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Cytotoxic and Metastatic Effects of Anatolian Propolis and Chemotherapeutic Agents (DOX, TAM, CLB) in 2D and 3D Breast Cancer Models

Anadolu Propolisi ve Kemoterapötik Ajanların (DOX, TAM, CLB) 2B ve 3B Meme Kanseri Modellerindeki Sitotoksik ve Metastatik Etkileri

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Abstract

This study aims to comparatively investigate the cytotoxic and metastatic effects of Anatolian propolis and chemotherapeutic agents (Doxorubicin, Tamoxifen, Chlorambucil) on two-dimensional (two-dimensional) and three-dimensional (three-dimensional) breast cancer cell cultures. The triple-negative MDA-MB-231 breast cancer cell line was cultured in two-dimensional and three-dimensional models. Anatolian propolis was prepared using ethanol extraction and applied to the cells alone or in combination with chemotherapeutic agents (Doxorubicin, Tamoxifen, Chlorambucil) at their respective half maximal inhibitory concentration (IC50) doses. Cytotoxicity was assessed using the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, while cell migration and invasion were evaluated using wound healing and invasion assays. Propolis, when combined with chemotherapeutic agents, significantly inhibited cell proliferation and migration ($p<0.001$). Tamoxifen alone exhibited a half maximal inhibitory concentration value of 0.5 micromolar, whereas Tamoxifen + Propolis (40 µg/mL) more effectively suppressed proliferation and migration ($p<0.001$). For Chlorambucil applications, the half maximal inhibitory concentration

value was determined as 10 micromolar, and Chlorambucil + Propolis (40 µg/mL) reduced cell viability while suppressing metastatic activity ($p<0.001$). In three-dimensional cultures, Chlorambucil + Propolis (80 µg/mL) disrupted spheroid integrity, preventing cancer cell dissemination ($p<0.01$). Doxorubicin exhibited a half maximal inhibitory concentration value of 5 micromolar, and Doxorubicin + Propolis (40 µg/mL) increased cell death ($p<0.001$). In three-dimensional cultures, Doxorubicin + Propolis (80 µg/mL) further inhibited cell invasion by breaking down spheroid structures ($p<0.001$). Across all combination groups, propolis enhanced the efficacy of chemotherapeutic agents and significantly suppressed cell migration and invasion ($p<0.001$). The pronounced effects observed in three-dimensional cultures suggest that propolis may act as a potent anti-metastatic agent within the tumor microenvironment. These findings indicate that propolis may serve as a complementary agent to enhance chemotherapy sensitivity; however, further preclinical and clinical studies are required to confirm its clinical applicability.

Keywords: Propolis, Doxorubicin, Tamoxifen, Chlorambucil, MDA-MB-231

Özet

Bu çalışma, Anadolu propolisi ile Doksorubisin, Tamoksifen ve Klorambusil gibi kemoterapötik ajanların iki boyutlu (2B) ve üç boyutlu (3B) meme kanseri hücre kültürleri üzerindeki sitotoksik ve metastatik etkilerini karşılaştırmalı olarak değerlendirmeyi amaçlamaktadır. Üçlü negatif MDA-MB-231 meme kanseri hücre hattı, 2B ve 3B modellerde kültürlenmiştir. Anadolu propolisi etanol ekstraksiyonu ile hazırlanmış ve hücrelere tek başına ya da kemoterapötik ajanlarla birlikte, bu ajanların yarı maksimal inhibitör konsantrasyon (IC50) dozlarında uygulanmıştır. Sitotoksisite, 2,3-bis-(2-metoksi-4-nitro-5-sülfofenil)-2H-tetrazolyum-5-karboksanilid (XTT) testi ile değerlendirilmiş; hücre göçü ve invazyonu ise yara iyileşme ve invazyon testleriyle analiz edilmiştir. Propolis, kemoterapötik ajanlarla birlikte uygulandığında hücre proliferasyonu, göçü ve invazyonunu anlamlı düzeyde baskılamıştır ($p<0.001$). Tamoksifen'in IC50 değeri 0.5 mikromolar olarak belirlenmiş, 40 µg/mL propolis ile uygulandığında proliferasyon ve göç üzerindeki baskılayıcı etkisi artmıştır. Klorambusil için IC50 değeri 10 mikromolar olarak tespit edilmiş ve 40 µg/mL propolis ile hücre canlılığı azalmış, metastatik aktivite bastırılmıştır. 3B kültürlerde 80 µg/mL propolis ile uygulanan Klorambusil, sferoit bütünlüğünü bozarak kanser hücrelerinin yayılımını engellemiştir ($p<0.01$). Doksorubisin'in IC50 değeri 5 mikromolar olarak bulunmuş; 40 µg/mL propolis ile kombinasyonu hücre ölümünü artırmıştır ($p<0.001$). 3B modellerde 80 µg/mL propolis ile uygulanan Doksorubisin, sferoit yapıları parçalayarak invazyonu daha da azaltmıştır ($p<0.001$). Tüm kombinasyon gruplarında propolisin kemoterapötik ajanların etkinliğini artırdığı ve hücre göçü ile invazyonu anlamlı düzeyde baskıladığı gösterilmiştir. 3B kültürlerdeki belirgin etkiler, propolisin tümör mikroçevresinde güçlü bir anti-metastatik ajan olarak rol oynayabileceğini göstermektedir. Bu bulgular, propolisin kemoterapi duyarlılığını artırabilecek tamamlayıcı bir ajan olarak değerlendirilebileceğini ortaya koymakta; ancak klinik uygulanabilirliğini doğrulamak için ileri düzey preklinik ve klinik çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: Propolis, Doksorubisin, Tamoksifen, Klorambusil, MDA-MB-231

