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The effect of natural products combination on MCF-7 cells exceeds tamoxifen therapeutic dose effects *in vitro*

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Abstract: Cancer remains to be one of the most severe sicknesses globally. Cases have kept rising over the years. Breast cancer (BC), which is among the leading types of cancers and predominantly affects women, is the second leading cause of cancer mortality. Researchers have developed interventions over the years; however, the BC survival rate has not improved since the 1980s. This has created the need for novel drug interventions that would manage and treat BC more effectively. This study focused on using a combination of natural product extracts such as phytoestrogen (*Ziziphus jujube*) and Tannin nanoparticles (NP99) together, which we have referred to as (Z.NP99) and tamoxifen (Tam) as one of the leading BC drugs since the 70s, in treating BC. The effectiveness of Tam if used alone in the treatment and if combined with Z.NP99 was evaluated using MCF-7 cells *in vitro*. The findings showed that the combination treatment of Z.NP99 affected the proliferation and viability of MCF-7 cells more than Tam at a 10 µg/mL dose. Moreover, Z.NP99 with Tam stimulated the maximum reduction of MCF-7 proliferation and viability in a time-dependent manner. Furthermore, Tam and Z.NP99 augmented the DNA fragmentation percentage combined with the upregulation of the apoptotic genes. Additionally, the results showed that the apoptotic impact of Z.NP99 and Tam on MCF-7 cells may be intermediated by down-regulating some genes such as *Claudin-1* followed by down-regulating mRNA expression of *MMP-9*, *VEGF*, and *BCL-2* genes of treated cells. Combining Tam with Z.NP99 considerably enhanced the effectiveness of conventional therapy. As a result, this study suggested that the Z.NP99 was ideal for developing effective natural treatments that would improve BC outcomes.

Introduction

Breast cancer (BC) remains one of the most severe health issues among women worldwide (Testa *et al.*, 2020). According to the International Agency for Research on Cancer (IARC), BC incidences in females of all ages stood at 47.8 per 100,000 population in 2020, while the age-standardized mortality rate (ASMR) stood at 13.6 per 100,000 population (Globocan, 2020a). Furthermore, IARC Saudi Arabia reported an age-standard incidence rate (ASIR) of 28.8 per 100,000 and an ASMR of 8.9 per 100,000 population (Globocan, 2020b). Likewise, the US cancer statistics cite breast, lung, and colorectal cancers as the most prevalent among women in the country during 2020. They account for 50% of all cancer cases, and among these, 30% of the cases are of BC (Siegel *et al.*, 2020). Cancer prevalence among women presents the urgent need to develop appropriate prevention and therapeutic approaches. This begins with developing robust knowledge about the cellular and molecular origin of the tumor.

Without this information, creating solid preventive frameworks for BC will be nearly impossible. Breast cancer is a highly heterogeneous illness for its histology, epidemiology, and molecular properties (Kennecke *et al.*, 2010). It is clustered into molecular traits, namely hormone receptor activation (estrogen and progesterone receptors), human epidermal growth factor receptor 2 (HER2), and/or BRCA (Testa *et al.*, 2020) BC with estrogen receptor (ER) positivity accounts for approximately 80% of cases (Lumachi *et al.*, 2015). It was logical to use a combined hormone-chemotherapy treatment with the aim of attaining additive and synergistic outcomes. This approach was necessary because BC comprises a mixed population of receptor-positive and receptor-negative cells (Osborne, 1998). Tamoxifen (Tam) (Nolvadex, ICI Pharma, Wilmington, Delaware) hormone-chemotherapy treatment for BC was approved in 1970 (Osborne, 1998; Harper and Walpole,

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1966; Klaab *et al*., 2018). The approval of Tam was linked to its antiestrogenic elements, effectiveness in adjuvant therapy, and its capacity to prevent estrogen receptor-positive (ER+) BC, which is common in over 69% of BC patients.

Nonetheless, utilizing Tam as a therapy also depicts some restrictions on its effectiveness, including stability, solubility, toxicity, delivery to target sites, and possible side effects (Glassman *et al*., 2017). Tam has numerous side effects; it impairs glucose and lipid metabolism, thereby increasing diabetes incidence (Klöting *et al*., 2020), causes hypertriglyceridemia, which could lead to acute pancreatitis (Tey *et al*., 2019), and is linked to ovarian cysts and elevated amounts of estradiol in premenopausal women (Goeminne *et al*., 2019). Despite its side effects, some patients present resistance to Tam. The resistance occurs in up to 30% of cases, thereby progressing to secondary tumors and eventual mortality over a specific period. Thus, it is critical to apply alternative methods for cancer treatment. One of the most important aspects of cancer therapy is the combination of several treatments to specifically target cancer-inducing or cancer-sustaining pathways to provide the most effective results (Yap *et al*., 2013; Blagosklonny, 2004). Traditional mono-therapeutic techniques do not distinguish between healthy and cancerous cells, so they ultimately destroy both types of cells. Patients who receive chemotherapy may experience multiple side effects and risks, as well as a significant reduction in immune function as a result of drug effects on bone marrow cells and increased susceptibility to infections (Partridge *et al*., 2001; LeBaron *et al*., 1988). Importantly, combination therapy can be toxic if it includes chemotherapeutic agents, but the toxicity is significantly reduced because different pathways are targeted. Therefore, the therapeutic dosages of each drug are reduced because these drugs act synergistically or additively (Albain *et al*., 2008; Mokhtari *et al*., 2013). Furthermore, Combination therapies may be able to kill cancer cells while inhibiting their toxic effects on healthy cells at the same time. The possible cause is when one of the drugs in the combination may have antagonistic effects, concerning cytotoxicity, on another drug in normal cells (Blagosklonny, 2005). Thus, Tam is combined with various other natural and medicinal products as extracts, for instance, phytoestrogen or in the form of functionalized nanoparticles.

Plants remain a censorious source in developing medicines that cure different types of illnesses. Scientists remain considerably interested in plants because they contain little toxicity to the tissue, are low-cost, and have fewer side effects concerning the standard chemotherapy treatment (Khan *et al*., 2019). Currently, at least 60% of anticancer medicines emerge from natural compounds (Mohammed *et al*., 2019). One of the predominant plants is the *Ziziphus jujube*, a phytoestrogen belonging to the family Rhamnaceae, generally known as annep, in Saudi Arabia. The plant is rich in biological and phytochemical properties and can cure various diseases, such as ulcers, asthma, and depression. It also has anti-inflammatory, antioxidant, and antimicrobial activities (Batool *et al*., 2019). Induction of apoptosis using *Z. jujube* aqueous extract has depicted promising results in killing cervical and breast cancer cells (Pandey and Poonia, 2018).

Fruit methanol extracts have been reported to suppress the proliferation of cancer cells and produce antioxidant effects (Pillai, 2020). Our previous study has demonstrated the positive effects of phytoestrogens on BC treatment through work synergistically with chemotherapeutic drugs like Tam (Klaab *et al*., 2018).

