJI concentration, there is a significant increase in the population of cells falling under the late apoptotic stage, thereby suggesting that RAJI is pivotal in inducing programmed cell death in TNBC cell lines. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Determination of Intracellular ROS Levels

ROS production is considered to be an important hallmark of apoptosis; hence, the TNBC cell lines MDA-MB-231 and MDA-MB-468 treated with three different concentrations of RAJI - (10, 20, and 30 µg/ml in MDA-MB-231 cells and 12.5, 25, 37.5 µg/ml in MDA-MB-468 cells) were subjected to ROS level analysis via DCFDA staining. These results clearly indicate that there is a significant increase in ROS levels with an increase in RAJI concentration, thereby suggesting that RAJI promotes cell death via ROS generation. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Evaluating the Mitochondrial Membrane Potential

ROS production has been proven to greatly influences mitochondrial integrity in TNBC cells. To evaluate the role of RAJI in damaging mitochondrial integrity in the TNBC cell lines MDA-MB-231 and MDA-MB-468 after RAJI treatment, the cells were stained with Hoechst and JC-1, and the results distinctly emphasize that with an increase in RAJI concentration, the mitochondrial membrane was greatly compromised, which eventually led to cell death. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Colorimetric Assessment of Caspase Levels

Determining caspase levels proved to be another significant experiment in studying apoptosis in tumor cells. The TNBC cell line MDA-MB-468, when treated with RAJI, vividly substantiates that with an increase in the concentration of RAJI, a distinct increase in caspase levels was observed, eventually confirming cell death in the RAJI-treated cells. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Quantitative Real-Time analysis

To further explore the effect of RAJI in MDA-MB-231 cells, important genes involved in the *AKT/PI3K* signalling pathway, such as *Akt, PTEN, mTOR,* and *Cyclin D1*, were investigated using real-time PCR. Quantitative gene expression analysis revealed elevated levels of *Akt*, *PTEN*, mTOR, and *Cyclin D1* in untreated cells, which were subsequently found to be significantly downregulated upon RAJI treatment, ultimately resulting in apoptosis and cell death. Concurrently, there was a marked upregulation in the *Bcl2* genes, which proved that upon RAJI treatment, the cells swiftly underwent apoptosis, eventually leading to cell death, as depicted in Figure 9. Untreated cells served as controls, and cells treated with doxorubicin served as the reference standard.

Tumour volume reduction in xenograft study

The In-vivo antitumor effects of RAJI when administered alone and in conjunction with doxorubicin against MDA-MB-231 cells injected into BALB/c nude mice were assessed.

In the current study, neither a significant decrease in body weight nor any deleterious effects were observed. However, a significant reduction in both tumor weight and volume was observed. Macroscopic assessment of the reduction in tumor size in the animals of different groups post-sacrifice is depicted in Figure10A.

A significant reduction in tumor weight in the different groups was also recorded, where the group treated with the combination therapy displayed better results when compared to RAJI and doxorubicin when administered individually. The untreated and control groups had a tumor weight of 188.66 ± 11.7 mg, the RAJI-treated group had an average of 125.33 ± 17.09 mg, and the doxorubicin-treated group had an average of 47 ± 11.3 mg. However, a distinct reduction in the tumour weight was observed in the RAJI + doxorubicin group where the tumour weight was only 20.66 ± 2.51 mg, signifying that the combination therapy yielded better results as depicted in Figure10b.

Similarly, when the tumor volume was calculated, the results were consistent with the tumor weight observations. The RAJI + doxorubicin treated group had a mean tumour volume of 10.66 ± 2.25 mm^3 in comparison to the RAJI alone group (151.86 ± 41.82 mm^3), doxorubicin alone group (34.85 ± 6.81 mm^3) and the control group (437.46 ± 93.74 mm^3) as displayed in Figure10c.

Discussion

Although chemical medications are essential in the battle against breast cancer, serious side effects are associated with their use. The development of resistance, toxic side effects, and negative impacts on patients' quality of life highlights the need for further research into more focused and less hazardous treatment choices. Improving the effectiveness and tolerability of breast cancer treatments is still the aim of the pharmaceutical realm, as it continues to research and create alternative medicines. With these considerations in mind, RAJI was synthesized as a potential panacea for treating the most aggressive form of breast cancer, TNBC, with better therapeutic outcomes and fewer side effects when administered at low concentrations.

Several studies have shown that piperidine is extremely efficient in the treatment of various cancers. A recent study using MCF-7 and HeLa cell lines showed that piperidine prevents cell proliferation, thus signifying its cytotoxic potential [17]. Another study indicated that propylene-tethered isatin dimers had far stronger anti-cancer effects in MCF-7 cell lines than the gold standard, doxorubicin [18], proving that isatin derivatives are cytotoxic against human cancer cells [19]. The results of the cytotoxicity evaluation of RAJI in the current study via the MTT assay correlate with the aforementioned findings, thus emphasizing that RAJI exhibits higher toxicity against human TNBC cells and relatively low toxicity against normal cell lines (as mentioned in Figure 1), thereby suggesting that RAJI is a potential candidate for cancer therapy against TNBC.

The AO/EtBr staining protocol was used to analyze apoptosis and cellular morphology in MDA-MB-468 and MDA-MB-231 cells [20]. Cell death and membrane damage were observed in RAJI-treated MDA-MB-231 cells after staining, as depicted in Figure 2. A recent study of isatin derivatives in HCT-116 cells was in line with our findings, where untreated cells revealed deep green fluorescence, implying the presence of live cells with a prominent nucleus. In contrast, varying dosages resulted in early apoptosis (orange staining) and late apoptosis (red staining) in HCT-116 cells [21]. Cell migration assays play a crucial role in cancer prognosis [22]. Upon treatment with RAJI, a significant reduction in cell migration was observed compared with doxorubicin, as shown in Figure 3. Another study utilized an isatin derivative to check its proliferation in HepG2 cells and reported that it inhibited the migration of HepG2 cells upon treatment [18].