Abbreviations: 2D, Two-dimensional; 3D, Three-dimensional; DOX, Doxorubicin; TAM, Tamoxifen; CLB, Chlorambucil; TNBC, Triple-negative breast cancer; ER, Estrogen receptor; PR, Progesterone receptor; HER2, Human epidermal growth factor receptor 2; IC_{50}, Half maximal inhibitory concentration; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; FBS, Fetal bovine serum; ATCC, American Type Culture Collection; RPMI-1640, Roswell Park Memorial Institute Medium; BG, Bee Gum

1. INTRODUCTION

Breast cancer is one of the leading causes of cancer-related mortality, particularly among women, with the highest fatality rates. According to GLOBOCAN 2020 data, breast cancer has been identified as the most diagnosed malignant tumor worldwide, with over 2.2 million new cases recorded (Sung et al., 2021). Breast cancer is divided into distinct subtypes according to the expression levels of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Each subtype possesses unique molecular features, which influence differences in disease progression and responsiveness to treatment (Rouzier et al., 2005).

Triple-negative breast cancer (TNBC) is a unique form of breast cancer defined by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. Representing approximately 20% of all breast cancer cases, TNBC is known for its aggressive nature, exhibiting higher metastatic potential and poorer survival rates compared to other breast cancer subtypes (Kuo et al., 2017).

Doxorubicin (DOX) is an anthracycline derivative and a widely used chemotherapeutic agent for the treatment of various cancers, including breast, lung, stomach, ovarian, thyroid cancers, and pediatric malignancies (Arcamone et al., 1969; Cortes-Funes & Coronado, 2007; Weiss, 1992). Tamoxifen (TAM) is a selective estrogen receptor modulator (SERM) that antagonizes the effects of estrogen in breast tissue. Due to these properties, it is widely used in clinical applications for the treatment of breast cancer (Ali et al., 2016). Chlorambucil (N, N-bis(2-chloroethyl)-p-aminophenyl butyric acid, CLB) is an FDA-approved DNA alkylating agent used in the treatment of chronic lymphocytic leukemia, lymphomas, and advanced-stage ovarian and breast cancers (Ganta et al., 2008).

Various treatment modalities, including surgery, chemotherapy, and radiotherapy, are used alone or in combination for the treatment of breast cancer. However, identifying new and effective therapeutic agents alongside existing treatment strategies is crucial for improving disease management. Apitherapy, the use of bee products for therapeutic purposes, has been practiced since ancient times and is increasingly recognized as a medical adjunct by modern scientific authorities. Among the most extensively studied bee products in apitherapy are honey, propolis, pollen, and royal jelly (Oršolić & Jazvinšćak Jembrek, 2022).

Propolis (bee glue, bee gum) is a natural resinous mixture produced by honeybees (Apis mellifera) from various plant sources, including leaves, flower buds, and tree bark (Iqbal et al., 2019). Propolis can be obtained from various botanical sources, and its chemical composition varies depending on geographical region, local flora, and collection time. Consequently, this variability is a key factor in determining the biological and pharmacological properties of propolis (Stojanović et al., 2020). Over 300 distinct compounds have been detected in propolis. Among these are phenolic acids, flavonoids, terpenes, lignans, amino acids, fatty acids, vitamins, and minerals (Kasote, Bankova, & Viljoen, 2022; Popova et al., 2017). Although GC-MS analysis was not performed within this study, previous studies on Anatolian propolis have identified major bioactive flavonoids such as chrysin, galangin, and pinocembrin (Kartal, Kaya, & Kurucu, 2002; Uzel et al., 2005). These flavonoids have been associated with cytotoxic effects against various cancer cell lines by promoting apoptosis and inhibiting proliferation in previous studies. Research has shown that propolis is a significant natural agent in combating infections due to its antibacterial, antifungal, antiviral, and anti-inflammatory properties (Zulhendri et al., 2022). Additionally, due to its wound-healing properties, tissue regeneration support, and immunomodulatory effects, propolis is gaining increasing interest in dermatology and reconstructive medicine (Yang et al., 2022).

