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Isatin, a monoamine oxidase inhibitor, sensitizes resistant breast cancer cells to tamoxifen *via* MAO-A/HIF1α/MMPs modulation

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One of the biggest obstacles to treating breast cancer effectively is chemotherapy resistance, which emphasizes the need for innovative therapeutic approaches. An important factor in tumor progression is the mitochondrial enzyme monoamine oxidase-A (MAO-A). In the development of anticancer drugs, isatin (1*H*-indole-2,3-dione), a MAO inhibitor obtained from *Isatis microcarpa*, has shown great promise. This study assessed isatin's ability to fight resistance in tamoxifen-resistant LCC2 breast cancer cells, both by itself and in combination with tamoxifen. Chromatographic techniques were used to extract and purify isatin, which was subsequently examined for cytotoxicity, cell cycle arrest, colony formation, and migratory inhibition. Isatin and tamoxifen together dramatically decreased cell viability, prevented migration, stopped the advancement of the cell cycle, and repressed proliferation. Using qRT-PCR, gene expression analysis showed that important indicators for treatment resistance and metastasis, including MAO-A, HIF-1α, TWIST, MMP2, MMP9, and ABCB1, were downregulated. ELISA-based protein expression analyses further validated the modification of proteins linked to migration and apoptosis, including BAX, BCL2, and caspases 3, 8, and 9. The ATP-binding cassette transporter ABCB1, which is intimately linked to multidrug resistance, was similarly impacted by the isatin–tamoxifen combination. In conclusion, our findings demonstrate that isatin, alone or in combination with tamoxifen, exerts significant anticancer effects in tamoxifen-resistant breast cancer cells by promoting apoptosis, cell cycle arrest, and suppression of resistance-associated pathways. These effects may involve modulation of MAO-A and HIF-1α signaling, highlighting MAO-A as a lesser-studied but promising target in breast cancer.

Introduction

Globally, breast cancer ranks high in the frequency of cancer diagnoses among women, accounting for approximately 15% of all cancer-related deaths.[1] Endocrine therapy, particularly the use of tamoxifen, has been a cornerstone in the treatment of hormone receptor-positive [HR+] breast cancer for over three decades.[2] Tamoxifen, a selective estrogen receptor modulator [SERM], exerts its effects by attaching to estrogen receptors [ERs] on cancer cells, thereby inhibiting estrogen-mediated signaling pathways and inducing apoptosis.[3]

However, despite the initial efficacy of tamoxifen, many patients eventually develop resistance to the treatment, leading to disease progression and recurrence.[4] The mechanisms underlying tamoxifen resistance are complex and multifactorial, involving alterations in ER expression and signaling, activation of growth factor signaling pathways, and epigenetic modifications.[5,6] Furthermore, the development of tamoxifen resistance is often associated with a more aggressive and metastatic phenotype, highlighting the need for novel therapeutic strategies to overcome resistance.[7]

Monoamine oxidase A [MAO-A] is a flavin-containing enzyme that mediates the degradation of monoamine neurotransmitters, including serotonin, dopamine, and norepinephrine.[7] Increasingly, evidence supports the link between the MAO-A isoenzyme and several types of cancer, notably prostate cancer,[8,9–12] hepatocellular carcinoma,[13,14] brain tumor [gliomas],[15] classical Hodgkin's lymphoma,[16] colorectal cancer,[17] lung cancer,[18–20] and breast cancer.[21] The MAO-A enzyme is implicated in causing DNA damage[22,23] and oxidative stress.[23] Furthermore, it might contribute to tumor formation *via* the production of reactive oxygen species [ROS] during its enzymatic activity.[24] Elevated MAO-A levels lead to increased generation of harmful substances like hydrogen peroxide and aldehydes,[25] which can trigger oxidative stress, resulting in mitochondrial damage, significant lipid membrane damage, and DNA damage,[22] potentially contributing to cancer development.

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Notably, oxidative stress has been associated with both depression and cancer.[24,26]

Elevated MAO-A levels increase reactive oxygen species (ROS), which inhibits prolyl hydroxylases (PHDs) and leads to the stabilization of HIF1α, even under non-hypoxic conditions. This stabilized HIF1α is a central point for promoting tumor aggression. It directly increases the expression of matrix metalloproteinases (MMPs), which help break down tissue and enable invasion, while also boosting the expression of TWIST1. TWIST1, in turn, promotes epithelial-to-mesenchymal transition (EMT), a process that makes cancer cells more mobile and invasive. This interconnected pathway—where MAO-A activates HIF1α, which then upregulates MMPs and TWIST1—drives the increased invasiveness and metastasis seen in prostate cancer cells.[27]

Monoamine oxidase inhibitors [MAOIs] have shown promising potential in treating resistant cancers through multiple mechanisms such as reducing the activation of androgen receptors; they can contribute to the anti-proliferative effects of antiandrogen treatment in both types of prostate cancer cells.[28] Kushal *et al.*,[29] reported that inhibiting MAO-A reduces the advancement of glioma that resists temozolomide (TMZ), which in turn extends how long individuals survive. The mechanism of the effects of MAO-A inhibitors involves decreased proliferation; reduced small blood vessels along with more macrophages had moved into the area. In an earlier study indicated that MAO-A inhibitors could be beneficial for treating breast cancer due its ability to act as antiproliferative, antimigratory, and synergistic anticancer agents in the treatment of breast cancer.[30]

Natural product-derived compounds represent a rich source of novel therapeutic agents. *Isatis microcarpa*, a plant traditionally utilized in medicinal practices, has been identified as a source of an oxidized indole derivative, isatin [1*H*-indole-2,3-dione], which is present in mammalian tissues and body fluids and is known for its wide range of pharmacological effects.[31] Isatin derivatives have demonstrated significant inhibitory effects on both monoamine oxidase isoforms, with some compounds showing IC_{50} values below 1 μM. For instance, competitive inhibition of MAO was observed with 4-chloroisatin [K_i = 0.311 μM] and 5-bromoisatin [K_i = 0.033 μM].[32]

This study endeavors to elucidate the therapeutic potential of isatin isolated from *Isatis microcarpa* as a MAO inhibitor, increasing the sensitivity of resistant MCF-7 breast cancer cells [LCC2] to tamoxifen focusing on cytotoxicity, cell cycle, apoptotic machinery, and the interplay between MAO-A, HIF-1α, TWIST, MMP2, MMP9, and MDR-1. This research may provide new insights into overcoming tamoxifen resistance, reducing chemo-toxicity through decreasing their doses and improving breast cancer treatment outcomes.

Methods

Plant materials

Isatis microcarpa's aerial parts were gathered from El Arish, North Sinai during 2024. After botanical identification at the Desert Research Centre Herbarium, the plant material underwent preparation, which included cleaning, shade-drying, and grinding into a fine powder.