Nanoparticles are considered smart vehicles to transport across various barriers and increase the interaction of therapeutic agents with BC cells, significantly improving the survival rate and forming an integral part of current BC treatment (Gao *et al*., 2020). Currently, researchers are exploring the development of a natural compound that will model nanoparticles to augment bioavailability and lessen the dose necessary to attain a therapeutic effect and the ability to target particular tissues or organs (Rizvi and Saleh, 2018).

Polyphenols remain the most important natural product compounds that can be produced as nanoparticles.

Examples include condensed tannin. Previous studies have also shown that tannin extract has antimicrobial, antioxidant, antigenotoxic, and anti-tyrosinase properties (Maisetta *et al*., 2019; Kwon, 2018). This proves that nanomedicine using natural products contributes to developing more efficient modalities for cancer cure and prevention.

Our earlier works highlighted the impact of Tam, *Z. jujube*, and tannin nanoparticles (NP99) on MCF-7 breast cancer cells. One study examined the individual effect of *Z. jujube* and Tam as well as the combinatory impact (Klaab *et al*., 2018). More recent work also evaluated the cytotoxic effects of Tam and tannin nanoparticles on MCF-7 cells, individually and jointly (AlMalki *et al*., 2021).

In this evaluation, Tam, a standard therapeutic agent of ER+ BC, *Z. jujube* extract, a phytoestrogen, and tannin nanoparticles were used jointly on MCF-7 BC cells *in vitro*. The study also examined the potential molecular processes through which these agents affect resistance, proliferation, and apoptotic pathways. They all augmented the sensitivity of anticancer drugs.

MCF-7 cell line has been chosen for the present study because this work is extended from our previous investigations that applied MCF-7 cells and used phytoestrogen extracts (Klaab *et al*., 2018; AlMalki *et al*., 2021). Additionally, MCF-7's popularity stems from its excellent hormone sensitivity due to the expression of the estrogen receptor (ER+), making it a crucial model for hormone research (Levenson and Jordan, 1997).

Keeping estrogen receptor expression in cultured cells is particularly challenging, thus leading to a high proportion of ER-negative cell lines rather than ER-positive ones (Novaro *et al*., 2003).

Materials and Methods

Materials and reagents

Penicillin-Streptomycin Solution (10.000 Units/mL penicillin, 10.000 µ/mL streptomycin), Dulbecco's Modified Eagle Medium (DMEM), 0.25% trypsin with

1 mM EDTA (1 X) and fetal bovine serum (FBS) were obtained from (Gibco by Life Technologies, USA), Trizol reagent (Ambion RNA by Life Technology, USA). Gene expression Master Mix (FIREPol®), phosphate-buffered saline (PBS) (Gibco by Life Technologies, USA), 0.4% Trypan blue (Sigma-Aldrich), and MTT (Bio Basic Inc., Canada). All other chemicals used were of a high grade.

Cell line

The MCF-7 cell line was originally sourced from American Type Culture Collection. The MCF-7 cells were cultured and plated at 1 × 106 cells/well in 6-well plates containing a complete DMEM culture medium with 10% FBS and 1% penicillin-streptomycin antibiotics and were allowed to attach overnight at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air (Walle *et al.*, 2007; Klaab *et al.*, 2018). These cells were treated on day 0, after 24 h in basal medium with a medium containing various treatments (experimental groups), and then 24, 48, and 72-h incubation. The experiments were divided using groups of treatments summarized in Fig. 1.

Tamoxifen drug

Pure tamoxifen drug from EBEWE Pharma®-UK was obtained from King Abdul-Aziz Specialist Hospital, Taif, Saudi Arabia. Tam was dissolved in absolute ethanol to make a stock solution of 10 mg/mL. The stock solution was further diluted in 1.0 mg/mL in 10% ethanol before being added to the culture. The second process was necessary due to the low solubility of tamoxifen in water. The culture gave an ultimate concentration of 10 µg/mL medium (Ichikawa *et al.*, 2008).

Natural products preparation

Ziziphus jujube extract

The extract of *Z. jujube* was acquired as capsules, each with 500 mg of pure Jujube fruit extract without any additives. The extract was purchased from Source Naturals, Amazon (GMP, FDA, Registered FA City, USA). Pure Jujube extract was dissolved in warm distilled water for 30 min. It was prepared freshly, just before the treatment (Klaab *et al.*, 2018).

Tannin nanoparticles (NP99)

Fifty milliliters of purified water and propylene glycol were heated at 70°C in a 1:1 ratio in a beaker. Ethylenediaminetetraacetic acid (EDTA, 0.5%) and poloxamer 407 (pluronic F-127, 0.5%) were then added to the solution while continuously stirring, and the mixture was cooled to 50°C. It was further cooled to 40°C, and the NP99 was added into the carbopol gel and mixed evenly to create a viable gel. The NP99 was dispersed into the carbopol gel to obtain the final concentration of NP99 at 1.5%, while the ultimate concentration of carbopol was maintained at 2%.

The NP99 powder was diffused in sterilized water to come up with varied concentrations to examine its cytotoxic effect on MCF-7 cells, which showed a half-maximal inhibitory concentration (IC_{50}) value at 18 µg/mL (AlMalki *et al.*, 2021).

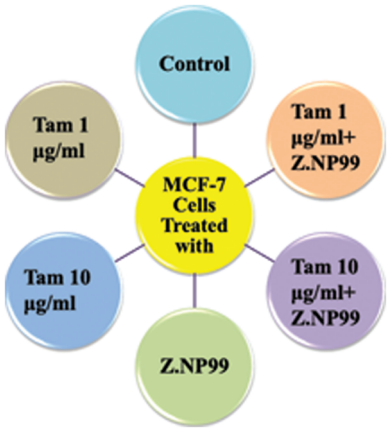

FIGURE 1. The diagram shows the treatment groups and experiment design.

Methods

Cell proliferation assay

Counting of proliferating cells was done using Trypan blue staining. The living and healthy cells are not colored because they are not able to absorb trypan blue. Consequently, dead cells are visible under a microscope as blue. Following the removal of the culture medium, attached cells were rinsed in PBS. Immediately after trypsinization, centrifuge at 2500 rpm for 7 min at 4°C, dispose of the media, and 1 mL of fresh media was added. Using a microcentrifuge tube, add 10 µL of the cell suspension along with 100 µL of the dye "Trypan blue," and apply 10–20 µL of the mixture to the Heamocytometer slide then monitored carefully under a microscope (Strober, 2001).

Detection of cell viability

The 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium (MTT) colorimetric assay was used to assess the viability and cytotoxicity of MCF-7cells induced after the treatment of Z.NP99 and Tam as it is used to test the activity of mitochondrial dehydrogenase producing purple formazan salt crystals (Mosmann, 1983; Wen *et al.*, 2019). The cells were seeded onto the 96-well microtiter plates and treatments were performed separately and in combination.