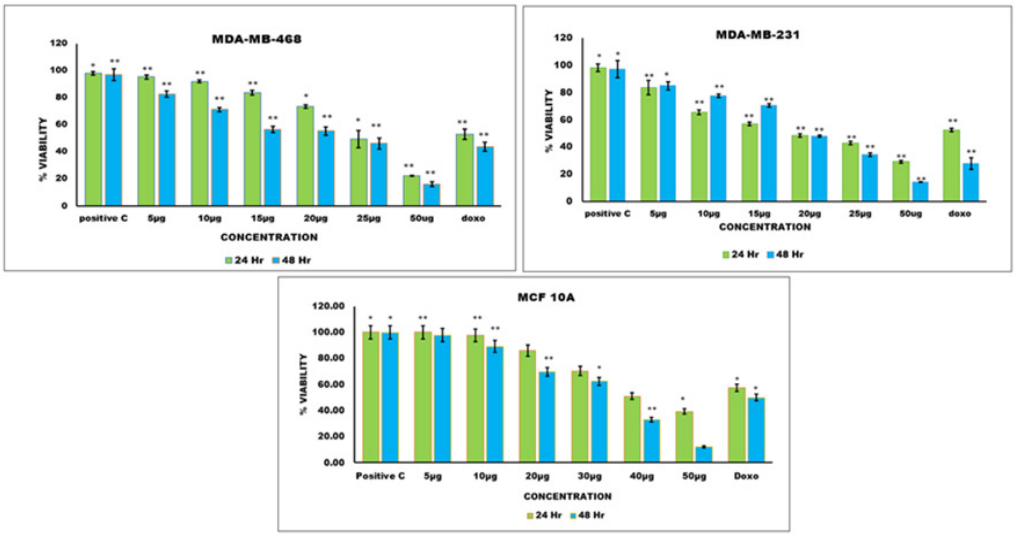
Flow cytometric analysis of MDA-MB-468 and MDA-MB-231 cells treated with different concentrations of RAJI revealed distinct cell cycle distribution patterns. Minimal cell death or DNA fragmentation was observed, with a predominant cell population in the G0/G1 phase, suggesting potential cell cycle arrest (Figure 4). Similarly, another study showed that sericin treatment induced G0/G1 cell cycle arrest and promoted cellular apoptosis in MDA MB 468 cells via the *PI3K/Akt* Pathway [23]. Both compounds, RAJI and sericin, exhibited similar effects on cell cycle dynamics, with a predominant population of cells arrested at the G0/G1 phase and minimal cell death, indicating potential cell cycle modulation. Another experiment was performed using eupatorin in MCF-7 and MDA-MB-231 cells, and the results indicated that eupatorin treatment demonstrated anti-proliferative activity by inducing cell cycle arrest at sub Gθ/G1 phase in a time-dependent manner in MCF-7 and MDA-MB-231 cells, similar to the findings of the current study [24].

Annexin V is a family of proteins known for their ability to bind specifically to phosphatidyl serine molecules that are exposed to the outer leaflet of the plasma membrane, which is an indicator of apoptotic cells [25]. Results from the present study were in agreement with another analogous study that reported 98.4% of live cells in the control sample (untreated cells), whereas on treatment with Pyrazole derivatives, a significant increase in early and late apoptosis was noticed [26]. The observed fluorescent patterns align with those of the present study, as depicted in Figure 5, thus ensuring the reliability of the present study in analyzing apoptotic cell populations in TNBC cells.

Examination of ROS levels is crucial for screening antitumor drugs [27]. A dose-dependent response was observed when MDA-MB-468 and MDA-MB-231 cells were treated with RAJI at different concentrations, and the ROS levels were examined, as shown in Figure 6. This elevation in ROS levels aligns with a recent finding that suggests a correlation between cytotoxicity and ROS production in tumor cells. This elevation in ROS production was observed when cells were subjected to a combination treatment with gallic acid and low-level radiation, rather than gallic acid treatment alone [28].

Mitochondria membrane potential plays a critical role in understanding cellular stress, apoptosis, and various metabolic disorders [29]. Mitochondria are vital organs that synthesize ATP via oxidative phosphorylation [30]. A recent study used ampilopsin to explore the mitochondrial membrane potential in TNBC cells. Several studies have reported that ampelopsin is a potent anti-tumor agent [31]. Apoptosis in MDA-MB-231 and MCF-7 cells was confirmed to be associated with mitochondrial dysfunction and loss of mitochondrial membrane potential. When both cells were treated with ampilopsin, a decrease in red fluorescence was observed, which indicated malfunctioning of the mitochondrial membrane potential [32]. The observed fluorescent pattern closely aligns with the current experimental data, as shown in Figure 7, which suggests that both the drug moieties might have similar anti-tumor properties against TNBC cells, as they may share certain common cellular pathways.

The objective of caspase evaluation was to confirm the activation of caspases belonging to the cysteine protease family. The pivotal function of apoptosis has a substantial impact on its regulation, as it initiates apoptosis by cleaving specific cellular proteins [33]. The present study demonstrated a dose dependant increase in cell mortality upon subjection to RAJI treatment, which closely aligns with another study, which a 20% increase in caspase 3 protein expression was observed in cancer cells along with a 10% decrease in HDF cells. Moreover, a significant increase in pro-apoptotic genes has been



Cell line	IC_{50} conc (µg/ml)
MDA MB 468	25
MDA MB 231	20
MCF-10A	40

Figure 1. Cytotoxic Potential of RAJI in 3 Different Cell Lines, 2 TNBC cell lines - MDA-MB-231 & MDA-MB-468 and a normal epithelial breast cell line MCF 10A.

observed [34]. Congruence in the activation of apoptotic pathways provides strong and compelling evidence for sharing a common mechanism for inhibiting cancer cell growth. This reinforces the reliability of the current study, thereby validating RAJI's therapeutic efficiency of RAJI in combating TNBC, as depicted in Figure 8.