Extraction and the purification of isatin from *Isatis microcarpa*

The leaves of *Isatis microcarpa* were dried and ground into a powder [600 g]. This powder underwent Soxhlet extraction with chloroform [$CHCl_3$], and the resulting extract was concentrated using a rotary vacuum evaporator. The residue was then dissolved in dichloromethane [CH_2Cl_2] and ran through a silica gel column to eliminate pigments. Following concentration, the solution was separated using silica gel column chromatography [5 × 50 cm, 60–120 mesh] with a gradient of methanol in dichloromethane as the eluent [flow rate: 2.5 mL min^{-1}]. Fractions [250 mL] were collected, concentrated, and analyzed by thin-layer chromatography [TLC] [20 × 20 cm with 0.2 mm thickness, silica gel GF254, Merck, Darmstadt, Germany] using a petroleum ether/ethyl acetate [3 : 2] mobile phase. An orange-red pure compound was obtained, recrystallized from ethyl acetate [melting point 200 °C, yield 5.75 g, Rf 0.4]. A UV detector set to a wavelength of 295 nm was used to detect the samples. The pure compound was further analyzed using ^1H and ^{13}C NMR for structural identification.[33,34]

Cell lines

The tamoxifen-resistant MCF-7/LCC2 cell line was kindly provided by Dr. Robert Clarke (Office of Technology Commercialization, Georgetown University, Washington, DC, USA) under a material transfer agreement between Georgetown University and Cairo University. It was cultivated using 1% penicillin–streptomycin and 5% charcoal FBS in Eagle's minimum essential medium [EMEM]. The metastatic breast cancer cells MCF-7 were received from the Egyptian Company for Production of Vaccines, Sera, and Drugs [VACSERA] and growth maintenance was done in DMEM media supported with 10% FBS and 1% penicillin–streptomycin. All cell lines were maintained under a humidified atmosphere with 5% CO_2 at 37 °C.

Chemicals and reagents

We purchased tamoxifen [TAM] from Pharmaceuticals Company of Amriya, Alexandria, Egypt. Just prior to use, TAM and isatin were diluted in a series in medium. All tissue culture components, solvents, and reagents were supplied exclusively by Sigma Aldrich [St. Louis, MO, USA]. We sourced our fetal bovine serum, PBS, penicillin/streptomycin solution, DMEM, and trypsin–EDTA from ThermoFisher [Waltham, Massachusetts, United States].

In vitro evaluation of cytotoxicity

The sulforhodamine-B [SRB] based cell proliferation assay was used to evaluate the antineoplastic properties of isatin and tamoxifen [TAM] in LCC2 and MCF-7 cells.[35] The cells

were seeded at a density of 3×10^3 cells per well in 96-well microtiter plates. Before being drug-incubated, they were given a full day to connect. Following that, the cells were exposed to varying amounts of TAM [from 0–100 μM] and isatin [from 0–200 μM]. Fixation and staining of the cells were done using 20% trichloroacetic acid and 0.4% SRB dye, respectively, following a 48 hour incubation period. The ELISA microplate reader [TECAN Sunrise™, Germany] was used to spectrophotometrically quantify each well's optical density [O.D.] at 570 nm. Cell survival as a percentage was computed, and the IC_{50} [half-maximal inhibitory concentration] value of each drug was determined using sigmoidal dose–response curve-fitting models [Graph Pad Prism software, version 7.03].

Cell cycle analysis

1/2 IC_{50} of TAM, IC_{50} of isatin, and a combination of both were applied to LCC2 cells. Control cells were cells that had not received any treatment. The cells were fixed in 70% ethanol after 48 hours and incubated for 30 minutes at 4 °C. They were then rinsed twice with PBS, treated with RNase I, and allowed to rest in the dark for an hour at room temperature. A FACSCalibur flow cytometer [Becton Dickinson, Sunnyvale, CA, USA] was used to analyze the DNA content. Using computer software for the Dean and Jett mathematical analysis, the DNA histogram produced by flow cytometry was acquired.[36]

Colony formation efficiency

A clonogenic assay was used to show that isatin and TAM can prevent the proliferation of LCC2 cells. In a 6-well plate, 1×10^3 LCC2 cells were plated and exposed to 1/2 IC_{50} TAM, IC_{50} isatin, and a mixture of the two for 48 hours. After that, new media were added, and the cells were left to develop and multiply for ten days at 37 °C. The colonies were then stained with 0.5% crystal violet and preserved with methanol. An inverted microscope (Olympus IX70) was used to photograph and count the 20-cell colonies.[37]

Wound healing assay

LCC2 cells were cultured to create confluent monolayers in 6-well plates. A 200 μL pipette tip was quickly wrapped vertically through the cell monolayer after being firmly pressed against the top of the tissue culture plate. The cells were then treated with 1/2 IC_{50} of TAM, IC_{50} of isatin, and a mixture of both for 48 hours. To evaluate the healing effect, images of cell migration into the scratched area taken at 0 and 48 hours after treatment were analyzed using ImageJ software.[38]

RNA isolation and gene expression levels of MAO-A, TWIST, ABCB1, HIF1α, MMP9 and MMP2 by RT-qPCR

LCC2 cells grown in T25 flasks were treated with 1/2 IC_{50} of tamoxifen, IC_{50} of isatin and combination of both for 48 h. The cellular total RNA was then isolated from LCC2 cells using an miRNeasy mini kit [Qiagen, Hilden, Germany] following the instructions. ThermoFisher Scientific's NanoDrop-2000 spectrophotometer was then used to determine the concentration and purity of the resulting RNA. A Quantitect RNA reverse transcription kit [Qiagen, Hilden, Germany] was used to reverse-transcribe whole RNA. A QuantiNova SYBR Green PCR Kit [Qiagen, Hilden, Germany] was used to perform PCR for mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase [GAPDH] was used as a housekeeping gene to normalize the data of the PCR. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative RNA expression levels.[39] The sequences of primers used are shown in Table 1.

Evaluation of protein expression by enzyme-linked immunosorbent assay [ELISA]

ELISA kits were used to spectrophotometrically measure the protein levels of caspase 3, caspase 8, caspase 9, Bax, BCL2, MMP2, and MMP9 in a cell lysate at 450 nm (Cloud-Clone Corp, USA, North America SEA626Hu, MYBIOSOURCE MBS452285, Abcam ab119508, Elabscience E-EL-H0562, Elabscience E-EL-H0114, Elabscience E-EL-H1445, and Elabscience E-EL-H6075, respectively, following the instructions' guidelines). Cells grown

Table 1 Sequences of primers used in real time PCR

Gene	Sequence of the primer	Accession number	Annealing temp. [°C]
MMP2	Sense: 5′-AGACAGTGGATGATGCCTTTGC-3′ Antisense: 5′-GGAGTCCGTCCTTACCGTCAAA-3′	NM_001302510.2	55
MMP9	Sense: 5′-TTCCAAACCTTTGAGGGCGA-3′ Antisense: 5′-CAAAGGCGTCGTCAATCACC-3′	NM_004994.3	55
Twist	Sense: 5′-GCCAGGTACATCGACTTCCTCT-3′ Antisense: 5′-TCCATCCTCCAGACCGAGAAGG-3′	NM_000474.4	60
ABCB1	Sense: 5′-GCT GTC AAG GAA GCC AAT GCC T −3’ Antisense: 5′-TGC AAT GGC GAT CCT CTG CTT C-3′	NM_001348945	60
HIF1α	Sense: 5′-TATGAGCCAGAAGAACTTTTAGGC-3′ Antisense: 5′-CACCTCTTTTGGCAAGCATCCTG-3′	NM_001530.4	60
MAO-A	Sense: 5′-CTGATCGACTTGCTAAGCTAC-3′ Antisense: 5′-ATGCACTGGATGTAAAGCTTC-3′	NM_001270458.2	60
GAPDH	Sense: 5′-ACCCACTCCTCCACCTTTGA-3′ Antisense: 5′-CTGTTGCTGTAGCCAAATTCGT-3′	NM_001357943.2	55

in 75 cm^3 flasks were cultured to reach 70% to 80% confluence; after that, they were treated for 48 hours with 1/2 IC_{50} of TAM, IC_{50} of isatin, and a combination of both. A protease inhibitor-containing RIPA lysis buffer was then used to lyse the treated and control cells. The experiment was conducted three independent times, with two repetitions for each concentration. In relation to the matching protein content, the activity was computed.