After incubation at different time courses (24, 48 & 72 h), the medium was removed, and 40 µL MTT solution (5 mg/mL) was added to each well for 4 h at 37°C with a 5% CO_2 incubator. After that, the MTT solution was removed and replaced with 140 µL of dimethyl sulfoxide (DMSO) to solubilize the formed formazan. The absorbance was measured using a plate reader (MR-96A) at a test wavelength of 540 nm. The experiments were done in quadruplicate and are representative of at least three independent experiments (Rasul *et al.*, 2011a; Rasul *et al.*, 2011b). The inhibition of percentage growth was determined using the formula below:

$$\text{Inhibition\%} = [\text{A540 (control)} - \text{A540 (treated)}]/\text{A540 (control)} \times 100].$$

DNA fragmentation

Programmed cell death (apoptosis) was evaluated for natural products Z.NP99 and Tam using BioVision's Quick Apoptotic

TABLE 1

Summary of the sequence of the primers used in PCR amplification obtained from previous research

Genes	Forward primers	Reverse primers
GAPDH	5′-GTCTCCTCTGACTTCAACAGCG-3′	5′-ACCACCCTGTTGCTGTAGCCAA-3′
BCL-2	5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′	5′-GGTGCCGGTTCAGGTACTCAGTCA-3′
Bax	5′-CCTGTGCACCAAGGTGCCGGAACT-3′	5′-CCACCCTGGTCTTGGATCCAGCCC-3′
VEGF	5′-CTGGATCCATGGCAGAAGGAGGAG-3′	5′-GAATTCAGACCGCCTCGGCTTGTC-3′
Cludin-1	5′-CAGCTGTTGGGCTTCATTCTC-3′	5′-ATCACTCCCAGGAGGATGCC-3′
MMP-9	5′-CGTCGTGATCCCCACTTACT-3′	5′-AACACACAGGGTTTGCCTTC-3′

DNA Ladder Detection Kit (Bio-Vision, Life Science SOURCE^{TM}, USA) as indicated in (Klaab *et al.*, 2018; AlMalki *et al.*, 2021). DNA was extracted from MCF-7 cells, and DNA fragments were examined using the Kit. The DNA pellet was dissolved in a 30 µL DNA suspension buffer, and samples were then loaded into an agarose gel of 1.5% containing 0.5 µg/mL ethidium bromide. The DNA bands were examined under ultraviolet radiation and the extent of DNA fragmentation was then evaluated and estimated colorimetrically at 575 nm using Diphenylamine (DPA) according to the methods described by Burton (1956) and Perandones *et al.* (1993).

Gene expression

The effects of the treatments were examined in terms of the mRNA expression of apoptotic genes such as *BCL-2* and *Bax*.

Claudin-1, vascular endothelial growth factor (*VEGF*), and matrix metalloproteinase-9 (*MMP-9*) genes were also investigated. In brief, the total isolated RNA was extracted with the TRIzol reagent (Ambion RNA by Life Technology, USA), and its stability was checked by gel electrophoresis. The isolated RNA was reverse transcribed to generate the first-strand cDNA using 2 µg RNA and reverse transcription reagents (Maxime RT PreMix kit) from iNtRON Biotechnology, Korea. The polymerase chain reaction (PCR) reactions were conducted as described by Sun *et al.* (2013) using a thermal cycler (PXE 0.5 Thermo) and primer pairs described in Table 1 that were introduced in previous studies, to amplify *GAPDH* (Livak and Schmittgen, 2001), *BCL-2* and *Bax* (Khodapasand *et al.*, 2015), *Claudin-1* (Akasaka *et al.*, 2010), *VEGF* (Lee *et al.*, 2004), and *MMP-9* (Darakhshan *et al.*, 2013). The PCR products were separated in Tris-Borate-EDTA buffer on a 1.5% (w/v) agarose gel and visualized on a UV Trans-illuminator. The DNA bands were then scanned, and signal intensities were quantified under the Gel-Pro software (version 3.1 for Windows 3). The ratio between the target genes-amplification product and the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was calculated to standardize the original variation in sample concentration and endogenous control gene for reaction efficiency (Raben *et al.*, 1996).

Statistical analysis

All statistical analysis was done using the SPSS11 program.

Duncan's multiple tests were used to determine the significance of the differences between the treated groups after the One way-ANOVA test (Walter and Duncan, 1969).

The values of the results are displayed using Mean + SE. The result was statistically significant if $p < 0.05$.

Results

Impact of Z.NP99 and Tam treatments on proliferation and viability percentage of MCF-7 cells

The MCF-7 cells were cultured and treated with specific doses of Z.NP99 combination at IC_{50} dose (*Ziziphus* 1 mg/mL and NP99 18 µg/mL) *in vitro*. The Z.NP99 was mixed with Tam in either a therapeutic dose of 10 µg/mL or 1 µg/mL. After treatment with Z.NP99, the MCF-7 cell growth plummeted after a day, two, and three days ($p < 0.05$), compared to untreated cells and those treated solely with Tam at the exact dosage of 10 or 1 µg/mL.

The extreme cells growth inhibition occurred in the treatment group of Z.NP99 combined with 10 µg/mL Tam by about 55%, 63%, and 73% after 1, 2, and 3-day, exposure, respectively (Table 2). Furthermore, ZNP99 lessened MCF-7 cell viability in a time-dependent manner (Table 3). Interestingly, Tam at 1 µg/mL when combined with Z.NP99 significantly inhibited the proliferation and viability of MCF-7 cells more than Tam alone at the therapeutic dose, 10 µg/mL (Tables 2 and 3). The most significant cell reduction was about 25.67% for Tam 10 µg/mL with Z.NP99 treatment.

Nonetheless, the peak inhibitions in proliferation and viability proportions were observed after treatment for three days (Tables 2 and 3).

Changes induced in MCF-7 cells treated with Z.NP99 and Tam

As observed above, Z.NP99 and Tam impacted MCF-7 cells' proliferation and viability in different ways. On the one hand, the growth of untreated MCF-7 cells can be delineated as asymmetrical multilateral fusiform. The cells appeared bright, clear, refractive, and fully stretched, with intact membrane and large nuclei under an inverted microscope, as shown in Figs. 2a and 2b. On the other hand, cells treated with Tam individually (Figs. 2c and 2d), Z.NP99 alone (Figs. 2e and 2f), and in combination with Z.NP99 (Figs. 2g and 2h) depicted considerably reduced cellular crowding, had fragmented nuclei, round shape, shrunken, increased in internal complexity, and showed apoptotic appearance.

