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RESEARCH ARTICLE

Chemotherapeutic activity of silymarin combined with niclosamide in *MCF-7* human breast cancer cell line

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ABSTRACT

Background: Breast cancer is the most commonly diagnosed cancer in women. The recurrent tumor is a critical problem, responsible for patient mortality. There is a vital need to study new drug combinations that may have anticancer effects on the signaling pathways that drive tumor dormancy or recurrence. **Aims:** This study examined the anti-tumor effects of the silymarin and niclosamide combination on the MCF-7 cell line. It also explored their impact on breast cancer stem cells (BCSCs). **Materials and Methods:** The MCF-7 cell line was treated with various concentrations of silymarin and niclosamide alone and in combination. The cells were incubated for 24 hr. and 48 hr. The results were compared to control and cisplatin-treated cells. The cytotoxicity was evaluated using the MTT assay. The study also measured the telomerase and ALDH1 expression by quantitative RT-PCR. Apoptosis evaluation was performed using flow cytometry. The CD44 and CD133 expressions on BCSCs were examined with the cellular morphological changes using the confocal laser scanning microscope. **Results**: Silymarin showed a significant anticancer effect when compared to the control. Still, it was not as effective as cisplatin. Niclosamide, in comparison to the control and cisplatin, showed a significant anticancer effect. The most potent combination was silymarin and niclosamide 1:2 ratio. It showed a significant anticancer effect compared to cisplatin, silymarin, and niclosamide. **Conclusion**: The study provides promising evidence for the silymarin and niclosamide combination as an adjuvant breast cancer therapy. The study supports that the combination treatment is a revolutionary strategy that targets CSCs by combining phytochemicals and repurposed pharmaceuticals, with anticipated therapeutic applications.

Keywords: ALDH-1; MCF-7; Niclosamide; Silymarin; Telomerase

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INTRODUCTION

One of the most pressing challenges in cancer therapy is tumor recurrence. Although many tumors regress in response to adjuvant chemotherapy, residual tumor cells are detected in most cancer patients' post-treatment. These residual tumor cells are believed to remain dormant for years before resuming growth, leading to tumor recurrence (Fahn et al., 1994). Considering that recurrent tumors are most often responsible for patient mortality, it is crucial to study new drug combinations that may target the signaling pathways that drive tumor dormancy or recurrence (Chang, 2016).

The traditional drug development process is both expensive and time-consuming. It takes about 1 billion dollars and 10 years to bring the drug to the market. Drug repurposing is a growing area. It looks for new applications for already existing drugs. Drug repurposing has an advantage over de novo drug discovery. This is because many drugs already have known pharmacokinetics, pharmacodynamics, and toxicity profiles. This knowledge hastens the assessment of the medication in the clinical trials (Satoh et al., 2016). Cancer stem cells (CSCs) are quiescent, but they can self-renew. They can also differentiate into different cell types. CSCs are also responsible for medication resistance (Yang et al, 2024).

This contributes to conventional therapy failure and enhanced migration (Pronoy et al., 2024). This may lead to tumor recurrence and metastasis (Allan & Alison, 2011; Deleo, 2012; Xia et al., 2023). Controlling CSCs will solve a great part of the problem. Silymarin is an old hepatoprotective drug. It consists of a family of flavonoids: silybin, isosilybin, silychristin, silydianin, and taxifoline. These are commonly found in the dried fruit of the milk thistle plant, *Silybum marianum*. Silymarin is recognized for its antioxidant and liver-protective effects. However, more studies are needed to explain its anticancer properties (Agarwal et al., 2006; Wadhwa et al.,2022). Silymarin can inhibit telomerase activity. It reduces the mRNA levels of telomerase reverse transcriptase (TERT) in the human leukemia cell line K562 (Faezizadeh et al., 2012; Koltai and Fliegel, 2022). Telomerase maintains the integrity of the chromosomal end structure. This allows continuous

cell replication. Telomerase is active in 80 to 90% of cancers, but it is undetectable in normal cells. This makes it a promising target for cancer therapy (Man et al., 2016; Lansdrop, 2022; Alanazi et al., 2024). Niclosamide is an old anthelmintic drug, approved for human use for over 50 years (Satoh et al., 2016). Niclosamide is a potent inhibitor of the aldehyde dehydrogenase-1 enzyme (ALDH1) (Li et al., 2014; Needham, 2024). ALDH1 is essential in the retinoid signaling pathway. This pathway involves retinoic acid (RA) to regulate gene expression, morphogenesis, and cell development (Allan and Alison, 2011; Koppaka, 2012; Duan et al, 2024). Besides, ALDH1 plays a self-protective role in normal cells and CSCs. It acts as a detox enzyme. This means that CSCs with high ALDH1 levels can resist chemotherapy (Deleo, 2012; Duong et al., 2024).