Statistical analysis

All data were presented as mean ± SD. Differences between the treated samples and untreated controls were examined by one-way analysis of variance [ANOVA] followed by the Tukey–Kramer multiple comparison tests. Statistical analyses were conducted using GraphPad Stat software, version 7.03 [GraphPad, San Diego, CA, USA]. A p value at 0.05 or less was determined to be statistically different.

Results

^{1}H-NMR and ^{13}C NMR spectral analysis of isatin

As shown in Fig. 1, isatin shows two doublets: one at δ 7.47 ppm, attributed to the H2 proton, and another at δ 6.86 ppm, corresponding to the H5 proton, in its ^{1}H-NMR [Bruker FT-NMR, 400 MHz, DMSO-d_6] spectrum. A singlet appears at approximately δ 11.03 ppm, attributed to the NH proton [H1]. At δ 7.05 ppm and 7.57 ppm, respectively, the protons H3 and H4 indicate triplets. In the ^{1}H-NMR spectrum, deprotonation of NH in the isatin moiety causes a downfield shift for the azanion's protons [H2, H3, H4, and H5]. The detailed ^{13}C NMR [Bruker FT-NMR, 400 MHz] chemical shifts for isatin in DMSO-d_6 are shown in Fig. 2. The two carbonyl carbons, C[2] and C[3], resonate at δ 159.82 ppm and δ 184.85 ppm, respectively. These significantly downfield shifts are characteristic of carbonyl functional groups, where the electronegativity of the oxygen atom leads to substantial deshielding of the carbon nucleus. The six aromatic carbons of the indole ring appear in the typical aromatic region: C[3a] at δ 118.24 ppm, C[4] at δ 125.13 ppm, C[5] at δ 123.23 ppm, C[6] at δ 138.84 ppm, C[7] at δ 112.67 ppm, and C[7a] at δ 151.16 ppm. The variations in the chemical shifts of these aromatic carbons are due to the influence of the carbonyl groups and the nitrogen atom within the ring, which affect the overall electron density distribution. The result is in agreement with the literature.[40,41] The results of ^{1}H- and ^{13}C-NMR elucidation led to the conclusion that isatin was successfully isolated with high purity from the leaves of *Isatis microcarpa* plant.

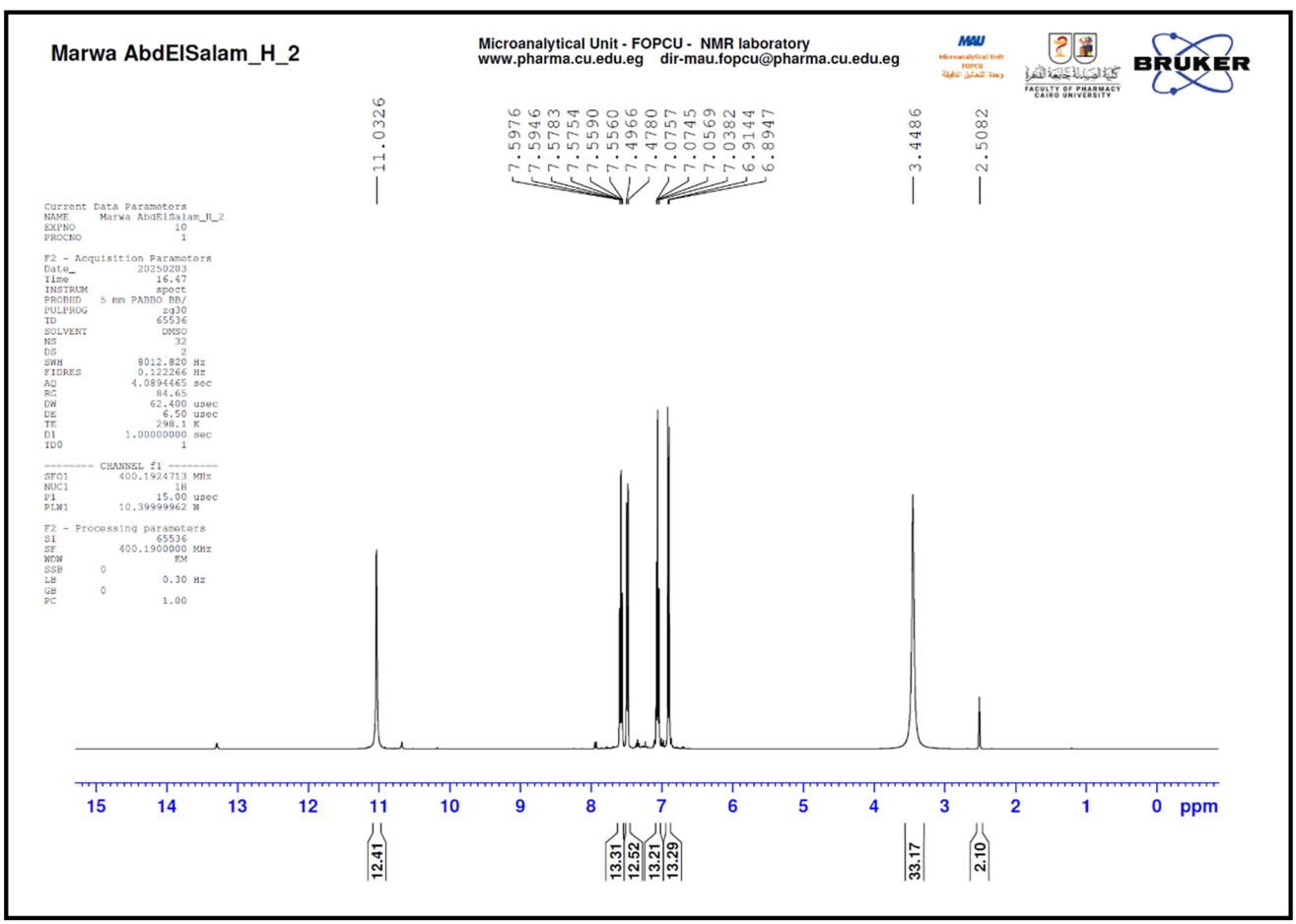

Fig. 1 ^{1}H NMR spectrum of the isatin compound.