Recent studies have demonstrated that propolis exhibits antitumor and antidiabetic activities by regulating cellular proliferation and reducing oxidative stress (El-Kersh, El-Ezz, Ramadan, & El-Kased, 2024). These findings suggest that the pharmacological potential of propolis extends beyond its traditional uses, making it a promising natural compound for the treatment of cancer, diabetes, and chronic inflammatory diseases. In recent years, there has been a significant increase in research investigating the chemotherapeutic and chemopreventive effects of propolis on key cellular processes involved in cancer development and progression, including apoptosis, autophagy, cytotoxicity, cell proliferation, angiogenesis, migration, and invasion (Altabbal et al., 2023; Hashemi, 2016; Patel, 2016). Recent findings indicate that

propolis can inhibit cancer cell proliferation, angiogenesis, and metastasis while inducing apoptosis (Nguyen et al., 2017; Oršolić & Jazvinšćak Jembrek, 2022; Pai et al., 2018).

In a study conducted by Xuan et al. (2014), propolis exhibited significant time- and dose-dependent cytotoxic effects on MCF-7 and MDA-MB-231 breast cancer cells. The study also demonstrated that propolis induced apoptosis and significantly inhibited migration in MDA-MB-231 cells (Xuan et al., 2014). In another study by Gogacz et al. (2023), propolis was found to significantly reduce cell viability in a dose-dependent manner in MDA-MB-231, MDA-MB-468, MCF-7, and T-47D breast cancer cells (Gogacz et al. 2023).

Two-dimensional (2D) and three-dimensional (3D) cell cultures are widely used in vitro model systems in cancer research. While 2D culture systems allow cells to grow in a single plane, 3D cultures better mimic the tumor microenvironment, particularly cell-cell and cell-matrix interactions, providing more physiologically relevant and clinically meaningful results (Fong et al. 2017; Lv et al. 2017). Therefore, in this study, both 2D and 3D culture models were utilized to comprehensively evaluate the effects of propolis and chemotherapeutic agents. To date, no study in the existing literature has extensively examined the effects of Anatolian propolis in combination with doxorubicin (DOX), tamoxifen (TAM), and chlorambucil (CLB) on the cytotoxic and metastatic properties of cancer cells. In this context, this study aims to contribute to the understanding of how natural compounds like propolis may influence cancer cell proliferation, migration, and invasion when used in combination with chemotherapeutic agents. Specifically, the study investigates the effects of Anatolian propolis, both individually and in combination with widely used chemotherapeutic agents—Doxorubicin, Tamoxifen, and Chlorambucil—at different concentrations on triple-negative breast cancer (TNBC) cells (MDA-MB-231) in two-dimensional (2D) and three-dimensional (3D) culture models.

2. MATERIALS and METHODS

2.1. Cell Culture and Models

The human breast cancer cell line MDA-MB-231 was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin-streptomycin) in a 37°C incubator with 5% CO_2. When the cells reached 80% confluence, they were passaged using 0.05% trypsin-EDTA. To examine cell growth in a three-dimensional (3D) culture environment, the hanging drop method (spheroid model) was utilized. A cell suspension was dispensed as 10 µL droplets onto the upper surface of a petri dish and incubated overnight at

37°C with 5% CO_2. Subsequently, the designated drug doses were added to each droplet, bringing the total volume to 20 µL. The cells were analyzed at 24, 48, and 72 hours using microscopy (Fong et al. 2017; Lv et al. 2017).

2.2. Procurement and Preparation of Test Substances

The chemotherapeutic agents used in this study—doxorubicin (DOX, D1515, Sigma-Aldrich, USA), tamoxifen (TAM, T5648, Sigma-Aldrich, USA), and chlorambucil (CLB, C105, Sigma-Aldrich, USA) —were obtained from commercial sources and used in high-purity powder form. The cell line MDA-MB-231 was obtained from the American Type Culture Collection (ATCC, USA). The following reagents were used in the study: RPMI-1640 (Capricorn Scientific, Germany), Cat. No: RPMI-A (500 mL), Trypsin-EDTA (Capricorn Scientific, Germany), Cat. No: TRY-2B, FBS (Capricorn Scientific, Germany), Cat. No: 10-FBS-16F, XTT (Biological Industries), Cat. No: 20-300-1000 (for 1000 assays), and Penicillin-streptomycin (Capricorn Scientific, Germany), Cat. No: PS-B. Anatolian propolis was collected from local beekeepers in Antalya, Turkey, in March 2024 and stored under dark and dry conditions until analysis. Prior to analysis, 100 mg of propolis was measured and placed into a 10 mL volumetric flask, then diluted to volume with 70% ethanol solution. To enhance solubility, the samples were vortexed and homogenized using a sonicator. The solution was then passed through 0.2 µm sterile filters to obtain a 40 µg/mL propolis concentration, which was stored at -20°C. In all experimental applications, the final ethanol concentration in the culture medium did not exceed 0.1%, a level considered non-toxic in cell culture studies. Vehicle control groups (ethanol + FBS) were included in each assay. Since no significant difference was observed compared to untreated controls, results are not shown. For cytotoxicity assays, a 40 µg/mL propolis concentration was prepared and diluted in a culture medium containing 3% FBS. The study utilized the triple-negative breast cancer cell line MDA-MB-231 (ATCC® HTB-26™), obtained from the American Type Culture Collection (ATCC, USA). The cells were provided by the Medical Pharmacology Laboratory at X University Faculty of Medicine and maintained under standardized cell culture conditions.