Numerous studies have indicated that the *PI3K/Akt/mTOR* signaling pathway, which is frequently overexpressed in human cancers, is abnormally modified in approximately 70% of BC [35-37]. Alterations or mutations in this pathway are present in 25% of primary TNBC cases, and potentially occur more often in TNBC. Inhibitors targeting this pathway have shown significant efficacy and clinical utility in the treatment of TNBC. This review outlines the activation mechanism of the *PI3K/Akt/mTOR* signaling pathway in TNBC and explores

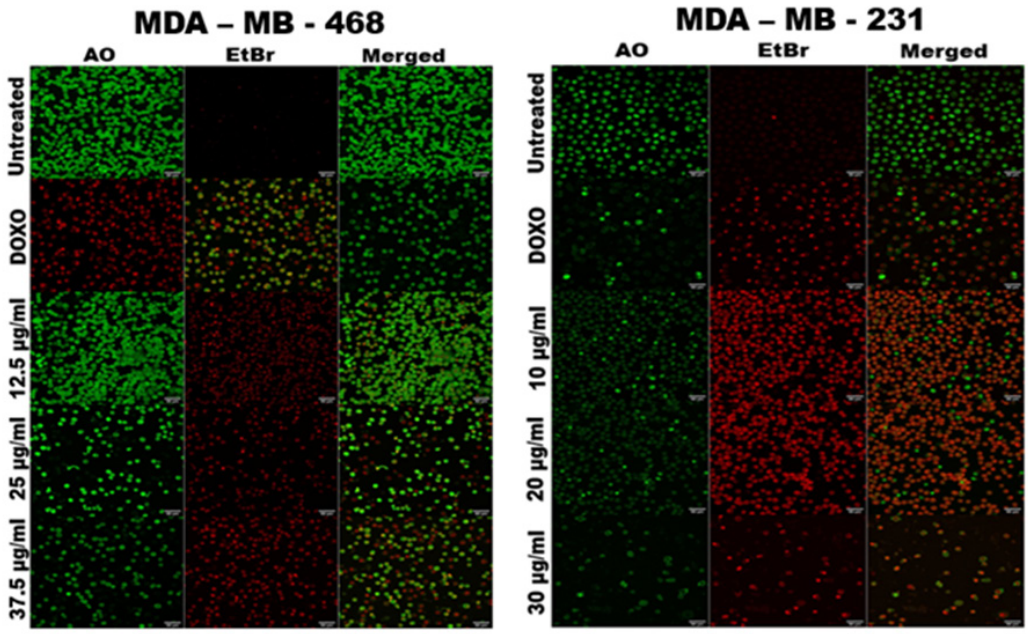


Figure 2. Live/Dead Staining of TNBC Cell Lines with Acridine Orange and Ethidium Bromide

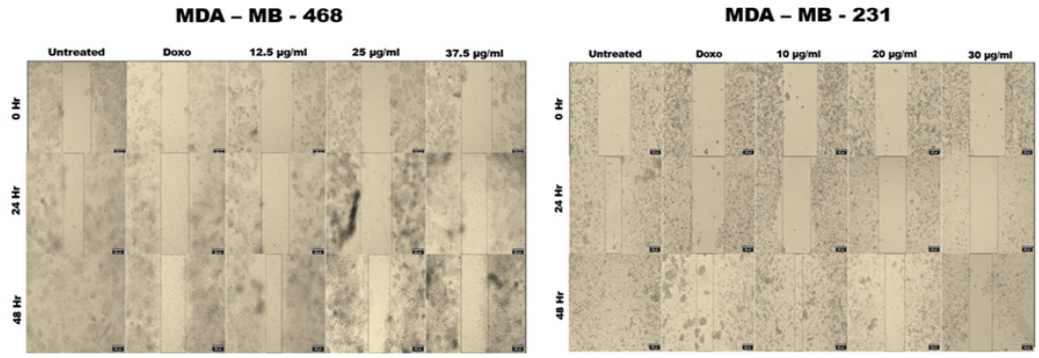


Figure 3. Effect of RAJI in Cell Migration in the TNBC Cell Lines where the Invasion was Measured by Wound Healing Assay

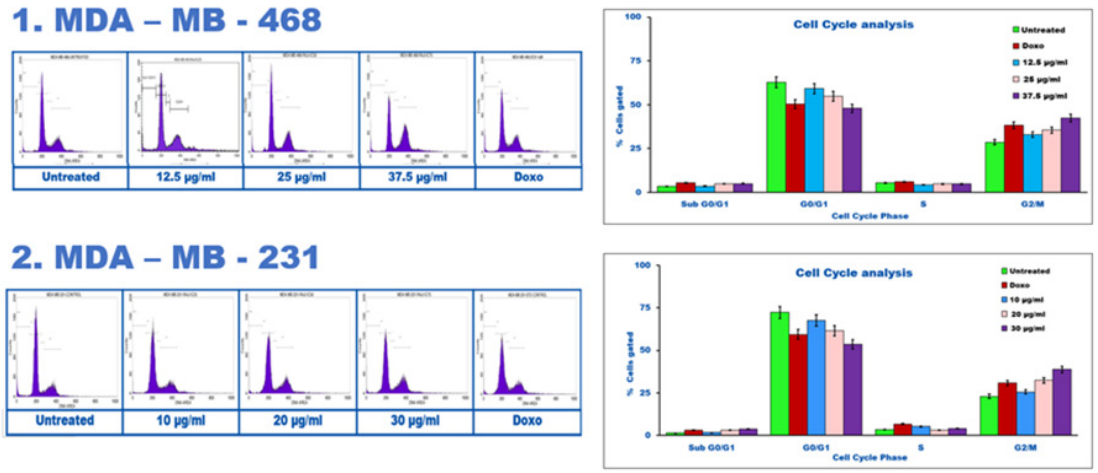


Figure 4. RAJI Induced Cell Cycle Arrest in TNBC Cell Lines where Cells Stained with PI in Different Concentrations of RAJI were Analysed via Flow Cytometry

its association with different TNBC subtypes. *AKT*, also known as protein kinase B, is a crucial effector molecule downstream of the *PI3K* pathway [38]. Upon *PI3K* activation, the accumulated PIP3 attracts intracellular PDK1 and *Akt* to the cell membrane. PDK1 phosphorylates *Akt* at Thr308, whereas mTORC2 phosphorylates *Akt* at Ser473, leading to full activation of Akt. In addition, PDK1 indirectly boosts mTORC2 activity. Activated *Akt* phosphorylates mTOR complex 1 (mTORC1), a key downstream effector that facilitates cell proliferation and oncogenic transformation [39, 40]. We observed upregulation in the expression of *Akt*, mTOR, and its downstream target cyclin D1 (Figure 9). Small-molecule inhibitors that suppress the *Akt*/mTOR pathway enhance the sensitivity of TNBC cells to apoptosis. Our findings demonstrated a significant decrease in *Bcl2* expression and