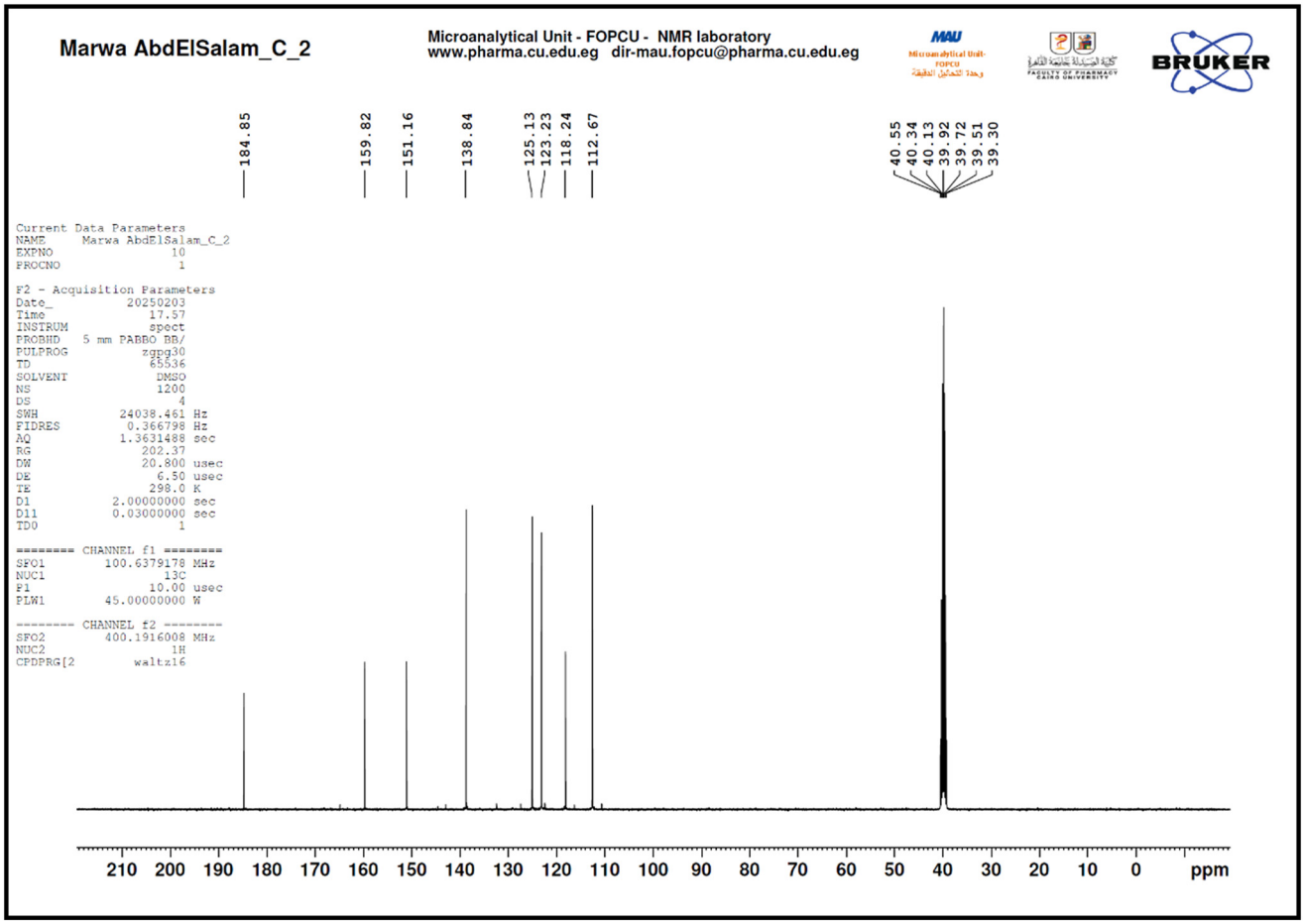

Fig. 2 ^{13}C NMR spectrum of the isatin compound.

Impact of isatin and TAM on LCC2 and MCF-7 cell line viability

To evaluate the impact of TAM and isatin on the viability of resistant LCC2 and MCF-7 cell lines, the cells were exposed to escalating doses of isatin [0–200 μM] and TAM [0–100 μM] and incubated for 48 hours. Then, the SRB test was used to examine cell proliferation. The findings showed that TAM and isatin decreased the MCF-7 and LCC2 cell viability in a dose-dependent drug reaction. According to our results, a TAM concentration of 68 μM and isatin concentrations of 40 μM and 81 μM were required to inhibit 50% of the viability of LCC2 and MCF-7 cells, respectively, as shown in Fig. 3 and 4, respectively.

Effect of single and combined treatments of isatin and TAM on LCC2 cell growth using colony formation assay

Using the colony formation assay, it was determined that isatin and TAM inhibited the growth of LCC2 cells. Fig. 5 illustrates how, in comparison to untreated cells, the colony counts of LCC2 cells treated with isatin and TAM or both were significantly reduced. Additionally, as compared to the cells treated with isatin and TAM alone, the combination treatment group showed the fewest colony numbers [p = 0.0033].

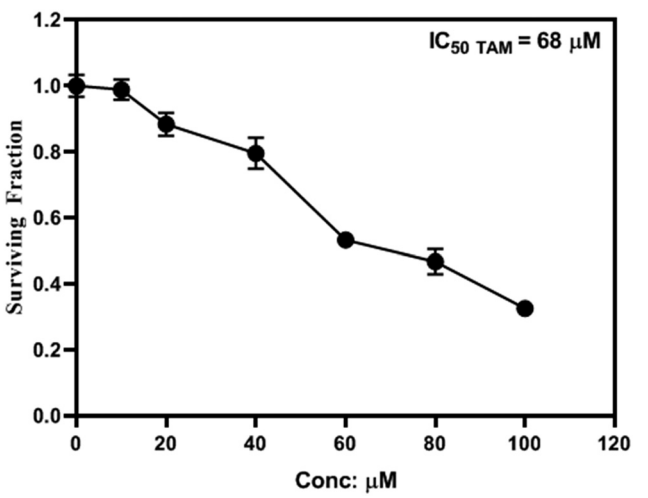

Fig. 3 Impact of tamoxifen on LCC2 viability. Cells were exposed to multiple dosage of tamoxifen for 48 h. SRB assay was used to assess cell viability. Data were expressed from three experiments and presented as mean ± SD.

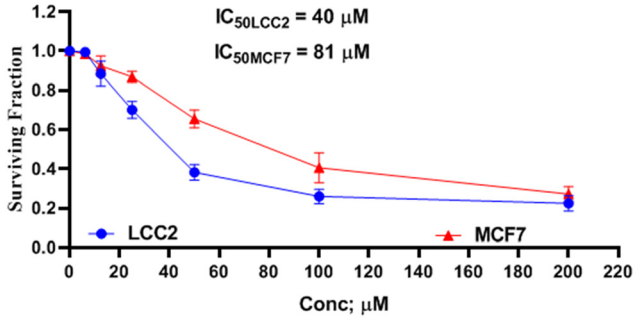

Fig. 4 Impact of isatin on LCC2 and MCF-7 cell viability. Cells were exposed to multiple dosage of isatin for 48 h. SRB assay was used to assess cell viability. Data were expressed from three experiments and presented as mean ± SD.

Impact of single and combined treatments of isatin and TAM on the LCC2 cell cycle

As demonstrated in Fig. 6A and B, a statistically significant increase [p value >0.0001] in G0–G1 cell population [93.59%] for the cells treated with combination of isatin and TAM compared to 76.73% for the untreated cells suggests that combined treatment causes cell cycle arrest at this phase and fewer cells are progressing to the S phase and G2-M phase, next stages of the cell cycle, potentially halting proliferation. Moreover, a reduction [p value >0.0001] in cell population in the S phase [5.5%] for isatin and TAM treated cells compared to 23.14% for controls was observed, which indicate that a lower percentage of cells in the S phase is undergoing DNA replication, suggesting that cell cycle progression is being inhibited. This supports the idea that isatin and TAM are blocking proliferation by preventing cells from entering or completing DNA synthesis.