2.3. Cytotoxicity Analysis

Cell viability was measured using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay kit (Biological Industries). The effects of propolis, doxorubicin, tamoxifen, and chlorambucil were examined in both 2D and 3D cell models. Cells were seeded into 12-well plates and incubated for 24 hours. After applying the predetermined

drug and combination doses, absorbance measurements were taken following incubation periods of 24, 48, and 72 hours. Absorbance was measured at 450 nm using a Biotek Microplate reader, with the XTT assay kit (Biological Industries). All tests were conducted in accordance with the manufacturer's protocols.

2.3.1. Cytotoxicity Experimental Groups

Group I (Control Group): Control cells cultured without the application of any test substances.

Group II: The cell line was treated with different concentrations of Tamoxifen (TAM) (0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM; 0.5 µM in 3D models).

Grup III: The cell line was treated with different concentrations of Chlorambucil (CLB) (2.5 µM, 5 µM, 10 µM, 25 µM; 2 µM in 3D models).

Grup IV: The cell line was treated with different concentrations of Doxorubicin (DOX) (1 µM, 2.5 µM, 5 µM, 10 µM, 25 µM; 5 µM in 3D models).

Group V (Combination Group-TAM): In 2D models, 40 µg/mL Propolis was combined with Tamoxifen (TAM) at concentrations of 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2.5 µM, and 5 µM. In 3D models, the combinations of 40 µg/mL Propolis+0.5 µM TAM and 80 mg/mL Propolis+0.5 µM TAM were applied.

Grup VI (Combination Group-CLB): In 2D models, 40 µg/mL Propolis was combined with Chlorambucil (CLB) at concentrations of 2.5 µM, 5 µM, 10 µM, and 25 µM. In 3D models, the combinations of 40 µg/mL Propolis+2 µM CLB and 80 mg/mL Propolis+2 µM CLB were applied.

Grup VII (Combination Group-DOX): In 2D models, 40 µg/mL Propolis was combined with Doxorubicin (DOX) at concentrations of 0.1 µM, 2.5 µM, 5 µM, 10 µM, and 25 µM. In 3D models, the combinations of 40 µg/mL Propolis+5 µM DOX and 80 mg/mL Propolis+5 µM DOX were applied.

In cytotoxicity assays, the IC50 value of propolis was determined to be 40 µg/mL, indicating the concentration at which 50% of cell viability was inhibited. Propolis was applied alone at concentrations of 40 µg/mL and 80 µg/mL, and its cytotoxic effects were evaluated in both 2D and 3D cell models.

2.4. Cell Migration Analyses

The wound healing assay was performed to evaluate the metastatic potential of the cells (Jonkman et al., 2014). After reaching 80% confluence, a wound was created in the cell cultures, followed by the addition of the designated agents. Cell migration was monitored under a microscope at 24 and 48 hours of incubation and analyzed using ImageJ software. Each experimental group was analyzed in triplicate unless otherwise stated.

2.5. Statistical Analysis

Data were analyzed using IBM SPSS 23.0. Normal distribution was assessed with the Kolmogorov-Smirnov test, and differences between groups were evaluated using the Mann-Whitney U or Kruskal-Wallis tests (p <0.05 was considered statistically significant).

3. RESULTS and DISCUSSION

In this study, the cytotoxic and metastatic effects of Anatolian propolis and chemotherapeutic agents (DOX, TAM, CLB) were comparatively evaluated in two-dimensional (2D) and three-dimensional (3D) MDA-MB-231 breast cancer cell cultures, both individually and in combination. Our findings indicate that the combination of propolis with chemotherapeutic agents significantly inhibits cell proliferation, migration, and invasion.

3.1. Findings from 2D Cell Culture

The effects of treatments on cell morphology, proliferation, and migration were first evaluated under standard 2D culture conditions (Figures 1–13).