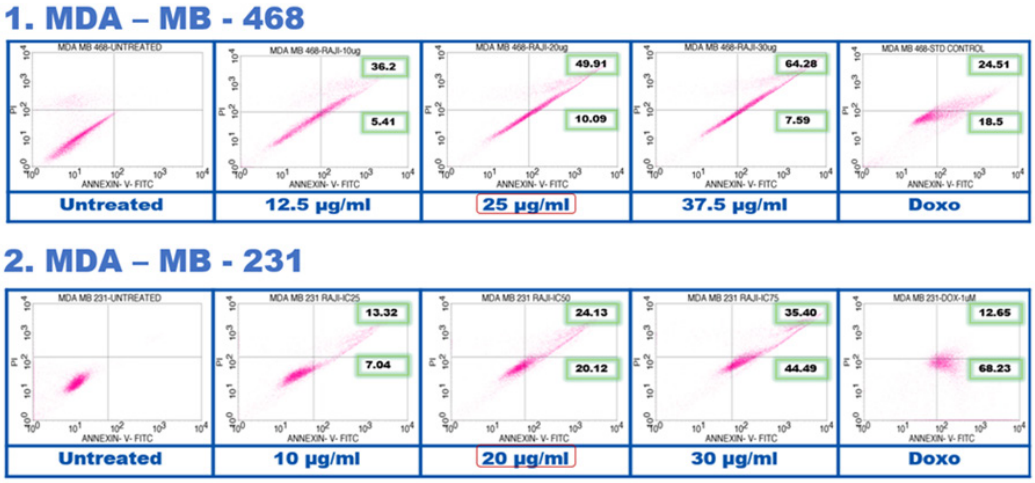


Figure 5. Flow Cytometric Analysis of Apoptosis by Staining Annexin-V-FITC in RAJI treated TNBC Cell Lines MDA-MB-231 & MDA-MB-468.

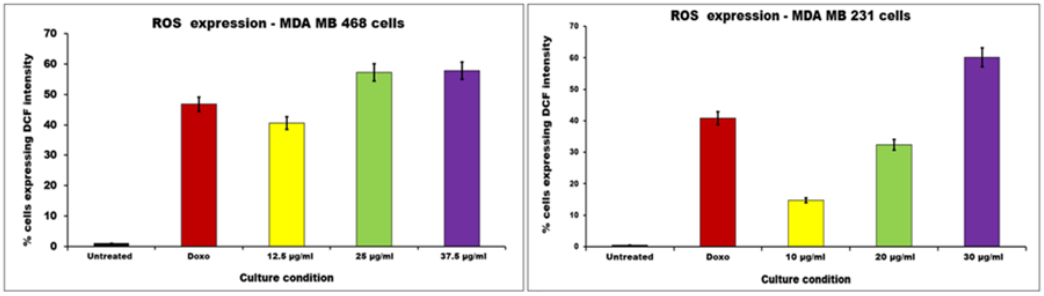


Figure 6. Flow Cytometric Analysis of ROS Levels in RAJI Treated TNBC Cell Lines MDA-MB-231 and MDA-MB-468.

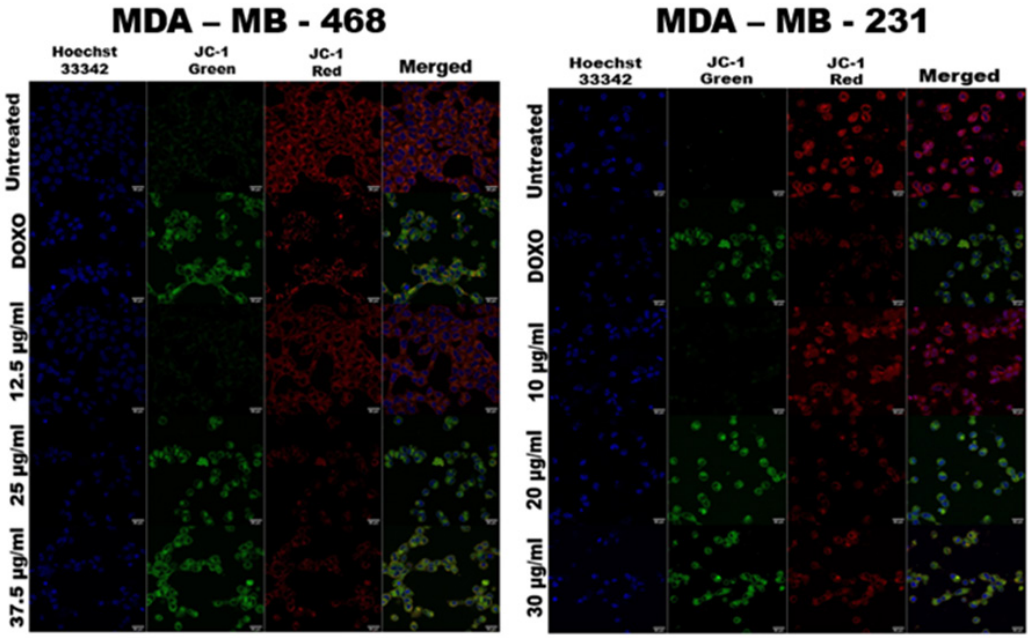


Figure 7. RAJI Treated TNBC Cell Lines MDA-MB-231 and MDA-MB-468 Imaged under Fluorescent Microscope Post Hoechst and JC-1 Staining.

an increase in *Bax* expression (Figure 9E and F) following RAJI administration. Published reports have shown that small-molecule inhibitors exert a profound effect on TNBC by inhibiting the *Akt*/mTOR pathway [38, 41-43]. We are convinced that suppression of *Akt*/mTOR signaling is responsible for the inhibition of TNBC.