In vitro effect of treatment with TAM, isatin, and combination of both on the inhibition of LCC2 cell migration

In vitro wound healing assay was used to assess the efficacy of treatment with TAM, isatin, and combination of both on inhibiting cellular migration and invasion as demonstrated in Fig. 7A. After scratching a monolayer of cells, the untreated cells at 48 h efficiently increase wound healing by decreasing the gap size of the wound, supporting the migration of cells. On the other hand, the LCC2 cells treated with the combination of TAM and isatin showed a significant reduction in gap closure compared with the control group at 48 h. The wound gap size showed a statistically significant increase in the combination group by 86.6% at $p < 0.0001$ compared with the control at 48 h, supporting their impact on inhibiting the migration of cells, as shown in Fig. 7B.

TAM and isatin combination reduces the expression levels of MAO-A, TWIST, ABCB1, HIF1α, MMP9 and MMP2 genes in LCC2 cells

The expression levels of MAO-A, TWIST, ABCB1, HIF1α, MMP9 and MMP2 were determined using qPCR to evaluate the potential impact of TAM and isatin combination therapy

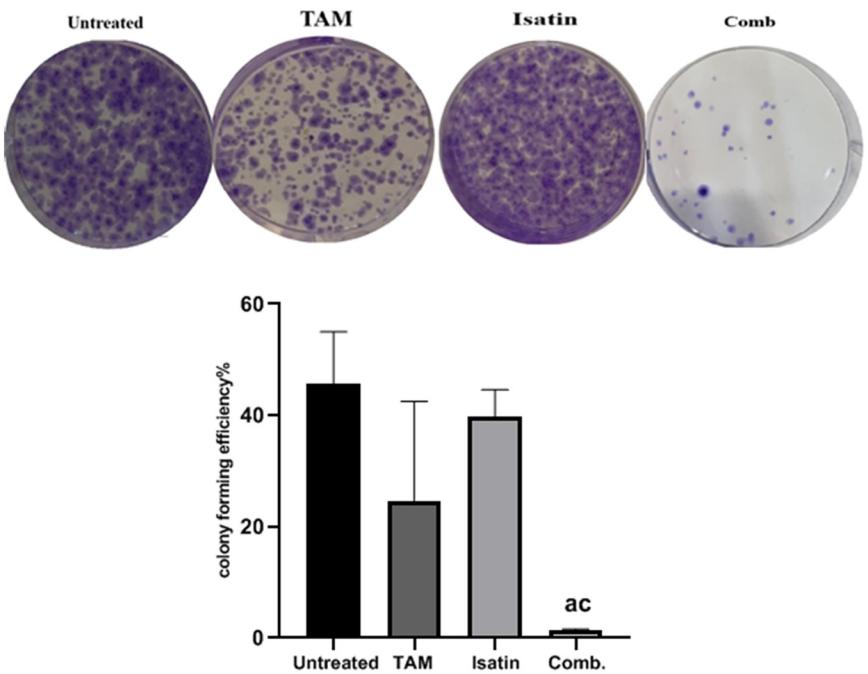

Fig. 5 Assessment of colony formation effectiveness in TAM-treated LCC2 cancer cells with and without isatin, as well as their combination. The mean ± SD [n = 3] is represented by each value. Data were statistically examined using the ANOVA test, "c": statistically significant different from LCC2 treated with isatin; "a": statistically significant different from untreated cells.

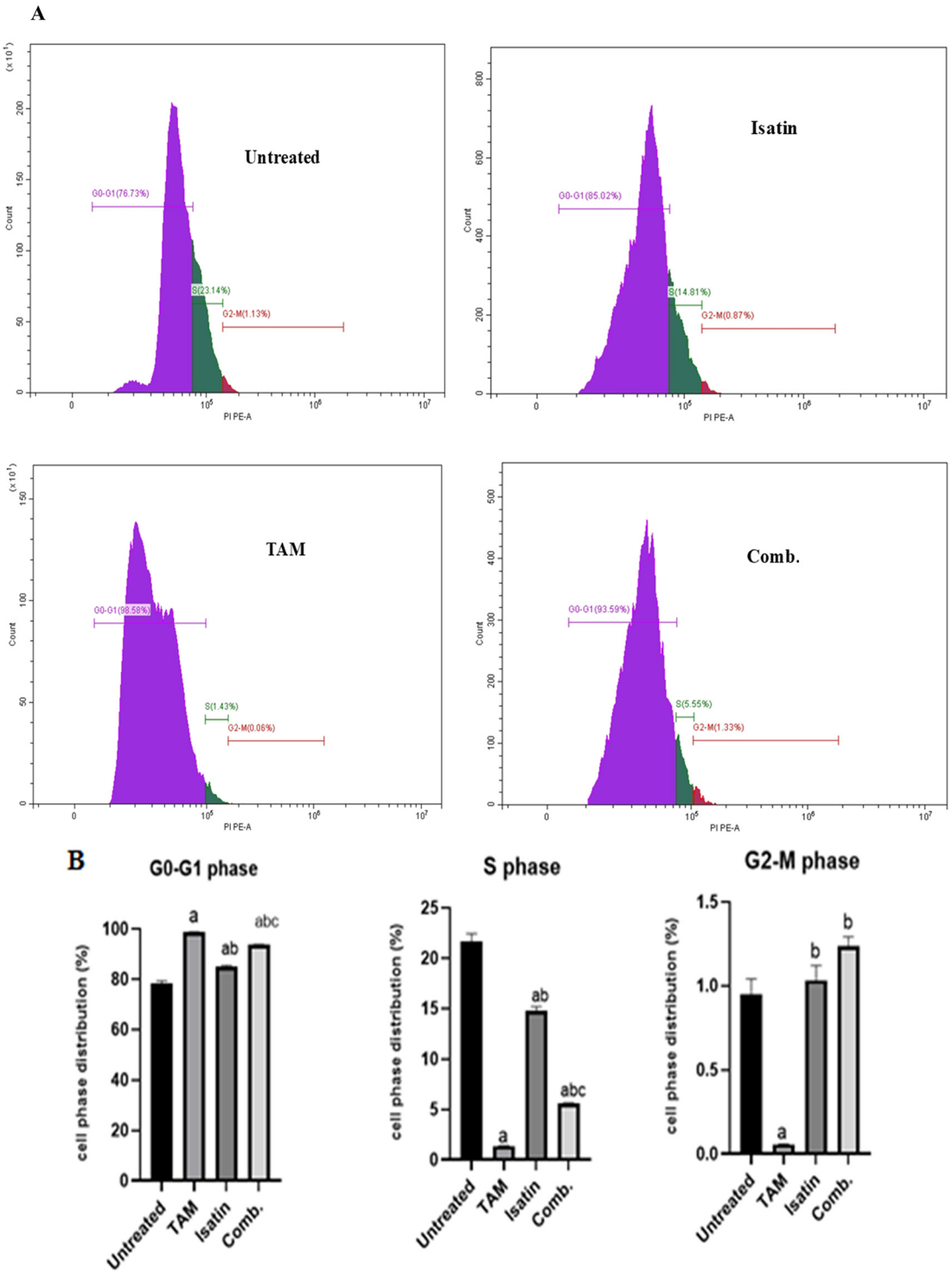

Fig. 6 A. Distribution of cell population in different cell cycle phases after cell treatment with TAM, isatin or combination of both. B. Histograms of cell population percentage accumulated in different cell cycle phases after treatment of cells with TAM, isatin or combination of both. Results are shown as mean ± SD. The one-way analysis of variance [ANOVA] test was used to analyze the data; "a": statistically significant different from untreated; "b": statistically significant different from LCC2 treated with TAM; "c": statistically significant different from LCC2 treated with isatin.