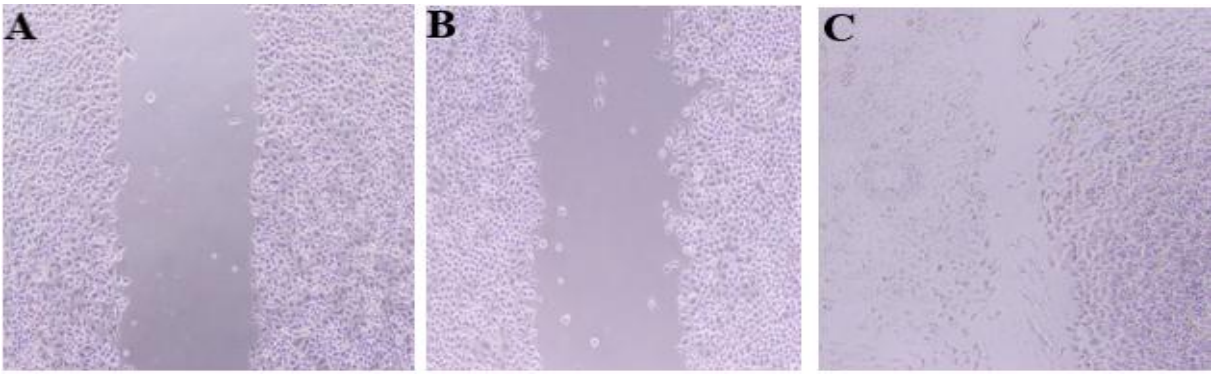

Figure 1. Cell morphology and proliferation of MDA-MB-231 in the control group at 24 hours (A), 48 hours (B), and 72 hours (C) (10X, light microscope).

In the control group, a noticeable increase in wound closure was observed at 48 hours. According to the wound healing assay results, cell migration began within 24 hours, and by 48 hours, the wound area had significantly closed (Figure 1). Compared to the control group, migration rates varied in the other experimental groups ($p < 0.05$).

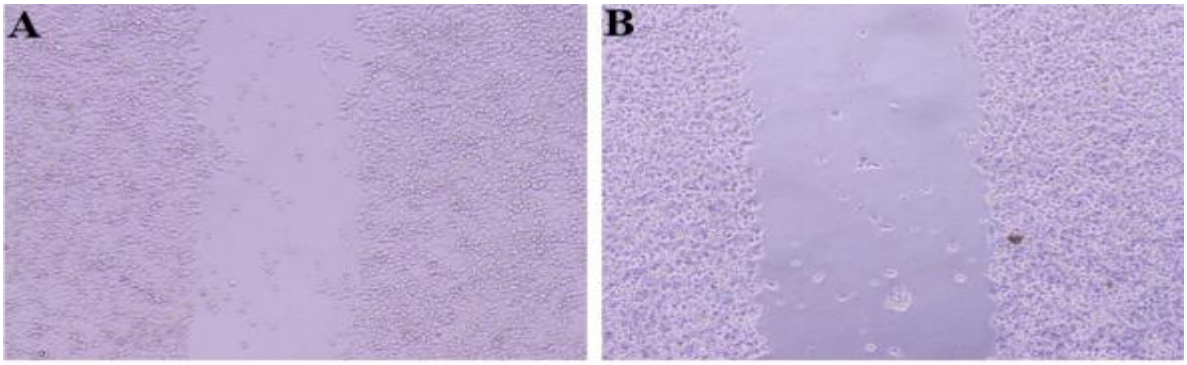

Figure 2. Cell morphology of MDA-MB-231 cells after 24 hours of treatment with 0.5 µM TAM (A) and 0.5 µM TAM+40 µg/mL Propolis (B) (10X, light microscope).

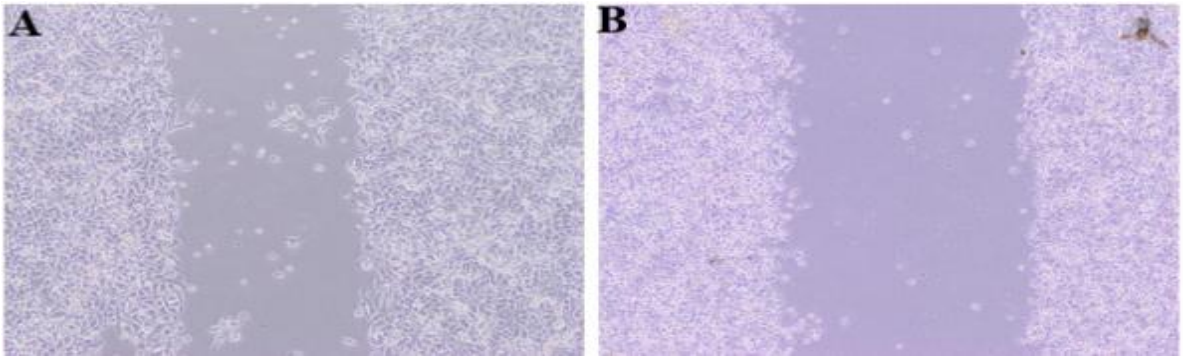

Figure 3. Cell morphology of MDA-MB-231 cells after 48 hours of treatment with 0.5 µM TAM (A) and 0.5 µM TAM+40 µg/mL Propolis (B) (10X, light microscope).