Breast cancer is now the most frequently diagnosed cancer, surpassing lung cancer. In 2022, there were 2,296,840 new cases of breast cancer in women globally. It is the fifth leading cause of mortality worldwide. According to estimates, 670,000 women died from breast cancer in 2022 globally (Siegel et al., 2020; Siegel et al., 2022; Cao et al., 2024).

This study focused on the possible anticancer effects of combining silymarin and niclosamide on the MCF-7 human breast cancer cell line. We investigated the possible underlying mechanisms of these effects. This included the MTT assay, evaluation of the RNA expression of telomerase and ALDH1 genes and studying apoptosis. The effects on breast cancer stem cells (BCSCs) were also studied by looking at the expression of CD44 and CD133 markers and observing any morphological changes.

Materials and Methods

This study was conducted at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. We conducted the CD44 and CD133 expression on BCSCs at The Children's Cancer Hospital Egypt 57357 (CCHE), El-Saida Zinab, Cairo, Egypt.

Drugs and Chemicals

Silymarin (Legalon®) was provided by Chemical Industries Development (CID), Giza, Egypt. The preparation contains 75.0–80.9% silymarin. This includes silibinin (26–31% in weight), isosilybin (10–14%), silychristin (12–14%), and Silydianin (14–17%). Niclosamide (Yomesan®) was provided by Alex. Co. for Pharmaceuticals, Alexandria, Egypt. Cisplatin (Unistin®) was provided by EIMC United Pharmaceuticals, Badr City, Egypt.

Cell Line Propagation

We obtained the MCF-7 human breast cancer cell line from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured using RPMI-1640 media (Lonza, Belgium). This media contained 10% inactivated fetal bovine serum (FBS) (Lonza, Belgium) and 50 µg/ml gentamicin (Lonza, Belgium). The cells remained at 37 °C in a humid environment with 5% CO_2 and underwent sub-culturing two to three times a week.

Clinical Tissue Specimens

Tumor tissues were collected from breast cancer patients after their initial surgery. These patients had not received any preoperative radiotherapy or chemotherapy. The samples were collected at Dar El-Salam Cancer Hospital (Hermel), Cairo, Egypt. We collected the primary breast cancer cells from fresh tumor tissues after surgery. The cells were cultured in RPMI-1640 media with 10% inactivated FBS and 50 µg/ml gentamicin. The cells remained at 37 °C in a humid environment with 5% CO_2. This is for identifying CD44 and CD133 expression in BCSCs by confocal laser scanning microscopy.

Study Design

The study included five groups: control, cisplatin, silymarin, niclosamide, and combination. The experimental design of the combination had six subgroups: silymarin and niclosamide at 1:1, 1:2, and 2:1 ratios, silymarin and niclosamide $IC_{50\%}$, silymarin and cisplatin at a 1:1 ratio, and niclosamide and cisplatin at a 1:1 ratio. The cells were incubated for 24 hr. and 48 hr. for each treatment.

Cytotoxicity Evaluation (MTT Assay)

MCF-7 cells were placed in Corning® 96-well plates at a density of 5 x 10^4 cells per well. They were then incubated for 24 hr. at 37 °C in a humid environment with 5% CO_2. The compounds were tested at different concentrations. Each concentration had three replicates. After incubating for 24 hr. and 48 hr., the number of viable cells was determined using the MTT assay. The media was removed and replaced with 100 µl fresh RPMI-1640 media. A volume of 10 µl of 12 mM MTT stock solution (Sigma-Aldrich, USA) was added to each well. This stock contains 5 mg of MTT in 1 ml phosphate-buffered saline (PBS) (Sigma-Aldrich, USA). The plates were incubated at 37 °C with 5% CO_2 for 4 hr. An aliquot of 85 µl of the media was removed from the wells, and 50 µl of dimethyl sulfoxide (DMSO) was added, followed by 10 min incubation at 37 °C. The optical density was measured at 590 nm using a microplate reader (SunRise, TECAN, Inc., USA). The $IC_{50\%}$ values were calculated from the dose-response curve plots

using GraphPad Prism Software (San Diego, CA., USA) (Mosmann, 1983; Duan et al., 2014; Gomha et al., 2015).