TABLE 2

The effects of Z.NP99 and/or tamoxifen (Tam) treatment on the proliferative percentage of MCF-7 cultured cells using the Trypan blue staining technique. The proliferative percentages of MCF-7 cells treated with Tam (10 μg/mL) were 70%, 57%, 40% after treatment for 24, 48 & 72 h, respectively, while the combination therapy with Z.NP99 recorded 45%, 37% & 27% proliferative percentages during the same exposure times

Numbers	Treatments	Proliferative percentage of MCF-7 cells after		
		24 h	48 h	72 h
1	Untreated cells	95.3 ± 0.75^{a}	97.54 ± 1.38^{a}	98.2 ± 0.69^{a}
2	Tam 1	81.60 ± 0.92^{b}	62.90 ± 1.67^{b}	57.80 ± 1.04^{b}
3	Tam 10	70.00 ± 0.87^{c}	57.10 ± 0.64^{c}	40.10 ± 0.57^{c}
4	Z.NP99	63.30 ± 0.75^{d}	49.00 ± 2.31^{ed}	40.10 ± 0.64^{c}
5	Tam 1 + Z.NP99	51.87 ± 1.10^{e}	44.00 ± 2.30^{d}	34.00 ± 1.73^{d}
6	Tam 10 + Z.NP99	44.60 ± 1.50^{f}	36.73 ± 1.09^{e}	26.90 ± 1.10^{e}

Note: Means with different superscripts (a, b, c, d, e and f) between groups in the same column are significantly different at $p < 0.05$. Cell numbers were counted and data are expressed as the percentage of untreated control. Z: *Ziziphus*, Tam 1 or Tam 10: Tamoxifen at 1 or 10 μg/mL.

TABLE 3

The effects of Z.NP99 and/or tamoxifen (Tam) treatment on the viability percentage of MCF-7 at various duration times using MTT assay. The viability percentages of MCF-7 cells treated with Tam (10 μg/mL) were 76%, 52% & 41% after treatment for 24, 48 & 72 h, respectively, while the combination therapy with Z.NP99 recorded 56%, 40% & 26% viable cells during the same exposure times

Number	Treatments	Viability percentage of MCF-7 cells after		
		24 h	48 h	72 h
1	Untreated cells	91.8 ± 1.04^{a}	95.23 ± 1.73^{a}	96.6 ± 0.92^{a}
2	Tam 1	88.12 ± 0.95^{a}	80.42 ± 0.81^{b}	70.32 ± 1.32^{b}
3	Tam 10	76.20 ± 0.69^{b}	51.83 ± 1.03^{c}	40.95 ± 0.63^{c}
4	Z.NP99	63.50 ± 1.27^{c}	48.63 ± 0.92^{cd}	33.07 ± 0.58^{d}
5	Tam 1 + Z.NP99	63.62 ± 2.07^{c}	46.16 ± 1.27^{cd}	35.86 ± 1.09^{d}
6	Tam 10 + Z.NP99	56.42 ± 0.81^{d}	40.33 ± 0.77^{d}	25.67 ± 0.98^{e}

Note: Means with different superscripts (a, b, c, d, and e) between groups in the same column are significantly different at $p < 0.05$. Cell numbers were counted and data are expressed as the percentage of untreated control. Z: *Ziziphus*, Tam 1 or Tam 10: Tamoxifen at 1 or 10 μg/mL.

DNA fragmentation percentage in MCF-7 cells treated with Z.NP99 and Tam

DNA fragmentation was performed on the harvested cells' DNA treated with Tam at concentrations of 10 & 1 μg/mL, Z.NP99, and the combination of Tam and Z.NP99 for 2 days.

Diphenylamine (DPA) was used to determine the level of DNA fragmentation colorimetrically. DNA profile comparisons on agarose gel electrophoresis were also observed. The migration of fragmented DNA was observed as a smear pattern (Fig. 3a).

The percentage of DNA fragmentation was computed (the rate of fragmented DNA divided by the whole amount of DNA) multiplied by 100, as shown in Table 4. The data showed that DNA fragmentation was observed in MCF-7 cells treated with Tam at the therapeutic dose and/or Z.NP99, but the fragmented DNA became overly dominant after Z.NP99 and Tam in both doses were combined (Fig. 3b).

These observations further supported the apoptotic changes appearing in the microscopic results as mentioned above. The low molecular DNA fragments could be easily detected on the lanes of the combination treatment, and this appearance is a typical characteristic of apoptosis.

Alteration of apoptosis regulating genes expression by Tam and Z.NP99 in MCF-7 cells

Effects on mRNA expression of apoptotic genes (Bax and BCL-2)

The study also sought to ascertain the impact of mRNA expression on apoptotic genetic coding, in particular *Bax* and *BCL-2*. *Bax* is a sturdy proapoptotic gene that leads to cytochrome c release from mitochondria, triggers the caspase pathway, and gives rise to apoptosis.

The *Bax* and *BCL-2* mRNA expression was examined in MCF-7 cells by RT-PCR after each treatment. This was necessary to analyze the effect of Tam, and in combination with Z.NP99, to activate apoptosis in treated MCF-7 cells.

As shown in Table 5 and Fig. 4, 1 μg/mL of Tam treatment-induced trivial *Bax* transcript level elevation when used discretely.

Nonetheless, 10 μg/mL of Tam treatment augmented *Bax*-mRNA expression at 65.5%, compared to untreated MCF-7 cells. *Bax*-mRNA elevation expression was 2.8 times higher than untreated MCF-7 cells (cells treated with 10 μg/mL of Tam and Z.NP99 combination). Conversely, Tam lowered *BCL-2* mRNA expression by approximately

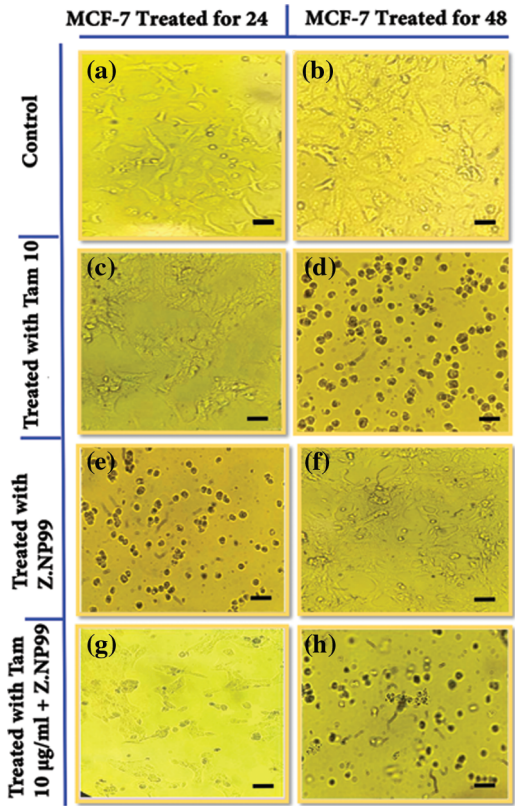

FIGURE 2. Inverted microscope images show changes induced in MCF-7 cells. (a & b) control groups after 24 & 48 h, respectively. The cells appeared bright, clear, refractive, and fully stretched with intact membranes and large nuclei. (c & d) cancer cells treated with tamoxifen (Tam) at a therapeutic dose (10 µg/mL) for 24 & 48 h, respectively. (e & f) treated cells with Z.NP99 for 24 & 48 h, respectively while (g & h) treated cells with the combination of Z.NP99 and Tam (10 µg/mL) for 24 & 48 h, respectively. The cells represent apoptotic characterizations with round shapes, and shrunken and fragmented nuclei. Magnification, X 200.