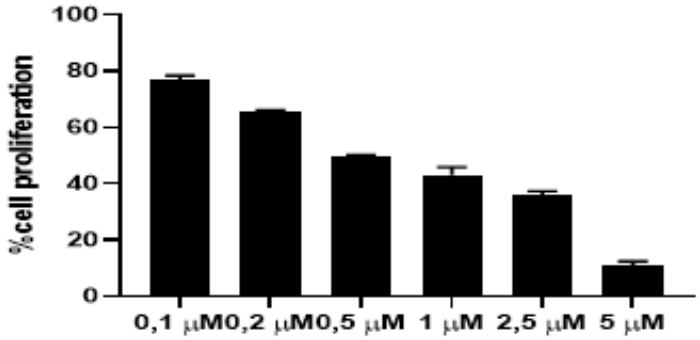

Figure 4. Effects of Tamoxifen (TAM) on MDA-MB-231 cell proliferation (48 hours, 0.1–5 µM).

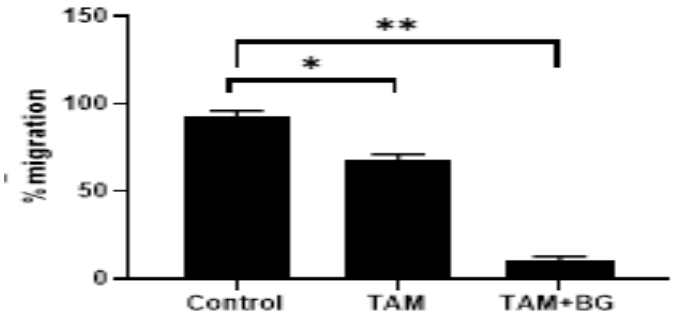

Figure 5. Effects of Tamoxifen (TAM) and TAM+Propolis (Bee Gum, BG) combination on MDA-MB-231 cell migration. Statistical significance between treatment groups is indicated: * $p < 0.05$, ** $p < 0.01$.

In this study, the cytotoxic and migratory effects of Tamoxifen (TAM) on the MDA-MB-231 cell line were evaluated. Cells were treated with 0.5 µM TAM alone and in combination with 40 µg/mL propolis. Cytotoxicity assays determined the IC50 value of TAM to be 0.5 µM. As shown in Figures 2–5, the combination of TAM and propolis significantly inhibited cell proliferation and migration compared to TAM alone ($p < 0.001$). These findings align with the study by Maria et al. (2017), which reported that the combination of TAM and DOX altered the metabolic profile of MDA-MB-231 cells by reducing phosphocholine levels (Maria et al., 2017). Similarly, Gogacz et al. (2023) reported that propolis inhibited proliferation in MDA-MB-231 cells (Gogacz et al., 2023). This suggests that propolis may exhibit a stronger anti-proliferative effect when used in combination with TAM. Additionally, the study by Liu et al. (2014) demonstrated that TAM promotes cell death by activating apoptotic mechanisms in ER-negative cells (Liu et al., 2014). This suggests that TAM can activate apoptotic pathways and that propolis may enhance this effect. However, some studies indicate that TAM may have limited efficacy in MDA-MB-231 cells. Majumdar (2012) reported that MDA-MB-231 cells exhibit lower sensitivity to TAM and that their cloning capacity is not significantly affected by TAM treatment (Majumdar, 2012).

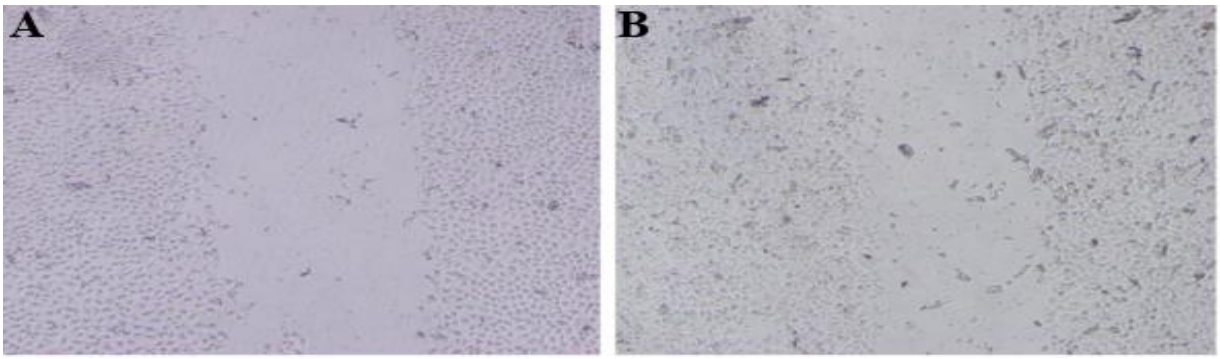

Figure 6. Cell morphology of MDA-MB-231 cells after 24 hours of treatment with 10 µM CLB (A) and 10 µM CLB+40 µg/mL Propolis (B) (10X, light microscope).