Based on the MTT assay results, half of the calculated $IC_{50\%}$ values were chosen for subsequent assays, which involved a 48 hr incubation period.

Evaluation of Telomerase and ALDH1 Expression on The RNA Level by Quantitative Real-Time PCR

The cell samples, each containing 5 x 10^5 cells, were treated with various drugs and incubated for 48 hr. The total RNA was extracted following treatment using miRNeasy® Mini Kit (QIAGEN, Hilden, Germany) (catalog no. 217004). The reverse transcription process was conducted for cDNA synthesis using QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany) (Catalog no. 205311). Quantitative Real-time PCR was performed to measure the relative expression levels of telomerase and ALDH1 genes using the QuantiTect® SYBR Green PCR Kit (QIAGEN, Hilden, Germany) (Catalog no. 204141). The primers used (Invitrogen, Thermo Fisher Scientific, USA) included: forward telomerase, 5′TGG GGC CCG AGG GCA GGC GG3′; reverse telomerase, 5′GCT CGC AGA GTC TCT GCA CA3′; forward β-actin telomerase, 5′GGC TGT ATT CCC CTC CAT CG3′; reverse β-actin telomerase, 5′CCA GTT GGT AAC AAT GCC ATG T3′; forward ALDH1, 5′CTG CTG GCG ACA ATG GAG T3′; reverse ALDH1, 5′GTC AGC CCA ACC TGC ACA G3′; forward β-actin ALDH1, 5′GCA TGG GTC AGA AGG ATT CCT3′; reverse β-actin ALDH1, 5′TCG TCC CAG TTG GTG ACG AT3′. β-actin was serving as the housekeeping gene. Each PCR reaction was conducted in triplicate. The thermal cycler for Applied Biosystems (Applied Biosystems, 7300 Real-Time PCR System, Foster City, CA, USA) was programmed accordingly. Fold changes relative to β-actin were determined using the comparative threshold method.

Apoptotic Rate Evaluation by Flow Cytometry

The apoptosis assay was conducted using BD Cycletest™ Plus DNA Reagent Kit (BD Biosciences, San Jose, USA). The cells were treated with various drugs and incubated for 48 hr. Following treatment, the cell sample, containing 5 x 10^5 cells, was suspended in a 10x citrate buffer, pH 6.0 (Sigma–Aldrich, USA). The cells were centrifuged at 400 xg for 5 min at room temperature. Subsequently, 250 µl of solution A, which contains trypsin in spermine tetrahydrochloride detergent buffer, was added to the pellets and allowed to incubate at room temperature for 10 min. Following this, 200 µl of solution B, containing RNase A and trypsin inhibitor in spermine buffer, was added. The sample was incubated at room temperature for 10 min. An aliquot of 200 µl of solution C, containing propidium iodide in spermine buffer, was added, and the sample was kept in the dark at 4 °C for 10 min. The BD FACSCalibur (BD Biosciences, San Jose, USA) was used to analyze the samples. The data interpretation was carried out using FACStation software.

Examination of The Expression of BCSC Markers, CD44 and CD133 by Confocal Laser Scanning Microscopy

A stock solution of MACS® BSA (Miltenyi Biotec GmbH, Germany) containing phosphate-buffered saline enriched with 10% bovine serum albumin was diluted in a ratio of 1: 20 using MACS® rinsing solution (Miltenyi Biotec GmbH, Germany). This buffer was kept cold at 2-8 °C. Following treatment with various drugs and a 48 hr incubation period, the cell sample containing 5 x 10^5 cells was centrifuged at 300 xg for 10 min. The cells were resuspended in 98 µl of the buffer. 2 µl of human CD44-APC antibodies and 2 µl of human CD133/1-PE antibodies were added (Miltenyi Biotec GmbH, Germany). The sample was then incubated in the dark at 2-8 °C for 10 min. Subsequently, the cells were washed with 1-2 ml of the buffer and centrifuged at 300 xg for 10 min. The resulting pellets were resuspended in an adequate amount of the buffer for subsequent analysis using a confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Germany) with ZEN microscopy software.

Statistical Analysis

Statistical analysis was conducted using SPSS version 22.0 software (SPSS Inc., Chicago, IL). Comparisons were analyzed using the ANOVA test. Results were expressed as mean ± SD. A significance level of $P < 0.05$ was used. All experiments were conducted in triplicate to confirm reproducibility.