31.2% compared to untreated cells. The results showed that Z.NP99 could lessen the expression of the anti-apoptotic gene *BCL-2*, elevate the proapoptotic *Bax* gene expression, and reduce the *BCL-2/Bax* ratio, as highlighted in Table 5 and Fig. 4.

Effects on mRNA expression of Claudin-1, VEGF, and MMP-9 genes

The effects of Z.NP99 and Tam demonstrated that they might induce cell apoptosis via modification of other associated genetic coding expressions. This hypothesis was verified by analytically examining Z.NP99 and/or Tam impact on the expression of *Claudin-1*, *VEGF*, and *MMP-9* genes by semi-quantitative RT-PCR and 2% gel electrophoresis of the products from cells treated with different treatments at the indicated concentration for 2 days. Further, on the one hand, after Tam treatment at 1 µg/mL, the results showed nugatory modifications in the mRNA expression of *Claudin-1* (1.011 ± 0.017), *VEGF* (2.686 ± 0.050), and *MMP-9* (1.104 ± 0.031) compared to the untreated cells (1.014 ± 0.058, 2.794 ± 0.057, and 1.118 ± 0.068) respectively as listed in Table 6. On the other hand, when Tam (1 µg/mL) was combined with Z.NP99 a significant reduction in the mRNA expression level of *Claudin-1* and *VEGF* (0.645 ± 0.026, 0.777 ± 0.044, respectively) has observed in MCF-7 treated cells for 48 h. These reductions are less than what was reported for the two genes when cancer cells were treated for two days with Tam at a therapeutic dose (Table 6).

Conversely, Tam (10 µg/mL) and Z.NP99 individually and jointly significantly reduced *Claudin-1*, *VEGF*, and *MMP-9* mRNA expression in MCF-7 treated cells after 2 days. The combination treatment of the drug and/or both extracts synergistically down-regulated the expression of these genes in MCF-7 treated cells, (Table 6 and Fig. 5).

The present data demonstrated that the endogenous expression of *Claudin-1* mRNA was meaningfully reduced by approximately 50% in MCF-7 cells treated with Z.NP99 extracts compared to untreated MCF-7 cells. The natural products mixture was more potent against MCF-7 cells than the conventional drug in the therapeutic dose as shown in Table 6 and Fig. 5. In addition, the co-treatment of BC cells with Z.NP99 and Tam (10 µg/mL) inhibited *Claudin-1*, *VEGF*, and *MMP-9* mRNA expression levels more than each one individually.

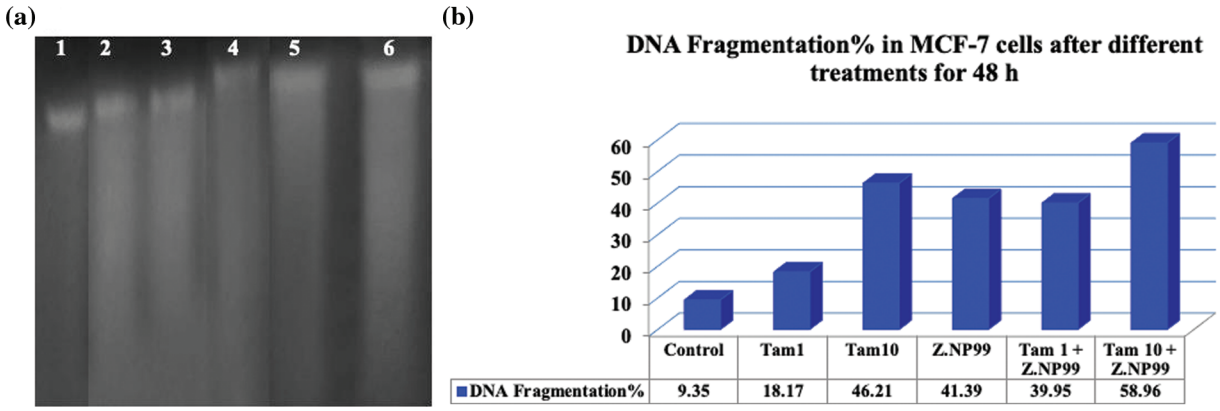

FIGURE 3. DNA fragmentation in MCF-7 cells after different treatments for 48 h. (a) DNA profile on agarose gel electrophoresis illustrates DNA fragmentation which was observed as a smear pattern induced in cancer cells: lane 1 control group, lanes 2 & 3 cells treated with tamoxifen (Tam) at 1 µg/mL and 10 µg/mL, respectively, lane 4 cells treated with Z.NP99, lanes 5 & 6 cells treated with Tam 1 + Z.NP99, and Tam 10 + Z.NP99, respectively. (b) DNA fragmentation is represented in a column chart. Results were obtained from three independent experiments and expressed as mean ± SEM.

TABLE 4

The effects of Z.NP99 and/or Tam treatment on the DNA fragmentation percentage induced in MCF-7 cells after 48 h

Number	Treatments	DNA fragmentation %	
		Mean ± SE	Change
1	Untreated cells	9.35 ± 0.37^{e}	0
2	Tam 1	18.17 ± 0.40^{d}	+8.82
3	Tam 10	46.21 ± 0.69^{b}	+36.86
4	Z.NP99	41.39 ± 0.80^{bc}	+32.04
5	Tam 1 + Z.NP99	39.95 ± 0.58^{c}	+30.60
6	Tam 10 + Z.NP99	58.69 ± 0.92^{a}	+49.34

Note: Means with different superscripts (a, b, c, d, and e) between groups in the same column are significantly different at $p < 0.05$.

TABLE 5

The effect of Z.NP99 and/or Tam treatment on the relative transcription levels of *Bax* and *BCL-2* genes in MCF-7 cells after treatment for 48 h

Number	Treatments	*Bax/GAPDH* Mean ± SE	*BCL-2/GAPDH* Mean ± SE	Ratio *BCL-2/Bax*
1	Untreated cells	0.520 ± 0.011^{d}	0.928 ± 0.016^{a}	1.785 ± 0.049^{a}
2	Tam 1	0.580 ± 0.032^{d}	0.710 ± 0.034^{b}	1.230 ± 0.029^{b}
3	Tam 10	0.861 ± 0.035^{c}	0.622 ± 0.029^{c}	0.722 ± 0.012^{c}
4	Z.NP99	0.851 ± 0.029^{c}	0.501 ± 0.040^{d}	0.589 ± 0.046^{d}
5	Tam 1 + Z.NP99	1.053 ± 0.10^{b}	0.716 ± 0.020^{b}	0.680 ± 0.020^{cd}
6	Tam 10 + Z.NP99	1.496 ± 0.06^{a}	0.523 ± 0.013^{d}	0.351 ± 0.029^{e}

Note: Means with different superscripts (a, b, c, d, and e) between groups in the same column are significantly different at $p < 0.05$. Z: *Ziziphus*, Tam 1 or Tam 10: Tamoxifen at 1 or 10 μg/mL.