Consistent with in In-vitro findings, RAJI administration hindered the growth of MDA-MB-231 xenografts in nude mice. The objective of integrating chemotherapeutic medications into novel combination

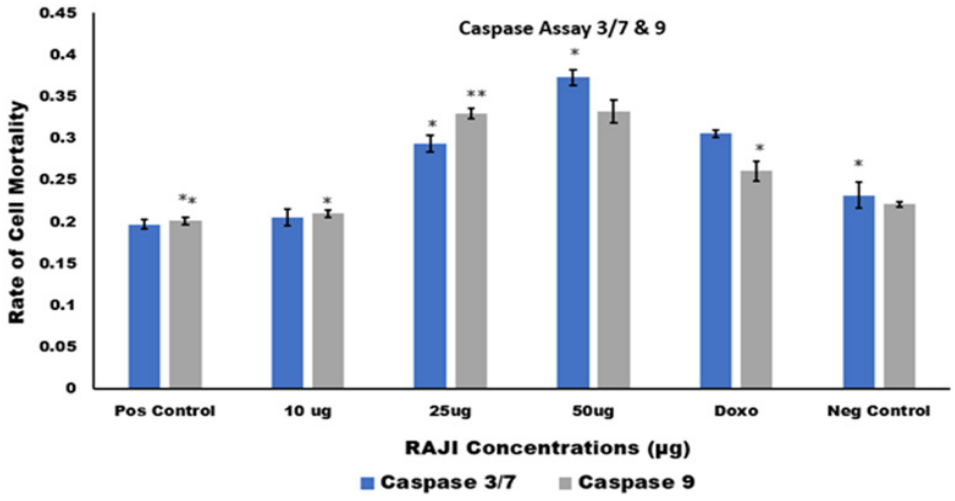


Figure 8. Estimation of Caspase 3/7 and 9 Activities in the RAJI treated TNBC Cell Line – MDA-MB-468.

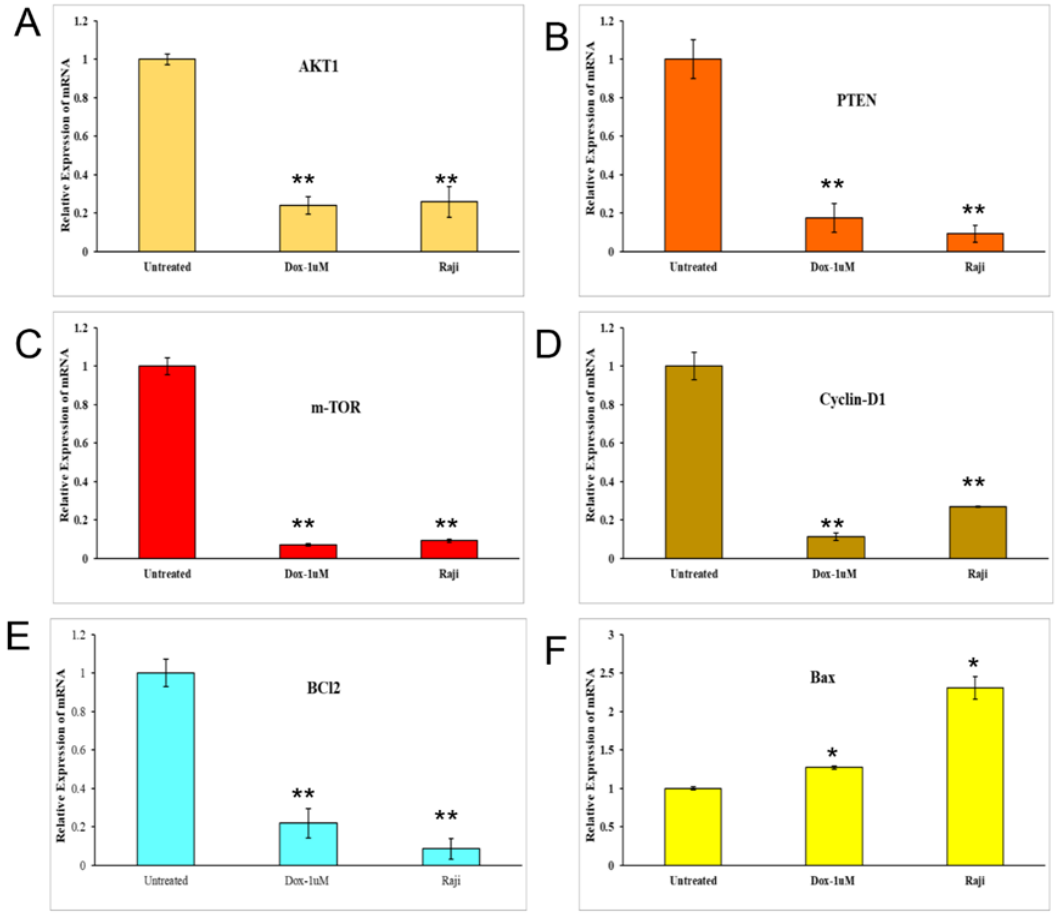


Figure 9. Relative mRNA Expressions ofGenes (A) *AKT1*, (B) *PTEN*, (C) *mTOR*, (D) *Cyclin D1*, (E) *BCl-2* and (F) *Bax* – Untreated, Doxorubicin and RAJI treated MDA-MB-231 cells.