Results

Cytotoxicity Evaluation

The $IC_{50\%}$ for silymarin was evaluated as 146 ± 18.17 µM and 98.83 ± 6.01 µM for 24 hr. and 48 hr. treatments, respectively. This represents a significant decrease of 32.31% for 48 hr treatment compared to 24 hr. treatment. Niclosamide displayed an $IC_{50\%}$ of 7.03 ± 0.25 µM for 24 hr. treatment and 1.75 ± 0.15 µM for 48 hr. There is a significant reduction of 75.11%. The $IC_{50\%}$ of cisplatin was evaluated as 29.80 ± 1.06 µM for 24 hr. and 7.33 ± 0.68 µM for 48 hr., reflecting a significant decrease of 75.41%. The findings suggest that niclosamide demonstrates greater cytotoxicity than cisplatin. The findings are illustrated in Figure 1.

The $IC_{50\%}$ for the silymarin and niclosamide 1:1 was 3.89 ± 0.47 µM for 24 hr treatment and 1.41 ± 0.2

µM for 48 hr. There is a significant reduction of 64.57%. For the silymarin and niclosamide combination 1:2, the $IC_{50\%}$ was 1.66 ± 0.09 µM and 0.64 ± 0.06 µM for 24 hr. and 48 hr. treatments, a substantial decrease of 61.45%. The $IC_{50\%}$ for silymarin and niclosamide combination 2:1 was 26.28 ± 1.68 µM for 24 hr. and 7.87 ± 1.32 µM for 48 hr treatments, with a significant decrease of 70.05%. Concerning the silymarin and niclosamide $IC_{50\%}$ combination, the $IC_{50\%}$ after 24 hr. was 32.13 ± 14.69 µM. After 48 hr., it was 7.55 ± 0.22 µM, revealing a significant decrease of 76.5%. For 1:1 cisplatin and silymarin combination, the $IC_{50\%}$ was 51.04 ± 7.55 µM and 14.87 ± 1.78 µM for 24 hr. and 48 hr treatments, respectively. A significant decrease of 70.87% was observed. The $IC_{50\%}$ for 1:1 cisplatin and niclosamide combination after 24 hr. was 16.44 ± 3.42 µM and 3.8 ± 0.36 µM after 48 hr. A significant decrease of 76.89% was observed.

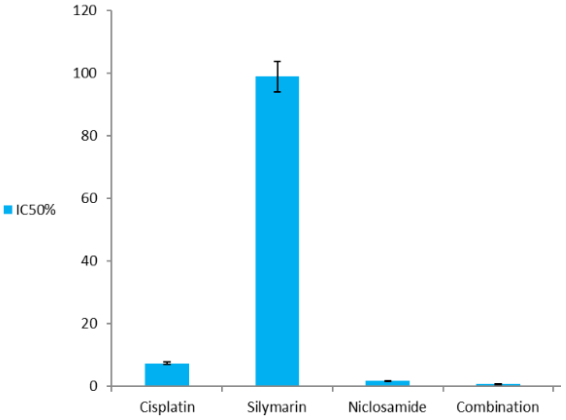

Figure 1: $IC_{50\%}$ values for cisplatin, silymarin, niclosamide, and silymarin and niclosamide combination 1:2. The figure represents the $IC_{50\%}$ values for cisplatin, silymarin, niclosamide, and silymarin and niclosamide combination 1:2 for the MCF-7 cell line after 48 hr. treatment. All assays were carried out in triplicate. The means ± SD are expressed.

A comparison of the $IC_{50\%}$ for the combinations silymarin and niclosamide 1:1, cisplatin and silymarin 1:1, and cisplatin and niclosamide 1:1 after 48 hr. treatment showed that the silymarin and niclosamide combination 1:1 exhibited the highest cytotoxicity. Furthermore, an analysis of the $IC_{50\%}$ after 48 hr. for the combinations silymarin and niclosamide 1:1, silymarin and niclosamide 1:2, silymarin and niclosamide 2:1, and silymarin and niclosamide $IC_{50\%}$ showed that silymarin and niclosamide 1:2 was the most cytotoxic, as illustrated in Figure 1.