on inhibiting the progression of breast cancer cells that are resistant to tamoxifen treatment. Our results showed that compared to the untreated cells, MAO-A, TWIST and ABCB1 showed minimal expression in the treated cells with combined TAM and isatin with p values = 0.009, 0.09 and 0.049, respectively (Fig. 8). In addition, combination of isatin and tamoxifen showed an antimigratory effect on LCC2 resistant cells and this is elucidated by measuring the

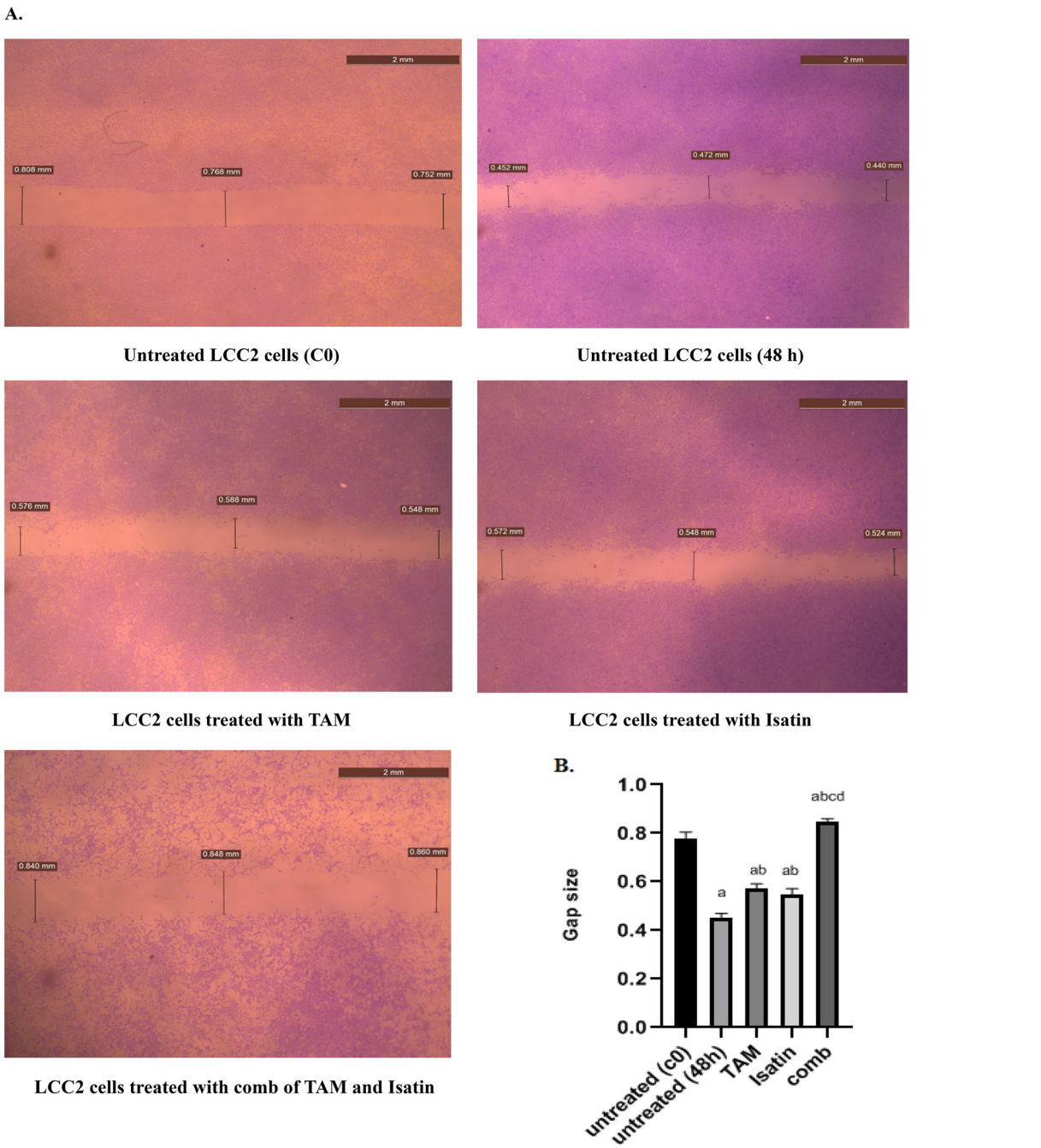

Fig. 7 Effect of treatment with TAM, isatin and combination of both on cell migration in LCC2 cells. Photos of wound healing assay showing the migration of LCC2 cells following treatment with TAM, isatin and combination of both [A]. Scratching was done using the tip of a 10 μL pipette. The graph illustrated the differences in the wound size of LCC2 cells after treatment with TAM, isatin and combination of both [B]. The results are presented as mean ± SD [n = 3]. The one-way ANOVA test was used to analyze the statistical differences between the groups; "a": statistically significant different from untreated at zero time; "b": statistically significant different from untreated after 48 hours; "c": statistically significant different from LCC2 treated with TAM; "d": statistically significant different from LCC2 treated with isatin.

expression of MMP9 and MMP2 genes using qPCR. MMP9 and MMP2 showed minimal expression in the treated cells with combined TAM and isatin compared with the untreated cells with p values of <0.0001 and <0.0001, respectively. Additionally, HIF1α showed a reduction in the combination group compared with the untreated group with a p value less than 0.0001 (Fig. 8).

Apoptotic and antimigratory effect of tamoxifen, isatin and their combination on LCC2 resistant cells

Some apoptotic and antimigratory marker proteins were evaluated using the ELISA technique to show the enhanced apoptotic and antimigratory effect of the combination of tamoxifen and isatin on LCC2 resistant cells. All proapoptotic