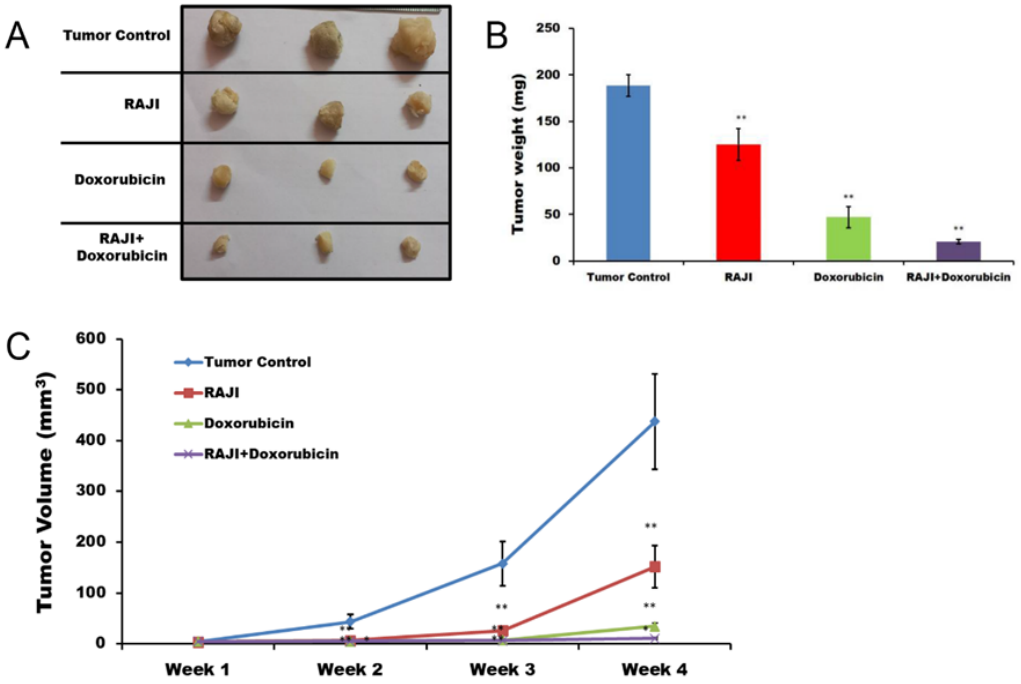


Figure 10. (A) Macroscopic visualization of tumour size reduction post treatment. (B) Significant reduction in the tumour weight in all the treated groups in comparison to the untreated or control group. The statistical difference is demoted as * ($p<0.05$). (C) Significant reduction in the tumour volume in all the treated groups in comparison to the untreated or control group when observed on a weekly basis. The statistical difference is demoted as * ($p<0.05$).

therapies is to mitigate adverse effects at minimal dosages [44]. Doxorubicin, a DNA-damaging agent (DDA), is a first-line chemotherapeutic drug for breast cancer treatment despite its severe adverse effects [16]. The current findings demonstrate that RAJI effectively decreased both the volume and weight of tumors in xenograft mice implanted with MDA-MB-231 cells, indicating a significant antitumor effect, as shown in Figure 10A-C. Additionally, the administration of RAJI alone or in combination with doxorubicin did not have any distinct adverse effects on the animals when visually observed. Hence, these data indicate that RAJI may be advantageous in therapeutic scenarios, whether employed alone or in conjunction with other conventional chemotherapeutic agents.

In conclusion, the efficacy of conventional therapeutic modalities, such as surgery, radiation, and chemotherapy, in the treatment of TNBC is limited by their inability to specifically target specific molecular targets. These methods have a variety of drawbacks, as their results are not optimistic. Consequently, there has been growing interest in exploring innovative strategies to efficiently address breast cancer treatment while minimizing adverse consequences. The present study demonstrated that RAJI exhibits promising outcomes by specifically targeting TNBC cells while inducing low harm to normal non-malignant breast epithelial MCF-10A cells. The findings from the present study highlight the similarity of cellular responses to RAJI with various other potential cytotoxic agents, thereby emphasizing the need for tailored therapeutic approaches based on other individual cellular contexts. Fluorescent microscopic visualization of RAJI-treated cells yielded encouraging findings pertaining to nuclear morphology, cell survival, mitochondrial depolarization, and apoptosis. A mutually reinforcing relationship was noted between the increase in RAJI concentration and increase in mitochondrial depolarization. The research conducted in this study demonstrated a positive correlation between the dosage of RAJI and the sensitivity of reactive oxygen species (ROS) levels upon exposure to MDA-MB-231 and MDA-MB-468 cells. In addition, dose-dependent upregulation of caspase 3/7 and 9 expression was observed, suggesting the onset of apoptosis in TNBC cells. Quantitative gene expression analysis demonstrated that RAJI treatment significantly downregulated the expression of important genes in the *AKT/PI3K* signalling pathway, namely *AKT, PTEN, mTOR,* and *cyclin D1* along with other apoptosis-associated genes, such as *Bax* and *Bcl-2* were regulated by RAJI in MDA-MB-231 cells. This shift in the gene expression profile suggests that RAJI induces apoptosis by suppressing pro-survival pathways and activating tumor suppressor mechanisms. In conjunction with the In-vitro studies, the In-vivo results showed that RAJI had anti-cancer effects in nude BALB/c mice, whether administered alone or in combination, and no substantial side effects were reported. Hence, these characteristics define RAJI as a promising candidate for BC treatment, warranting further investigations, such as its molecular mechanistic pathway of action, prior to its incorporation into the human population.

Author Contribution Statement

P.S.: Conceptualization, Methodology, Validation, Investigation, Writing - Original Draft, K.C.: Methodology, Validation, Investigation, S.K.A.: Validation, Methodology, Writing - Review & Editing, A.K.P.: Resources, Supervision, Project administration, Writing - Review & Editing.

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Data Availability Statement

All the original data presented in the study are included in the manuscript.

Ethical approval

The Institutional Animal Ethics Committee (IAEC) with approval no SVCOP/IAEC/014/2021-22 in accordance with the Committee for the Purpose of Control and Supervision of Experiments (CPCSEA), from Sri Venkateshwara College of Pharmacy, Andra pradesh, India

Conflict of Interest Statement

The authors have no conflict of interest to declare.

References

1. Aggarwal T, Wadhwa R, Gupta R, Paudel KR, Collet T, Chellappan DK, et al. Micrornas as biomarker for breast cancer. Endocr Metab Immune Disord Drug Targets. 2020;20(10):1597-610. https://doi.org/10.2174/1871530320666200428113051.
2. Mehrotra R, Yadav K. Breast cancer in india: Present scenario and the challenges ahead. World J Clin Oncol. 2022;13(3):209-18. https://doi.org/10.5306/wjco.v13.i3.209
3. Sadik SBS, Sivaprakasam P, Ramasami N, Pandurangan AK. The Molecular Mechanisms Involved in Suppressing Triple Negative Breast Cancer Using Natural Agents. In: Handbook of Research on Natural Products and Their Bioactive Compounds as Cancer Therapeutics. IGI Global; 2022. p. 45–71.
4. Chang-Qing Y, Jie L, Shi-Qi Z, Kun Z, Zi-Qian G, Ran X, et al. Recent treatment progress of triple negative breast cancer. Prog Biophys Mol Biol. 2020;151:40-53. https://doi.org/10.1016/j.pbiomolbio.2019.11.007
5. de la Cruz-Ku G, Chambergo-Michilot D, Torres-Roman JS, Rebaza P, Pinto J, Araujo J, et al. Neutrophil-to-lymphocyte ratio predicts early mortality in females with metastatic triple-negative breast cancer. PLoS One. 2020;15(12):e0243447. https://doi.org/10.1371/journal.pone.0243447
6. Sandhu GS, Erqou S, Patterson H, Mathew A. Prevalence of triple-negative breast cancer in india: Systematic review and meta-analysis. J Glob Oncol. 2016;2(6):412-21. https://doi.org/10.1200/jgo.2016.005397.
7. Anandasadagopan SK, Sivaprakasam P, Pathmanapan S, Pandurangan AK, Alagumuthu T. Targeting the Key Signaling Pathways in Breast Cancer Treatment Using