Evaluation of Telomerase Expression on the RNA Level

As illustrated in Figure 2, there is a significant decline of 45.83% in the relative expression of telomerase normalized to β-actin on the RNA level for silymarin compared with the control, but this was not the case when compared to cisplatin. Niclosamide showed a significant reduction of 79.17% relative to the control and 41.18% relative to cisplatin. Furthermore, for silymarin and niclosamide 1:2, significant reductions were noticed relative to cisplatin, silymarin, and niclosamide, amounting to 78.82%, 86.15%, and 64%, respectively.

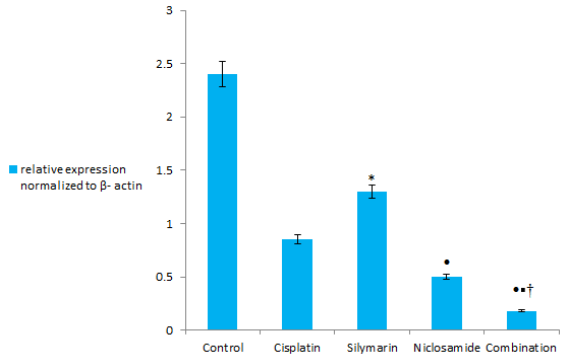

Figure 2: Relative expression of telomerase normalized to β-actin on the RNA level. The figure represents the relative expression of telomerase normalized to β-actin on the RNA level for the MCF-7 cell line after 48 hr. treatment. All assays were carried out in triplicate. The means ± SD are expressed. The symbols (*, •, ▪, †) indicate comparison to control, cisplatin, silymarin, and niclosamide, respectively (P ≤ 0.05).

Evaluation of ALDH1 Expression on the RNA Level

The relative expression of ALDH1 normalized to β-actin on the RNA level revealed a significant reduction of 50% for silymarin compared with the control, although this effect was not observed relative to cisplatin. Niclosamide resulted in a substantial decrease of 67.47% and 36%, respectively, compared with the control and cisplatin. Additionally, the combination treatment showed a significant reduction of 66.7%, 75.5%, and 47.9% when compared with cisplatin, silymarin, and niclosamide, respectively. These results are illustrated in Figure 3.

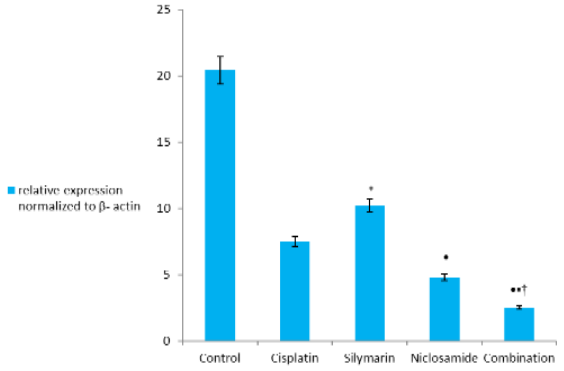

Figure 3: Relative expression of ALDH1 normalized to β-actin on the RNA level. The figure represents the relative expression of ALDH1 normalized to β-actin on the RNA level for the MCF-7 cell line after 48 hr. treatment. All assays were carried out in triplicate. The means ± SD are expressed. The symbols (*, •, ▪, †) indicate comparison to control, cisplatin, silymarin, and niclosamide, respectively (P ≤ 0.05).

Evaluation of The Apoptotic Rate

As depicted in Figure 4, a marked increase of 59.26% in the apoptotic rate was associated with silymarin relative to the control. This was not the case with cisplatin. Niclosamide demonstrated a substantial increase of 288.37% and 44.83%, respectively, when compared to the control and cisplatin. Furthermore, the combination treatment revealed a significant increase of 83.5%, 208.98%, and 26.7%, respectively, relative to cisplatin, silymarin, and niclosamide.

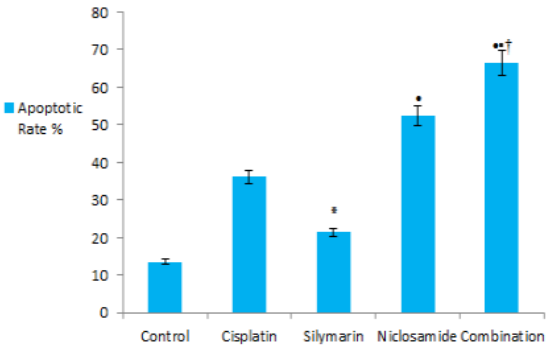

Figure 4: Apoptotic rate. The figure represents the apoptotic rate % for the MCF-7 cell line after 48 hr. treatment. All assays were carried out in triplicate. The means ± SD are expressed. The symbols (*, •, ▪, †) indicate comparison to control, cisplatin, silymarin, and niclosamide, respectively ($P \leq 0.05$).