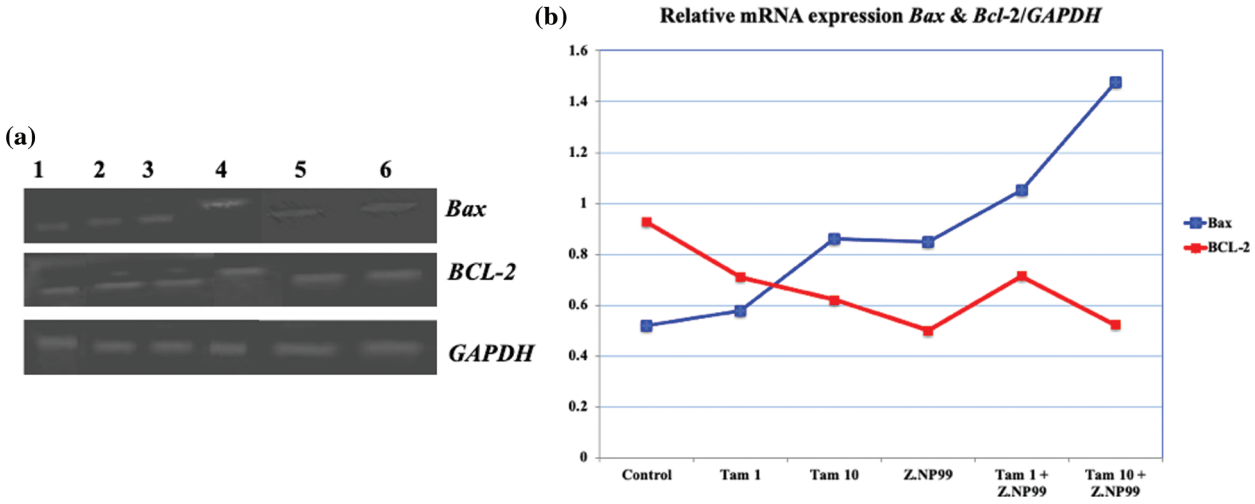

FIGURE 4. Photograph and histogram illustrate the effect of different treatments on the relative transcript level of apoptosis genes (Bax and BCL-2) in MCF-7 cells after 48 h. The figure showed that Z.NP99 individually and/or with tamoxifen (Tam) decreased the expression of the anti-apoptotic gene *BCl-2* and increased the proapoptotic Bax gene expression. (1) Control group cells were treated with (2) Z.NP99, (3) Tam 1, (4) Tam 10, (5) Tam 1 + Z.NP99, and (6) Tam 10 + Z.NP99. Results were obtained from three independent experiments and expressed as mean ± SEM.

TABLE 6

The effects of Z.NP99 and/or Tam treatment on the relative transcription level of *Claudin-1*, *VEGF*, and *MMP-9* genes in MCF-7 cells treated for 48 h

Number	Treatments	*Claudin-1* Mean ± SD	*VEGF* Mean ± SD	*MMP-9* Mean ± SD
1	Untreated cells	1.014 ± 0.058[a]	2.794 ± 0.057[a]	1.118 ± 0.068[a]
2	Tam 1	1.011 ± 0.017[a]	2.686 ± 0.050[a]	1.104 ± 0.031[a]
3	Tam 10	0.674 ± 0.043[b]	1.047 ± 0.027[b]	0.859 ± 0.031[c]
4	Z.NP99	0.554 ± 0.057[c]	0.851 ± 0.030[c]	0.776 ± 0.044[d]
5	Tam 1 + Z.NP99	0.645 ± 0.026[b]	0.777 ± 0.044[d]	0.990 ± 0.052[ab]
6	Tam 10 + Z.NP99	0.479 ± 0.046[d]	0.653 ± 0.054[e]	0.653 ± 0.030[e]

Note: Means with different superscripts (a, b, c, d, and e) between groups in the same column are significantly different at $p < 0.05$. Z: *Ziziphus*, Tam 1 or Tam 10: Tamoxifen at 1 or 10 µg/mL.

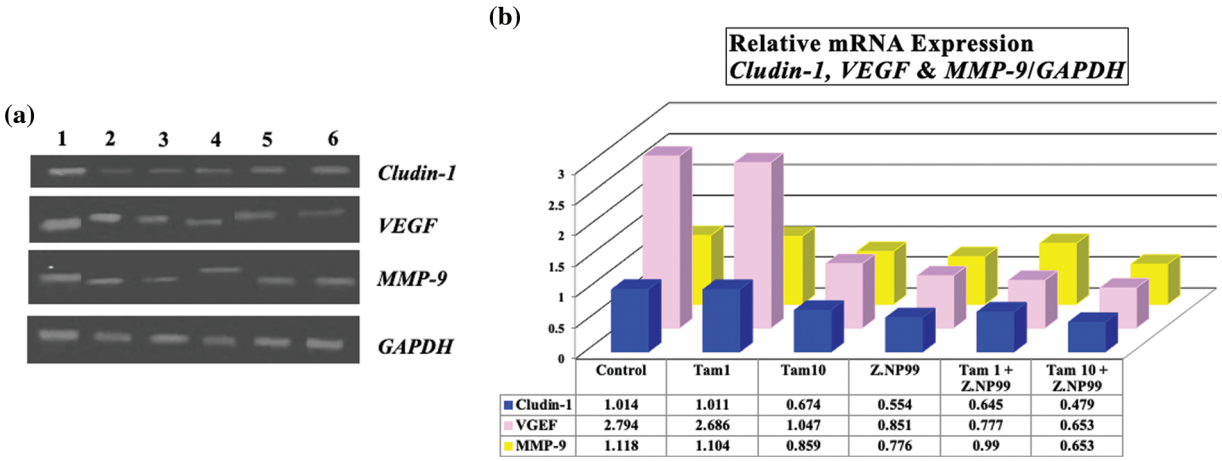

FIGURE 5. Photograph and histogram showing the effect of different treatments on the relative transcript level of *Claudin-1, VEGF*, and *MMP-9* genes in MCF-7 cells after 48 h. It is shown that Tam at 1 µg/mL induced insignificant changes in the mRNA expression of these three genes, while the combination of Tam 1 with Z.NP99 reduced the mRNA expression for all genes. Z.NP99 alone and/or with Tam 10 synergistically downregulated the expression of these genes and the maximum effect induced in MCF-7 cells treated with the therapeutic dose of Tam combined with Z.NP99. (1) Control group, then cells treated with (2) Z.NP99, (3) Tam 1, (4) Tam 10, (5) Tam 1 + Z.NP99 and (6) Tam 10 + Z.NP99. Results were obtained from three independent experiments and expressed as mean ± SEM.