Natural Agents. Plant-derived Bioactives: Chemistry and Mode of Action. 2020:137-83.
8. Santosh S, Rajagopalan MD, Pallavi BA, Rudramurthy GR, Rajashekar V, Sridhar KA, Swamy MK. Cancer therapies: current scenario, management, and safety aspects. InAnticancer Plants: Clinical Trials and Nanotechnology: Volume 3 2018 Jul 4 (pp. 1-25). Singapore: Springer Singapore.
9. Sivaprakasam P, Anandasadagopan S, Alagumuthu T, Pandurangan AK. Current update on natural agents against triple negative breast cancer. InResearch anthology on advancements in women's health and reproductive rights 2022 (pp. 318-335). IGI Global.
10. De Cicco P, Panza E, Armogida C, Ercolano G, Cirino G, Ianaro A. Current practices and awareness of anticancer plants in the traditional healthcare system. Anticancer plants: Properties and Application: Volume 1. 2018:1-36.
11. Akhtar MS, Swamy MK. Anticancer plants: mechanisms and molecular interactions. Berlin/Heidelberg, Germany: Springer; 2018.
12. Kumar S, Bal A, Das A, Loriya I, Khare S, Bhattacharya S, et al. Spectrum of pik3ca/akt mutations across molecular subtypes of triple-negative breast cancer. Breast Cancer Res Treat. 2021;187(3):625-33. https://doi.org/10.1007/s10549-021-06242-3
13. Reinhardt K, Stückrath K, Hartung C, Kaufhold S, Uleer C, Hanf V, et al. PIK3CA-mutations in breast cancer. Breast Cancer Res Treat. 2022;196(3):483–93. https://doi.org/10.1007/s10549-022-06637-w
14. Kakkar R. Isatin and its derivatives: a survey of recent syntheses, reactions, and applications. Medchemcomm. 2019;10(3):351–68. https://doi.org/10.1039/c8md00585k
15. Samarov AA, Riabchunova A V, Verevkin SP. Interactions between substituents in the benzene ring. Experimental and theoretical study of methoxy substituted acetophenones. J Chem Thermodyn. 2022;173:106847.
16. Hasanpourghadi M, Pandurangan AK, Karthikeyan C, Trivedi P, Mustafa MR. Mechanisms of the anti-tumor activity of Methyl 2-(-5-fluoro-2-hydroxyphenyl)-1 H-benzo [d] imidazole-5-carboxylate against breast cancer in vitro and in vivo. Oncotarget. 2017;8(17):28840.
17. Fu DJ, Liu SM, Yang JJ, Li J. Novel piperidine derivatives as colchicine binding site inhibitors induce apoptosis and inhibit epithelial-mesenchymal transition against prostate cancer pc3 cells. J Enzyme Inhib Med Chem. 2020;35(1):1403-13. https://doi.org/10.1080/14756366.2020.1783664
18. Zhang YZ, Du HZ, Liu HL, He QS, Xu Z. Isatin dimers and their biological activities. Arch Pharm (Weinheim). 2020;353(3):e1900299. https://doi.org/10.1002/ardp.201900299
19. Sathishkumar G, Logeshwaran V, Sarathbabu S, Jha PK, Jeyaraj M, Rajkuberan C, et al. Green synthesis of magnetic fe(3)o(4) nanoparticles using couroupita guianensis aubl. Fruit extract for their antibacterial and cytotoxicity activities. Artif Cells Nanomed Biotechnol. 2018;46(3):589-98. https://doi.org/10.1080/21691401.2017.1332635
20. Abdulkarim Alharbi S, Eldin Ahmed Abdelsalam K, Asad M, Alrouji M, Ahmed Ibrahim M, Almuhanna Y. Assessment of the anti-cancer potential of ephedra foeminea leaf extract on mda-mb-231, mcf-7, 4 t1, and mcf-10 breast cancer cell lines: Cytotoxic, apoptotic and oxidative assays. Saudi Pharm J. 2024;32(3):101960. https://doi.org/10.1016/j.jsps.2024.101960
21. Malaikolundhan H, Mookkan G, Krishnamoorthi G, Matheswaran N, Alsawalha M, Veeraraghavan VP, et al. Anticarcinogenic effect of gold nanoparticles synthesized from albizia lebbeck on hct-116 colon cancer cell lines. Artif Cells Nanomed Biotechnol. 2020;48(1):1206-13. https://doi.org/10.1080/21691401.2020.1814313
22. Shahi Thakuri P, Gupta M, Singh S, Joshi R, Glasgow E, Lekan A, et al. Phytochemicals inhibit migration of triple negative breast cancer cells by targeting kinase signaling. BMC Cancer. 2020;20(1):4. https://doi.org/10.1186/s12885-019-6479-2
23. Niu L, Yang S, Zhao X, Liu X, Si L, Wei M, et al. Sericin inhibits mda-mb-468 cell proliferation via the pi3k/akt pathway in triple-negative breast cancer. Mol Med Rep. 2021;23(2). https://doi.org/10.3892/mmr.2020.11779
24. Razak NA, Abu N, Ho WY, Zamberi NR, Tan SW, Alitheen NB, et al. Cytotoxicity of eupatorin in mcf-7 and mda-mb-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and induction of apoptosis. Sci Rep. 2019;9(1):1514. https://doi.org/10.1038/s41598-018-37796-w
25. Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ. Quantitation of apoptosis and necrosis by annexin v binding, propidium iodide uptake, and flow cytometry. Cold Spring Harb Protoc. 2016;2016(11). https://doi.org/10.1101/pdb.prot087288
26. Ashourpour M, Mostafavi Hosseini F, Amini M, Saeedian Moghadam E, Kazerouni F, Arman SY, et al. Pyrazole derivatives induce apoptosis via ros generation in the triple negative breast cancer cells, mda-mb-468. Asian Pac J Cancer Prev. 2021;22(7):2079-87. https://doi.org/10.31557/apjcp.2021.22.7.2079.
27. Vaneev AN, Gorelkin PV, Garanina AS, Lopatukhina HV, Vodopyanov SS, Alova AV, et al. In vitro and in vivo electrochemical measurement of reactive oxygen species after treatment with anticancer drugs. Anal Chem. 2020;92(12):8010-4. https://doi.org/10.1021/acs.analchem.0c01256
28. Khorsandi K, Kianmehr Z, Hosseinmardi Z, Hosseinzadeh R. Anti-cancer effect of gallic acid in presence of low level laser irradiation: Ros production and induction of apoptosis and ferroptosis. Cancer Cell Int. 2020;20:18. https://doi.org/10.1186/s12935-020-1100-y
29. Zorova LD, Popkov VA, Plotnikov EY, Silachev DN, Pevzner IB, Jankauskas SS, et al. Mitochondrial membrane potential. Anal Biochem. 2018;552:50-9. https://doi.org/10.1016/j.ab.2017.07.009.
30. Sadeesh EM, Lahamge MS, Malik A, Ampadi AN. Differential expression of nuclear-encoded mitochondrial protein genes of atp synthase across different tissues of female buffalo. Mol Biotechnol. 2025;67(2):705-22. https://doi.org/10.1007/s12033-024-01085-x
31. Bailly C, Waring MJ. Pharmacological effectors of grp78 chaperone in cancers. Biochem Pharmacol. 2019;163:269-78. https://doi.org/10.1016/j.bcp.2019.02.038
32. Li Y, Zhou Y, Wang M, Lin X, Zhang Y, Laurent I, et al. Ampelopsin inhibits breast cancer cell growth through mitochondrial apoptosis pathway. Biol Pharm Bull. 2021;44(11):1738-45. https://doi.org/10.1248/bpb.b21-00470
33. Tušar L, Usenik A, Turk B, Turk D. Mechanisms applied by protein inhibitors to inhibit cysteine proteases. Int J Mol Sci. 2021;22(3). https://doi.org/10.3390/ijms22030997
34. Özkan İ, Koçak P, Yıldırım M, Ünsal N, Yılmaz H, Telci D, et al. Garlic (allium sativum)-derived sevs inhibit cancer cell proliferation and induce caspase mediated apoptosis. Sci Rep. 2021;11(1):14773. https://doi.org/10.1038/s41598-021-93876-4.
35. Costa RLB, Han HS, Gradishar WJ. Targeting the pi3k/akt/mtor pathway in triple-negative breast cancer: A review. Breast Cancer Res Treat. 2018;169(3):397-406. https://doi.