Examination of The Expression of BCSC Markers, CD44 and CD133

As depicted in Figure 5A, the control exhibited a strong CD44 (red) and CD133 (green) expression, with no observable morphological alterations. In the case of cisplatin, the CD44 and CD133 were mildly expressed as illustrated in Figure 5B. The cells showed marked morphological transformations, featuring numerous protrusions, rough surfaces, many microvilli, and pseudopodia that reflected cellular swelling and membrane vesicle rupture. Additionally, some apoptotic cells were detected. Figure 5C demonstrated moderate CD44 and CD133 expression levels in the case of silymarin. The cellular morphology showed mild degenerative features, including protrusions, microvilli, and pseudopodia. Furthermore, the presence of cells with thin and rough membranes, along with some aggregations and swelling, suggests that these cells are likely to undergo rupture and apoptosis shortly. Regarding niclosamide, Figure 5D displayed reduced CD44 and CD133 expression levels, alongside pronounced morphological degenerations. Numerous cellular protrusions, microvilli, and pseudopodia were identified, indicating cellular swelling and rupture of the membrane vesicle. Observations also included cells with thin and rough membranes, along with substantial cytoplasmic aggregations and apoptotic fragments. In the combination treatment, CD44 expression was completely absent, and CD133 expression was markedly reduced, as depicted in Figure 5E The cellular morphology was extensively damaged, with apoptotic fragments being the most notable feature.

Discussion

Resistance to chemotherapy and radiation represents a critical challenge in cancer treatment strategies. CSCs can evade these treatment modalities, resulting in the formation of recurrent tumors or metastasis. This phenomenon is a significant factor in cancer-related mortality, contributing to nearly 90% of deaths (Fahn et al., 1994; Clark and Palle, 2016; Satoh et al., 2016). This study aimed to evaluate the potential anticancer effects of the silymarin and niclosamide combination on the MCF-7 cell line and BCSCs, and to elucidate the underlying mechanisms involved. The findings of this study indicate that silymarin has a more pronounced cytotoxic effect than the control.

This effect is likely due to a considerable decrease in the expression of telomerase and ALDH1. In addition, the apoptotic effect of silymarin further validates these results. The moderate expression of CD44 and CD133 on BCSCs, along with evidence of cellular degeneration, highlights the cytotoxic effect of silymarin on BCSCs. The real-time viability analysis of the MCF-7 cell line under silibinin treatment confirmed our results. The xCELLigence system detected that silibinin treatment had a significant effect on both the proliferation and viability of MCF-7 cells (Jahanafrooz and Rinner, 2023). In addition, silymarin showed a significant cytotoxic effect on the 4T1 cell line, which is derived from mammary tumors in BALB/c mice, with the impact being contingent on the dosage and duration of treatment (Shariatzadeh et al., 2014). Hajighasemlou et al. (2014) demonstrated that silymarin inhibited the growth of two breast cancer cell lines, SK-BR-3 and BT-474, at different concentrations over 24, 48, and 72 hr. In addition, silymarin enhanced the cell death in both cell lines, aligning with our results.

According to Nasiri et al. (2013), silibinin exerted a cytotoxic effect on the T47D, a human breast cancer cell line, and suppressed telomerase gene expression in a time- and dose-dependent manner. These results align with our findings. Furthermore, Jiang et al. (2020) showed that silybin significantly reduced the expression levels of ALDH1A1 mRNA and proteins in prostate cancer cells, which is consistent with our findings. Our results also align with those of Katiyar et al. (2005), who reported that silymarin exerts a significant apoptotic effect that relies on p53. Furthermore, Kauntz et al. (2012) indicated that the apoptotic effect of silymarin is strongly related