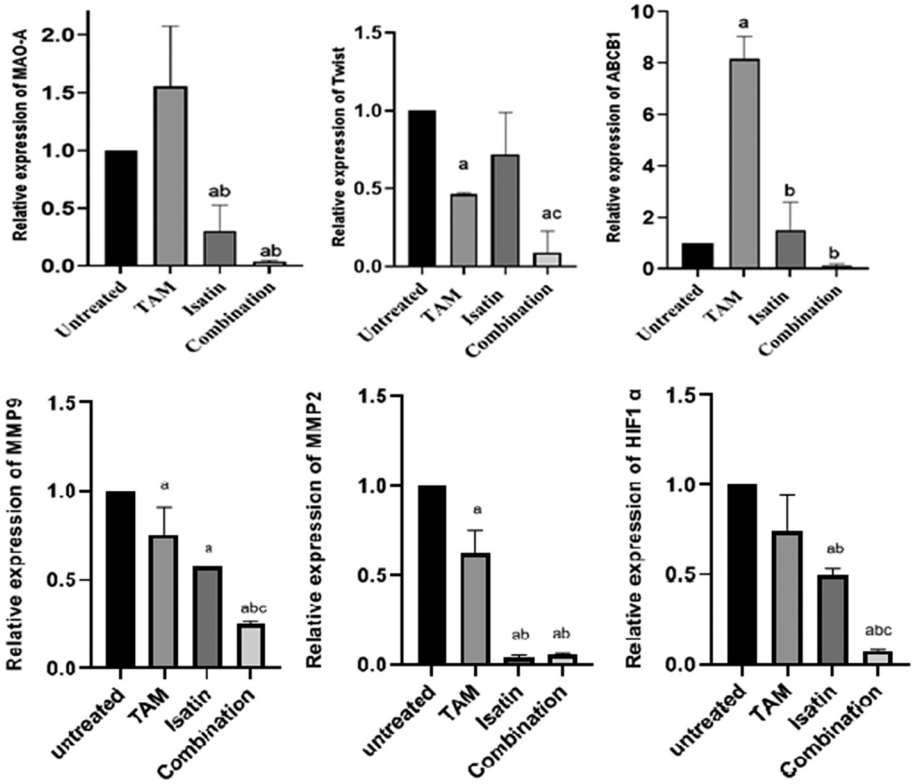

Fig. 8 The relative expression of MAO-A, TWIST, ABCB1, MMP9, MMP2 and HIF1α in treated LCC2 cells with TAM, isatin or a combination of both. Data are presented as mean ± SD. Data were statistically examined by ANOVA; "a": statistically significant different from untreated; "b": statistically significant different from LCC2 treated with TAM; "c": statistically significant different from LCC2 treated with isatin.

proteins [BAX, caspase 3, caspase 8 and caspase 9] showed high expression in the combination group compared with the untreated group with p value < 0.0001, p value < 0.0001, p value < 0.0001, and p value < 0.0001, respectively. Moreover, the antiapoptotic marker BCL2 was measured showing minimal reduction in the combination group compared with the untreated group with p value < 0.0001 (Fig. 9A). Results revealed that the combination of isatin and tamoxifen has a great apoptotic efficacy on LCC2 resistant cells, enhancing tamoxifen's therapeutic efficacy by sensitizing LCC2 cells to treatment. Furthermore, our study investigated the antimigratory effect of the combination of tamoxifen and isatin by measuring the protein level of both MMP2 and MMP9. Both proteins showed a significant reduction in the combination group compared with the untreated group with p value $<$ 0.0001 (Fig. 9B). Findings of the present study investigated the significant role of isatin to enhance the efficacy of tamoxifen on LCC2 resistant cells and increase the sensitivity of cells to therapy. Moreover, the combination of isatin with tamoxifen reduces the ability of cells to migrate and metastasize by reducing EMT markers such as MMP2 and MMP9.

Discussion

Breast cancer treatment encounters many hurdles including chemotherapy side effects, resistance to treatment and radiotherapy and chemotherapy expenses. Therefore, exploring new therapeutic approaches is an urgent necessity for breast cancer treatment. A mitochondrial flavin-containing enzyme, monoamine oxidase [MAO], plays a vital role in the oxidation of monoamine neurotransmitters. These enzymes are categorized into two isoforms: monoamine oxidase A [MAO-A] and monoamine oxidase B [MAO-B] that are involved in biogenic and dietary amine degradation.[42] MAO-A plays an important role in carcinogenesis, cardiovascular diseases, diabetes, and obesity and has a significant role in brain function.[27,43–45] Isatin [1*H*-indole-2,3-dione] represents an important therapeutic agent in drug discovery.[46] In humans, MAO-A was reported to be distributed in large quantities in peripheral tissues, body fluids and the central nervous system [CNS].[47] Naturally, Caribbean tumorigenic plant, *Melochia tomentosa*, was found to be the source of the substituted isatin: methoxy phenyl isatins; furthermore, 6-[3-methylbuten-2′-yl] isatin was found as a metabolite in *Streptomyces albus* fungi.[46] Isatin, a familiar monoamine oxidase [MAO] enzyme, is recognized as a reversible inhibitor.[48] MAO-A was reported to have an IC_{50} of around 12.3 μM, while MAO-B has an IC_{50} value of 4.86 μM.[48] In the current study, isatin was extracted and purified from the leaves of *Isatis microcarpa* that was collected from El Arish, North Sinai during 2024. Combination of isatin with other anticancer chemotherapeutic agents may exhibit a great benefit for breast cancer treatment through studying its impact to overcome drug resistance, increasing drug effectiveness, enhancing tolerability profile and reducing its adverse reactions.