Discussion

In our earlier investigations (Klaab *et al.*, 2018; AlMalki *et al.*, 2021), it has been reported that Tam, at the therapeutic dose of 10 µg/mL or at 1 µg/mL treatment, reduced the viability and proliferation percentage of MCF-7 cancer cells in respect to the control group in time and concentration-dependent manner and the maximum reduction was noticed after treatment for three days. The treatment with *Z. jujube* extract alone induced a significant reduction in the viability of the cell while combination treatment (*Z. jujube* + 10 µg/mL Tam) was dominant against MCF-7 cells (Klaab *et al.*, 2018).

Treatment with NP99 alone inhibited the growth of MCF-7 cells significantly, while co-treatment of 10 µg/mL Tam and 18 µg/mL of NP99 (IC_{50}) showed more reduction in the growth of the MCF-7 cells (AlMalki *et al.*, 2021).

Previous studies have reported *Z. jujube* as a valuable herb with extensive medicinal ingredients and have shown a considerable inhibitory impact on various cancer cell proliferation (Huang, 2008; Vahedi *et al.*, 2008; Plastina *et al.*, 2012). Tannin is a complex molecule with a binary performance. Hence, it is a radical scavenger, a chemopreventive agent, and an inducer of oxidative stress under certain conditions (Bouki *et al.*, 2013). Therefore, tannin displays anticarcinogenic characteristics (Katzenellenbogen *et al.*, 2007; Hamiza *et al.*, 2012; Majed *et al.*, 2015), proapoptotic action by producing reactive oxygen species, and the activation of intrinsic apoptotic pathways (Chen *et al.*, 2009; Chang and Wang, 2013; Chen *et al.*, 2014).

Moreover, combining *Z. jujube* and NP99 individually with Tam could considerably augment the drug's sensitivity, increase apoptotic activities and show a more prominent

reduction in the viability of MCF-7 cells (Klaab *et al.*, 2018; AlMalki *et al.*, 2021). The results of our previous investigations indicate that Tam at 10 μg/mL or 1 μg/mL, when combined with natural extracts, was more sensitive than when used alone (Klaab *et al.*, 2018; AlMalki *et al.*, 2021). This remains an essential observation because drug resistance is the main problem for cancer chemotherapy globally as the search for novel medication with reduced toxicity continues. Additionally, extensive evidence *in vitro* and *in vivo*, shows that the anticancer activity of phytoestrogen is due to apoptosis and cell cycle arrest.

The present data showed that combining Tam with Z. NP99 in IC_{50} values considerably inhibited MCF-7 cell growth and it was more effective and efficient than utilizing Tam alone in treating and managing ER+ BC *in vitro*. The combination treatment showed acute cytotoxic effects in MCF-7-treated cells compared with the untreated group.

Moreover, morphological investigations on MCF-7 have demonstrated that the treated cells were shrunk and became round in shape, and condensation and aggregation of the nuclear chromatin into dense masses beneath the nuclear membrane appeared after being treated with Tam (10 μg/mL) mixed with Z.NP99 for 48 h. Meanwhile, the features of the apoptotic process (as described above) were more pronounced when MCF-7 cells were treated with the combination treatments of Z.NP99 and/or Tam for 72 h. A considerable percentage of the anticancer drugs originating from plants or synthetic are known to cause DNA damage or suppress its replication by promoting apoptosis. During apoptosis, a specific nuclease, referred to as caspase-activated DNase (CAD) and pre-existed in living cells as an inactive complex, breaks the genomic DNA between nucleosomes and generates DNA fragments. This phenomenon has been used lengthily as a marker in cell death studies via apoptotic cell death (Wyllie, 1980; Nagata, 2000). MCF-7 cells treated with Z.NP99 showed a significant increase in DNA fragmentation percentage associated with modulating the gene expression of pro and anti-apoptotic genes like *Bax* and *BCL-2* at the mRNA level.

During apoptosis, damaged and unnecessary cells are removed from the body. Cancer cells are characterized by resistance to apoptosis, which is caused by the overexpression of proteins that block the process (Hanahan and Weinberg, 2000). To control this programmed cell death, pro-death proteins (like Bax) that cause cell death interact dynamically with pro-survival proteins (BCL-2 family). When the balance between these opposing proteins is shifted, malignant cells may be more susceptible to apoptosis (Chipuk *et al.*, 2010; Dewson and Kluck, 2010). The anti-apoptotic BCL-2 family has been shown to contribute to regulating mitochondria-mediated apoptosis (Chao and Korsmeyer, 1998). These anti-apoptotic proteins contain four conserved BCL-2 homology domains (BH1 to BH4) that form heterodimer complexes with members of the proapoptotic family, such as Bax, to prevent mitochondrial release apoptogenic molecules like cytochrome C to the cytosol (Chao and Korsmeyer, 1998; Oltvai *et al.*, 1993; Mazars *et al.*, 2005). Overexpression of BCL-2 triggers resistance to a variety of apoptotic stimuli, including chemotherapy drugs, in many hematological malignancies and solid tumors (Mazars *et al.*, 2005). Using Z.NP99 combination treatment, the anti- and proapoptotic proteins are selectively and differentially regulated to ameliorate the effects of apoptotic stimuli. As a result, the BCL-2 level in MCF-7 cells decreased in a time-dependent manner, while the level of Bax increased as well, resulting in the induction of apoptosis in MCF-7 cells, possibly through the activation of caspase-9 (Booth *et al.*, 2013; Boucher *et al.*, 2012).

Other related genes, such as *Claudin-1*, *VEGF*, and *MMP-9* in mRNA levels, have been examined.

Claudins are usually found in the cell membrane and primarily contribute to cell-cell adhesion (Tsukita *et al.*, 2001; Furuse *et al.*, 2002), and the function of *Claudin-1* may vary among dissimilar cell types. *Claudin-1* is localized to the cell membrane in T47 D cells and in the nucleus and cytoplasm in human colon cancers (Dhawan *et al.*, 2005).

Previous investigations have shown the link between *Claudin-1* expression and cellular resistance in tumors (Dhawan *et al.*, 2005; Yoon *et al.*, 2010).

In cancer cells, increased sensitivity to chemotherapy has been attributed to enhanced apoptosis via the extrinsic apoptotic pathway, and *Claudin-1* was identified as a regulator of these processes. In a similar way, *Claudin-1* may regulate these apoptotic pathways in BC (Zhou *et al.*, 2015). Precisely, cumulative evidence associating the deregulation of Claudins with tumorigenesis suggests that these proteins are essential in multiple cellular processes, including motility and invasion (Dhawan *et al.*, 2005; Agarwal *et al.*, 2005; Oku *et al.*, 2006; Todd *et al.*, 2015).

According to Akasaka *et al.* (2010), *Claudin-1* exerts anti-apoptotic activity and regulates B-catenin and E-cadherin expression and subcellular localization of MCF-7, but not the T47D cells. They also showed that *Claudin-1* expression increased in MCF-7 cells treated with 40 μM Tam, which disagrees with our data and may depend on the Tam concentration used in the treatment. Additionally, their results demonstrated that *Claudin-1* knockdown with or without Tam did not affect apoptosis-related proteins like *Bax, BCL-2, p53*, and *p21* (Akasaka *et al.*, 2010).