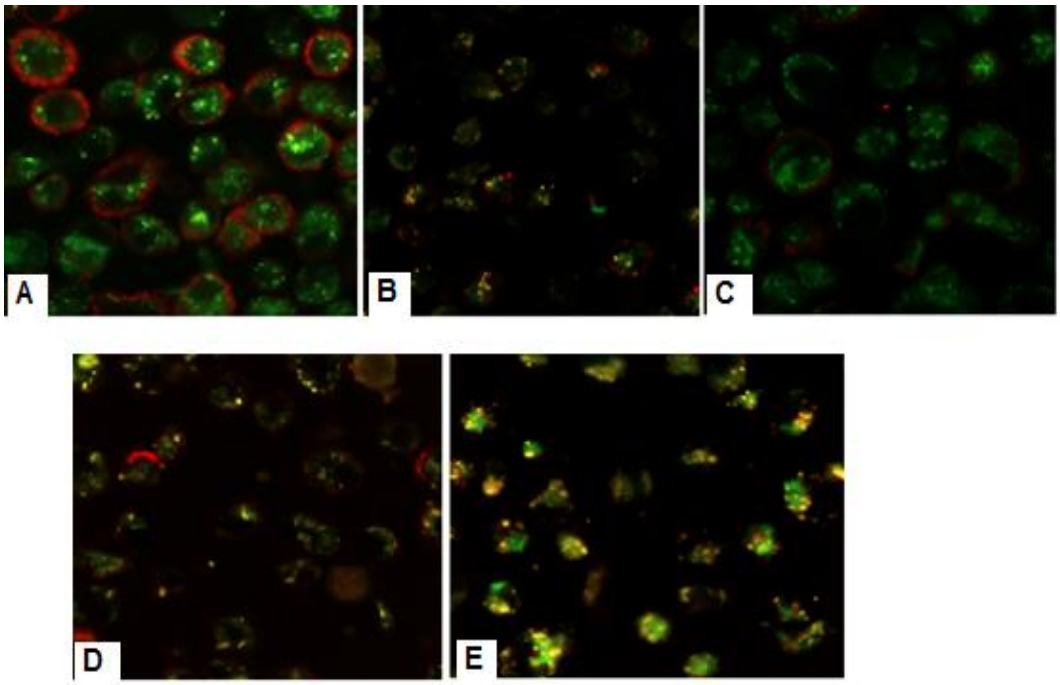

Figure 5: Confocal laser scanning microscopy images for CD44 and CD133 expressions in BCSCs. The figure represents confocal laser scanning microscopy images for CD44 (red) and CD133 (green) expressions in BCSCs after 48 hr. treatment: (A) control, (B) cisplatin, (C) silymarin, (D) niclosamide, (E) combination. All assays were carried out in triplicate.

to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). This is because silymarin promotes the up-regulation of the death receptors 4 and 5, thus increasing the number of receptors for TRAIL binding. Kim et al. (2011) showed that silymarin decreased CD44 expression, which is strongly correlated with high invasion and metastasis potential, and markedly elevated tumorigenic capacity as reported by Yan et al. (2013). In addition, Tehrani et al. (2021) revealed that the treatment of pancreatic CSCs with silybin encapsulated in polymersomes decreased the expression levels of CD24, CD44, and CD133.

This confirmed the effect of silymarin on BCSCs observed in the current study. Furthermore, silibinin decreased the viability of mammospheres derived from MCF-7, MDA-MB-231, and MDA-MB-468 cell aggregations in a dose-dependent manner. It also reduced the expression of genes associated with the epithelial–mesenchymal transition (EMT) as reported by Firouzi et al. (2022). They also reported a significant decrease in the expression levels of CD44 and CD133, corroborating the results of our study.

The existing evidence showed that silymarin has a promising role in breast cancer treatment. Its low toxicity, even at very high doses, along with the lack of adverse effects on normal cells, are critical factors that warrant further exploration of this drug. In summary, silymarin may serve as a valuable adjunct in breast cancer therapy. Niclosamide showed a significant decrease in the telomerase and ALDH1 expression levels relative to the control and cisplatin treatment. This finding can explain the cytotoxic effect of niclosamide, supported by the notable increase in the apoptotic rate. In addition, the significant decrease in the CD44 and CD133 expression in BCSCs with severe cellular degeneration reflects the marked cytotoxic effect on these stem cells. Furthermore, niclosamide exhibited cytotoxic effects on the four basal-like breast cancer cell lines (BLBC): 2LMP, SUM159, HCC1187, and HCC1143 as reported by Londoňo-Joshi et al. (2014). Niclosamide not only inhibited tumor growth in BLBC but also triggered apoptosis synergistically with a monoclonal antibody that selectively activated TRAIL death receptor 5.