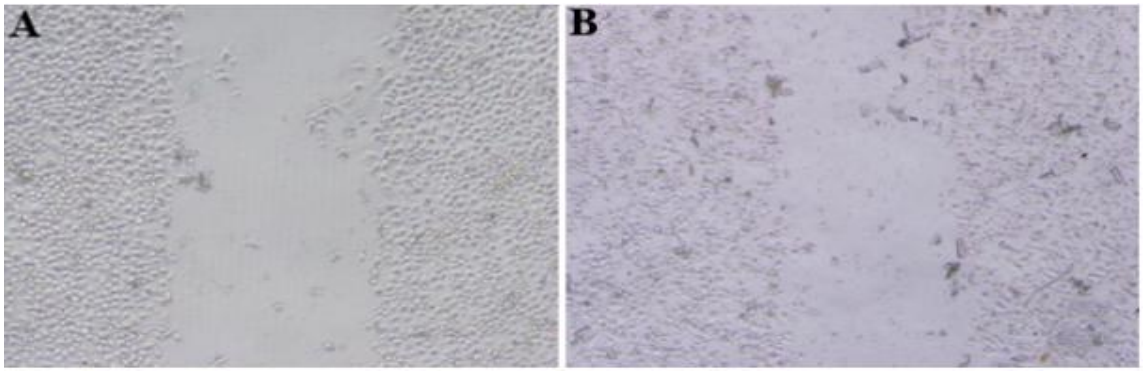

Figure 7. Cell morphology of MDA-MB-231 cells after 48 hours of treatment with 10 µM CLB (A) and 10 µM CLB+40 µg/mL Propolis (B) (10X, light microscope).

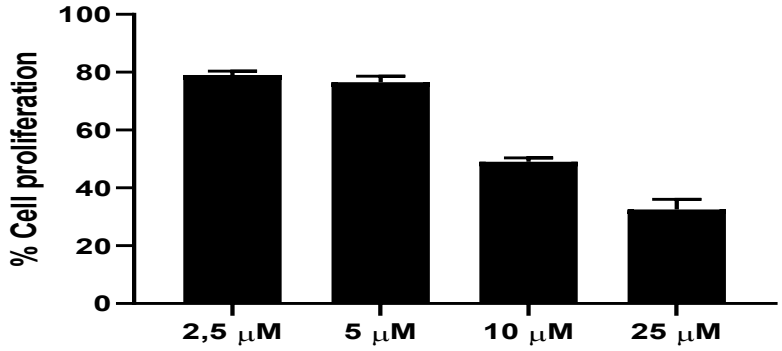

Figure 8. Effects of Chlorambucil (CLB) on MDA-MB-231 cell proliferation (48 hours, 2.5–25 µM).

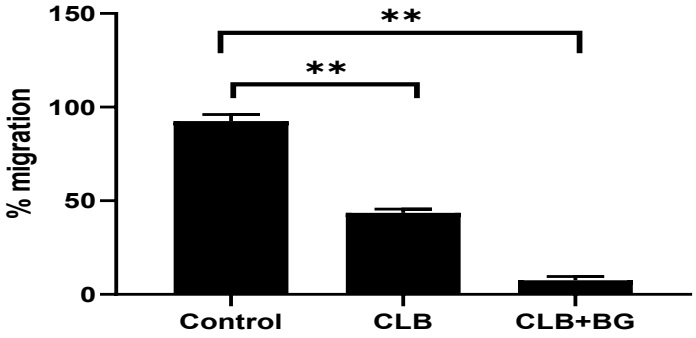

Figure 9. Effects of Chlorambucil (CLB) and CLB+Propolis (Bee Gum, BG) combination on MDA-MB-231 cell migration. Statistical significance between treatment groups is indicated: * $p < 0.05$, ** $p < 0.01$.

In our study, the cytotoxic and migratory effects of Chlorambucil (CLB) and 40 µg/mL propolis on the MDA-MB-231 cell line were evaluated. Cytotoxicity assays determined the IC50 value of CLB to be 10 µM. As shown in Figures 6–9, the combination of CLB and propolis significantly inhibited cell proliferation and migration compared to CLB alone ($p < 0.001$). These findings are consistent with the study by Xuan et al. (2014), which reported that propolis exhibits dose-dependent cytotoxic effects and reduces metastatic potential in MDA-MB-231 cells (Xuan et al., 2014). Additionally, Rouibah et al. (2021) demonstrated that propolis, when

combined with chemotherapeutic agents, reduces multidrug resistance and induces apoptosis by arresting the cell cycle in the S phase (Rouibah et al., 2021). On the other hand, another study reported that while CLB alone exhibits high cytotoxic activity, CLB conjugated to dendrimers demonstrates an even stronger anti-proliferative effect (Bielawski et al., 2011).