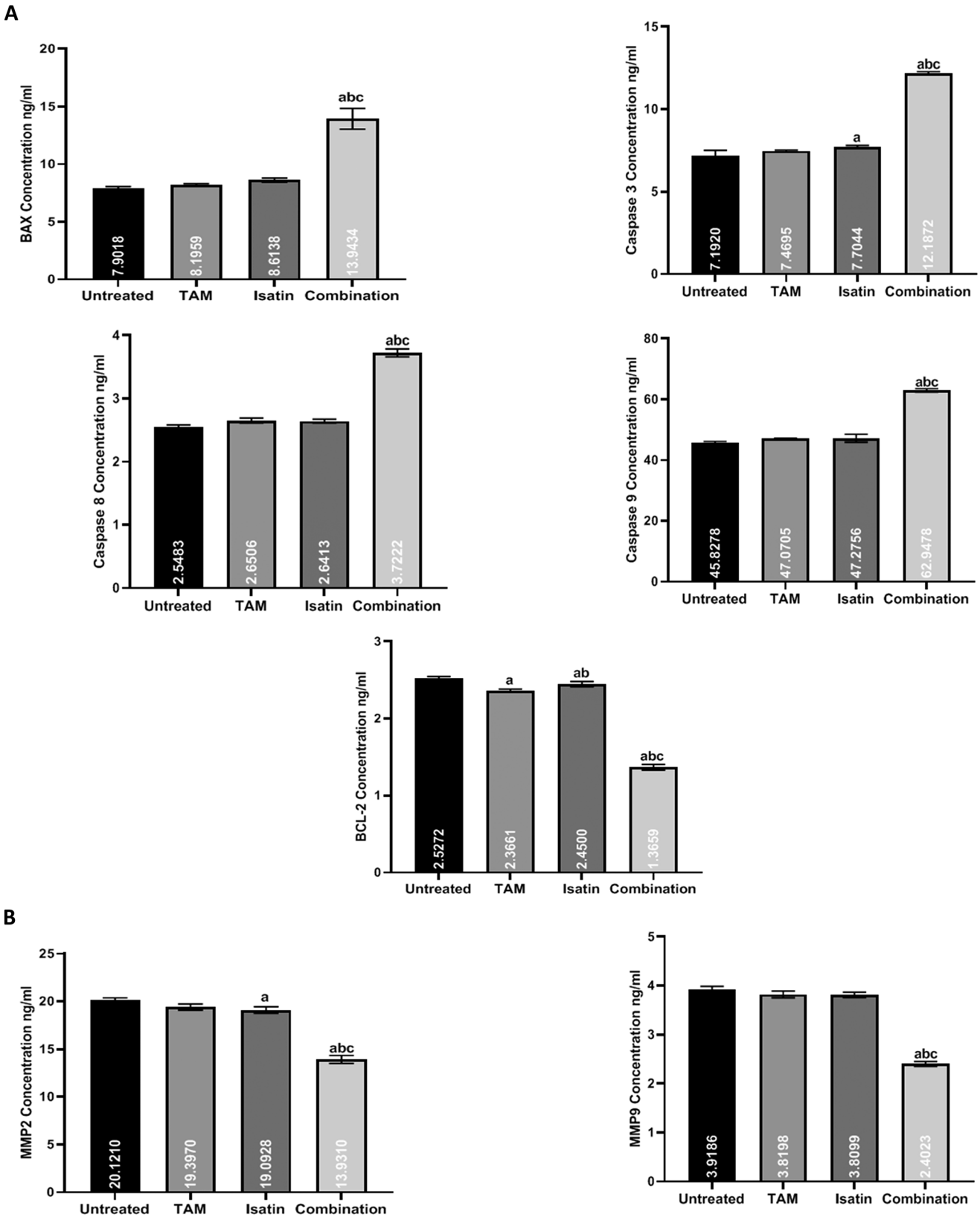

Fig. 9 The proapoptotic, antiapoptotic [A] and antimigratory [B] effect of TAM, isatin and combination of both on LCC2 resistant cells. Data expressed as mean ± SD. The one-way analysis of variance [ANOVA] test was used to analyze data; "a": statistically significant different from untreated; "b": statistically significant different from LCC2 treated with TAM; "c": statistically significant different from LCC2 treated with isatin.

Accordingly, combination of isatin and tamoxifen are adaptable and intriguing scaffolds for the development of new chemotherapeutic regimens for both drug-resistant and drug-sensitive breast cancers.

The current research aims to assess the potential efficacy of the MAO-A inhibitor [isatin] against tamoxifen resistant breast cancer MCF7 cells [LCC2] focusing on cytotoxicity, cell cycle, apoptotic machinery, and the interplay between MAO-A, HIF-1α, TWIST, MMP2, MMP9, and MDR. Our study showed enhanced down regulation of MAO-A, following treatment of resistant breast cancer cells to isatin (p value = 0.009). The reduction in MAO-A expression suggests that the combination of TAM and isatin may alter cellular metabolism and reduce pro-tumorigenic effects associated with MAO-A in LCC2 cells.

The MAO-A enzyme is recognized to be involved in oxidative deamination reactions causing the release of reactive oxygen species [ROS] that may eventually cause nucleic acid damage [DNA damage][44,49] and oxidative cell injury[49] leading to tumorigenesis.[50]

Over the last era, many studies connected the production of ROS caused by MAO-A with the development and progression of tumor. Patients with advanced prostate cancer [PCa] displayed increased expression of MAO-A.[51,52] In addition, proliferation of several types of cancer was explained due to oxidative stress and the resulting DNA damage caused by ROS.[53,54] Moreover, signaling pathways that involve MAO-A activity were linked to migration of cancer cells from their primary location to another tissue.[55]

Isatin showed several mechanisms for their antitumor activity, including apoptosis induction, angiogenesis inhibition, arrest of cell cycle, signaling pathway modulation, topoisomerase inhibition, and ROS production. Thus, isatin and its derivatives represent promising agents for cancer treatment. They showed many antineoplastic effects such as cell cycle arrest and cell proliferation inhibition.[56] In hypoxic environments, hypoxia-inducible factor alpha [HIF-alpha] promotes tumor proliferation and resistance by driving angiogenesis, invasion, metabolic reprogramming, and cell survival.[57] HIF-alpha increases resistance to apoptosis through induction of angiogenic factors, such as vascular endothelial growth factor [VEGF].[58] Previous study reported that down regulation of multidrug resistance gene MDR1/P-gp in colon cancer cells was linked to inhibition of HIF-1α.[59] The current study elucidated that the combination of isatin and tamoxifen can sensitize resistant breast cancer cells to tamoxifen *via* down regulation of the ABCB1 [MDR1] gene (p value = 0.049). Multidrug resistance was reported to be due to the upregulation of the transmembrane P-glycoprotein [P-gp] that is encoded by the adenosine triphosphate [ATP] binding cassette [ABC] transporter family.[60] Therefore, co-administration of antineoplastic drugs along with the inhibitor of P-gp is thus a novel approach for fighting MDR, as it significantly enhances drug efficacy.[61–63] In addition, our results revealed that the combined treatment causes cell cycle arrest in G0–G1 cell population [p value >0.0001] and fewer cells are progressing to the next stages of the cell cycle [S phase and G2-M phase], potentially halting proliferation.

Moreover, our study showed expression reduction in HIF1α and TWIST levels (p value less than 0.0001 and p value equal to 0.09, respectively). The transcription factor, TWIST, has a crucial role in tumor invasion, epithelial mesenchymal transition [EMT] and resistance to treatment. TWIST expression reduction suggests that the combination of tamoxifen and isatin may inhibit EMT, thereby reducing the potential for cancer cell migration and invasion. Furthermore, the combined effect of isatin and tamoxifen showed an antimigratory effect through minimal expression of genes and proteins of MMP9 and MMP2 compared with untreated cells with p value < 0.0001. Overall, the combination of TAM and isatin appears to have an anti-tumor effect in LCC2. Reducing the metabolic and pro-tumorigenic factors [MAO-A] suppresses metastasis related pathways [Twist/MMPs], decreasing HIF1α and migratory markers *e.g.*: MMP2 and MMP9 and decreasing drug resistance mechanisms [ABCB1/MDR1]. This suggests that isatin may enhance tamoxifen's therapeutic efficacy by sensitizing LCC2 cells to treatment and reducing metastasis potential.

Conclusion and future directions

In the present study, a tamoxifen resistant breast cancer cell [LCC2] *in vitro* model provided conclusive proof that the combination of isatin [MAO-A inhibitor] and tamoxifen played a crucial role in reducing the survival of resistant breast cancer cells and overcoming the development of resistance to tamoxifen. The main significant results of the current study can be outlined as follows: [1] the tamoxifen resistance in MCF-7 cells [LCC2] can be reduced through down regulation of the MDR1 gene that may be due to the inhibition of MAO-A caused by isatin which in turn caused the inhibition of HIF1α; [2] the isatin mechanism of action *via* down regulation of endogenous MAO-A/HIF1α/TWIST signaling can sensitize resistant MCF-7 cells toward tamoxifen and decrease epithelial mesenchymal transition [EMT]; [3] isatin enhances tamoxifen's therapeutic efficacy and stimulates apoptosis at non-cytotoxic concentrations *via* up regulation of proapoptotic proteins: BAX, caspase 3, caspase 8 and caspase 9 and down regulation of antiapoptotic protein BCL2; [4] combination of isatin and tamoxifen promotes cell cycle arrest in the G0–G1 phase inhibiting the survival of resistant tumor cells and sensitizing them to tamoxifen *via* preventing cells from entering or completing DNA synthesis; [5] EMT inhibition through down regulation of MMP expression [MMP2 and MMP9]. Building upon these promising findings, future research should prioritize several key areas. To further understand the precise binding interaction between isatin and MAO-A, as well as the relationships between MAO-A inhibition and the other genes under study, comprehensive mechanistic research is an essential next step. Furthermore, in order to confirm the effectiveness, safety, potential toxicity, and pharmacokinetics of the isatin–tamoxifen combination as adjuvant therapy,

in vivo studies are advised utilizing relevant animal models, such as patient-derived xenografts or syngeneic models of tamoxifen-resistant breast cancer.

Author contributions

All authors have an equal contribution. The published version of the manuscript has been read and revised by all authors.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

All data are available within the article.

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