Moreover, niclosamide showed a cytotoxic effect against ALDH-expressing cells from these four BLBC cell lines. These findings reinforce our results. Tahtouh et al. (2015) performed an in vitro invasion assay to assess the metastatic potential of the PLC/PRF/5 hepatic cancer cell line after treatment with niclosamide. The study revealed a significant decrease in the invasion of PLC/PRF/5 cells relative to the control. This was associated with the inhibition of signal transducer and activator of transcription (STAT3), protein kinase C (PKC), and telomerase, thereby supporting the conclusions of the present study. In addition, Wang et al. (2013) found that niclosamide down-regulated stem cell pathways. It inhibited the formation of breast cancer spheroids and induced apoptosis in these spheroids.

Animal studies also confirmed this therapeutic effect.

These findings correlated with our results. Furthermore, Moskaleva et al. (2015) reported that niclosamide not only inhibited molecular signaling pathways but also targeted mitochondria in cancer cells, leading to growth inhibition and apoptosis, whereas normal cells exhibited resistance to niclosamide. These findings are consistent with our results indicated in the present study. Moreover, niclosamide nanocrystals inhibited cell migration and decreased the expression of CD44 in BCSCs as reported by Fu et al. (2020). These results came in parallel with our findings. According to the findings of Arend et al. (2016), niclosamide selectively targeted the ovarian CSCs that expressed the surface protein CD133 and significantly reduced the expression of proteins in the Wnt/β-catenin, mTOR, and STAT3 pathways, aligning with the findings of the current study.

The accumulated experimental evidence suggests that niclosamide, through its multi-faceted targeting of pathways, is a promising candidate for breast cancer therapy. The outcomes pointed to silymarin and niclosamide combination 1:2 as the most potent, highly cytotoxic. This combination showed a significant reduction in the telomerase and ALDH1 expression levels. This explained the high cytotoxic effect and high apoptotic rate of this combination relative to cisplatin, silymarin, and niclosamide. Moreover, confocal scanning revealed the marked effect of this combination on BCSCs, where there was a notable decrease in CD44 and CD133 expression levels, accompanied by considerable changes in the cellular morphology. Chan et al. (2018) suggested that the combination of thioridazine, a dopamine receptor antagonist, and curcumin suppressed the spheroids from the ovarian cancer cells more effectively than either drug alone.

In addition, the combination of thioridazine and curcumin significantly promoted apoptosis in AMC-HN4, MDA-MB231, and U87MG cell lines without affecting human normal cells (Sachlos et al., 2012; Seo et al., 2017). Additionally, Montales et al. (2015) indicated that the combination of metformin and genistein, an isoflavone present in soybeans, effectively inhibited the formation and proliferation of colon CSCs spheroids, and this combination improved 5-FU efficacy. Chan et al. (2018) presented an innovative combination therapy approach that utilizes both phytochemicals and repositioned drugs to specifically target CSCs.

This strategy is noted for its efficacy, low toxicity, and affordability. Moreover, it is free from intellectual property constraints. These attributes ensure that these combinations are economically viable and more accessible than traditional chemotherapy and targeted therapies (Block et al., 2015; Sullivan et al., 2017; Wang et al., 2022). Dietary phytochemicals and repurposed pharmaceuticals are pleiotropic in contrast to the traditional anticancer therapies with unique molecular targets. Each has a wide range of cellular targets for molecular and cellular pathways that inhibit the proliferation, differentiation and resistance of CSCs (Mencher and Wang, 2005; Fojo, 2008; Emran et al., 2022; Duong et al., 2024). This novel combination is an innovative concept worthy of promotion (Chan et al., 2018; Jug et al., 2024). We advocate this novel strategy and look forward to clinical applications.

Abbreviations

ALDH1, aldehyde dehydrogenase-1 enzyme; BCSCs, breast cancer stem cells; BLBC, basal-like breast cancer; CSCs, cancer stem cells; EMT, epithelial–mesenchymal transition; FBS, fetal bovine serum; mTOR, mammalian target of rapamycin; PBS, phosphate-buffered saline; PKC, protein kinase C; RA, retinoic acid; STAT3, signal transducer and activator of transcription; TERT, telomerase reverse transcriptase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

Declarations

Ethics approval and consent to participate: The experimental procedures were approved by the Research Ethics Committee, Faculty of Pharmacy, Tanta University, Egypt, approval code: TP/RE/9/23p-0045

Availability of data and material: The data will be made available by the corresponding author on request.

Competing interests: The authors declare that there is no conflict of interest.

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