Another gene that we have investigated is *VEGF*, a key cytokine involved in tumor growth and development.

Various types of tumors grow invasively and noninvasively through angiogenesis, the formation of new blood vessels that supply oxygen, and nutrients to cancer tissues and remove the waste products. This process is largely mediated by the *VEGF* gene (Ferrara *et al.*, 2003; Kim *et al.*, 1993).

It is known that matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases. The human body expresses at least 23 of the 28 types of MMPs found in vertebrates (Cui *et al.*, 2017). In addition to proteolytic degradation of the extracellular matrix (ECM), alteration of the cell-cell and cell-ECM interactions, migration, and angiogenesis, they play a key role in tumor growth and invasion (Gialeli *et al.*, 2011). Furthermore, they influence the microenvironment

during cancer progression (Kessenbrock *et al.*, 2010). Apoptosis and anti-apoptosis can be induced by matrix-degrading enzymes. On the one hand, cancer cells become resistant to apoptosis and chemotherapy due to the proteolytic activity of the MMPs, which inactivates the Fas receptor in the cell surfaces (Strand *et al.*, 2004; Mitsiades *et al.*, 2001). Another possible strategy by which MMPs induce an anti-apoptotic effect is by indirectly activating serine/threonine kinase Akt/protein kinase B through receptors of endothelial growth factors (EGFR) and IGFR signaling cascades (Gialeli *et al.*, 2009; Kulik *et al.*, 1997). On the other hand, MMPs also can induce apoptosis through an indirect mechanism by altering the extracellular matrix composition (Sympson *et al.*, 1994).

Tumor angiogenesis requires *MMP-9*, which controls the bioavailability of *VEGF*, the most powerful angiogenic factor. For instance, pancreatic tumors are able to undergo an angiogenic switch when the MMP-9 enzyme releases sequestered VEGF to its receptor, VEGFR2 (Bergers *et al.*, 2000). BCL-2 is produced by the action of VEGF (Saleem *et al.*, 2013). Therefore, VEGF inhibits tumor cell apoptosis with its effects on the expression of *BCL-2* and angiogenesis (Pidgeon *et al.*, 2001).

The present study findings also showed that the Z.NP99 combination treatment noticeably contracted *VEGF* and *MMP-9* mRNA expression in MCF-7 treated cells for 48 h. It collectively down-regulated the expression of both genes in MCF-7 treated cells. Besides, Lee *et al.* (2004) observed a reduction in *VEGF* mRNA expression in MCF-7 cells treated with Tam (Lee *et al.*, 2004). This confirmed the current study findings that may explain the antagonistic effect of Tam or phytoestrogens on ER+ breast cancer.

Further, Nilsson *et al.* (2007) showed that estradiol induced a significant decrease in the expression level of this gene and it modulated the *MMP-2* and -9, and activity in BC cells. Therefore, the impact of estrogen and antiestrogen on MMPs has led to conflicting findings (Pilips and McFadden, 2004; Lymperatou *et al.*, 2013). Conversely, Darakhshan *et al.* (2013) reported that a decline in *MMP-9* expression was witnessed after treatment with tranilast alone and in the combined treatment with the Tam. This combination treatment can inhibit growth and proliferation, leading to the angiogenesis of MCF-7 cells. These findings agree with the current investigation results (Darakhshan *et al.*, 2013).

Taken together, Z.NP99 affected the expression of the *MMP-9* gene and reduced its production in MCF-7-treated cells. The presence of MMP-9 protein manages VEGF bioavailability which controls the expression of the anti-apoptotic, *BCL-2*, gene. Thus, we hypothesize that when *MMP-9* works in the anti-apoptotic pathway, its expression is abolished or reduced by the action of Z.NP99, followed by decrease in *VEGF*, which downregulates *BCL-2* expression and induces apoptosis in the treated cell. According to the current study finding, under the same conditions, the action of Z.NP99 on MCF-7 cells is higher than the effect of the Tam therapeutic dose (10 μg/mL). Consequently, the combination treatment of Tam 10 and Z.NP99 was more effective on cancer cells, as observed from the results.

Interestingly, reducing the Tam dose to 1 μg/mL had an insignificant effect on MCF-7 alone, but when it was combined with Z.NP99, it became more effective against cancer cells and represented the same effect as the therapeutic dose of Tam (10 μg/mL).

Based on the results, Tam alone is ineffective in treating and managing cancer. Instead, combining it with other plant extracts and nanoparticles like Z.NP99 agents is ideal because it minimizes the side effects of Tam by reducing the therapeutic dosage. The combination creates synergistic anticancer activities of the mixture treatment making Tam more effective. Thus, this treatment combination is ideal for BC treatment and management as well as future research in the field of oncology.

Finally, this study has some limitations, such as the investigation of the efficacy of Tam, Z.NP99, and the combination of long-term assays like clonogenic assay for evaluation of anticancer efficacy. It is the method of choice for determining the fate of cells after exposure to ionizing radiation. However, the clonogenic assay can also measure other agents that cause cytotoxicity. Colonies can be formed only by a small proportion of the seeded cells *in vitro*. Within one to three weeks following treatment, cells are seeded out in appropriate dilutions to form colonies. At least 50 cells are required to constitute a colony. It tests the ability of each cell in the population to undergo “unlimited” division. (Franken *et al.*, 2006). Additionally, several cancer cells expressing ER+ can be applied, such as MDAMB415, T47D, and CAMA1 (Dai *et al.*, 2017). This study can be carried out *in vivo* using animal models like mice or rats, which will give accurse data.

Conclusion

Tamoxifen has proven to be one of the most influential and widely used hormone-receptor-positive treatments for BC; some take it to prevent BC. The medicine has been accepted broadly because BC tumors have a high affinity for estrogen and progesterone, or even metastasize. Therefore, Tam, being an estrogen receptor regulator, plugs into specific proteins inside BC cells. Once inside the cells, the drug prevents cancer from accessing the hormones necessary for it to grow and spread. However, Tam has been less effective in preventing and treating BC. As a result, this study sought to examine how the drug can be boosted to attain top-level effectiveness in managing BC. The results were promising in BC treatment and further studies in oncology. In this case, nutritional phytochemicals could play a critical role in developing new BC drugs and highlight promising treatment options for the same type of cancer. The novel drugs could present therapeutic benefits by restraining the primary cellular tumorigenesis activities. This study highlighted the importance of herbs in exerting anti-carcinogenic impact due to their capacity to instigate apoptosis. If this is combined with chemotherapy, it will lead to developing a more effective and efficient approach to cancer treatment rather than utilizing a single agent. This research presented considerable dose reduction when

natural extracts and commercial drugs were utilized jointly to treat MCF-7 cells *in vitro*.

To our knowledge, the current work is the first one to show the anticancer properties of Z.NP99 (*Z. jujube* and Tannin) extracts in human breast cancer cells.

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Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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