org/10.1007/s10549-018-4697-y
36. Dey N, De P, Leyland-Jones B. Pi3k-akt-mtor inhibitors in breast cancers: From tumor cell signaling to clinical trials. Pharmacol Ther. 2017;175:91-106. https://doi.org/10.1016/j.pharmthera.2017.02.037
37. Zhang HP, Jiang RY, Zhu JY, Sun KN, Huang Y, Zhou HH, et al. Pi3k/akt/mtor signaling pathway: An important driver and therapeutic target in triple-negative breast cancer. Breast Cancer. 2024;31(4):539-51. https://doi.org/10.1007/s12282-024-01567-5
38. Haga Y, Higashisaka K, Yang L, Sekine N, Lin Y, Tsujino H, et al. Inhibition of akt/mtor pathway overcomes intrinsic resistance to dasatinib in triple-negative breast cancer. Biochem Biophys Res Commun. 2020;533(4):672-8. https://doi.org/10.1016/j.bbrc.2020.09.143.
39. Chen WM, Hu QY, Hou W, Chen MW, Chen YH, Tang JC. Nrf3 promotes the proliferation and migration of triple-negative breast cancer by activating pi3k/akt/mtor and epithelial-mesenchymal transition. Oncol Lett. 2023;26(4):443. https://doi.org/10.3892/ol.2023.14030
40. Tan HK, Moad AI, Tan ML. The mtor signalling pathway in cancer and the potential mtor inhibitory activities of natural phytochemicals. Asian Pac J Cancer Prev. 2014;15(16):6463-75. https://doi.org/10.7314/apjcp.2014.15.16.6463.
41. Hakeem AN, El-Kersh DM, Hammam O, Elhosseiny A, Zaki A, Kamel K, et al. Piperine enhances doxorubicin sensitivity in triple-negative breast cancer by targeting the pi3k/akt/mtor pathway and cancer stem cells. Sci Rep. 2024;14(1):18181. https://doi.org/10.1038/s41598-024-65508-0
42. Ganesan K, Xu C, Wu J, Du B, Liu Q, Sui Y, et al. Ononin inhibits triple-negative breast cancer lung metastasis by targeting the egfr-mediated pi3k/akt/mtor pathway. Sci China Life Sci. 2024;67(9):1849-66. https://doi.org/10.1007/s11427-023-2499-2
43. Ananda Sadagopan SK, Mohebali N, Looi CY, Hasanpourghadi M, Pandurangan AK, Arya A, et al. Forkhead box transcription factor (foxo3a) mediates the cytotoxic effect of vernodalin in vitro and inhibits the breast tumor growth in vivo. J Exp Clin Cancer Res. 2015;34:147. https://doi.org/10.1186/s13046-015-0266-y.
44. Dai X, Wang L, Deivasigamni A, Looi CY, Karthikeyan C, Trivedi P, et al. A novel benzimidazole derivative, mbic inhibits tumor growth and promotes apoptosis via activation of ros-dependent jnk signaling pathway in hepatocellular carcinoma. Oncotarget. 2017;8(8):12831-42. https://doi.org/10.18632/oncotarget.14606.