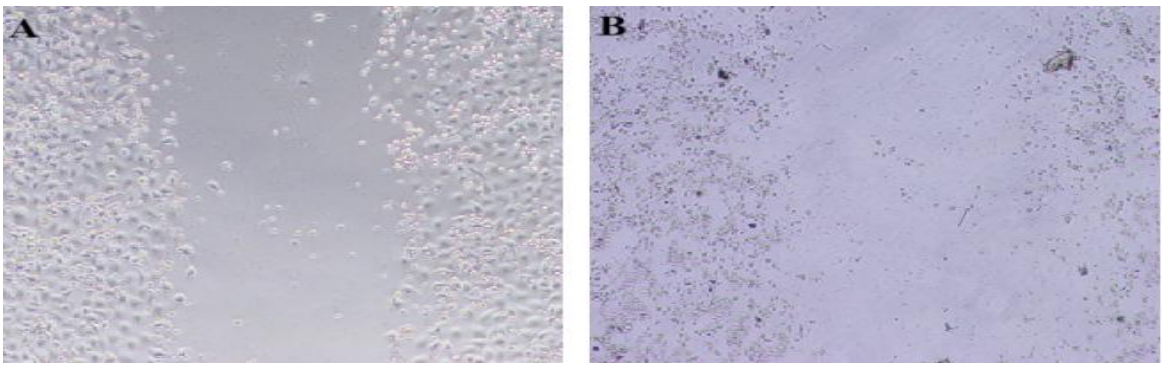

Figure 10. Cell morphology of MDA-MB-231 cells after 24 hours of treatment with 5 µM Doxorubicin (DOX) (A) and 5 µM DOX+40 µg/mL Propolis (B) (10X, light microscope).

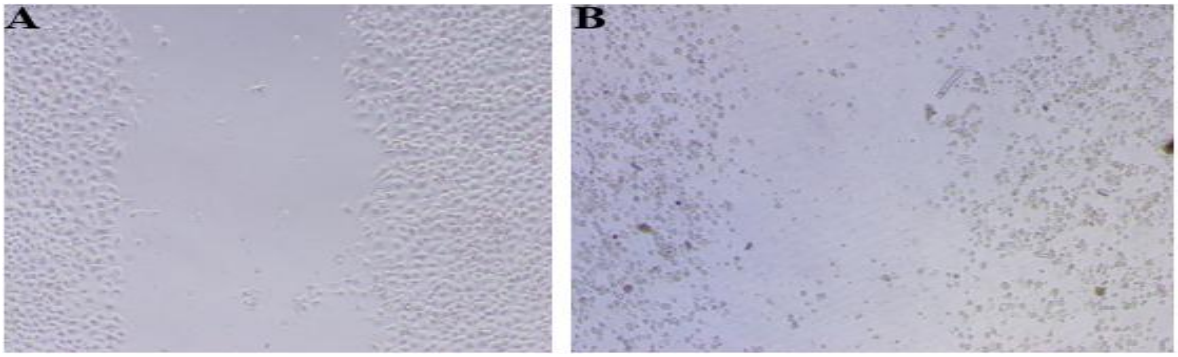

Figure 11. Cell morphology of MDA-MB-231 cells after 48 hours of treatment with 5 µM Doxorubicin (DOX) (A) and 5 µM DOX + 40 µg/mL Propolis (B) (10X, light microscope).

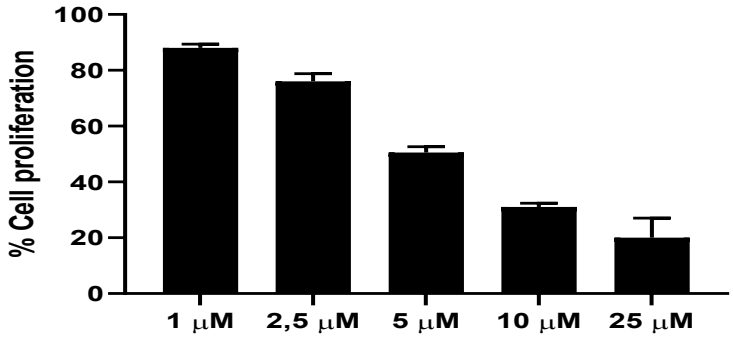

Figure 12. Effects of Doxorubicin (DOX) on MDA-MB-231 cell proliferation (48 hours, 1–25 µM).

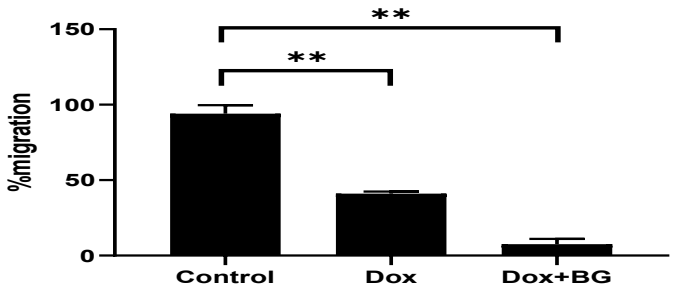

Figure 13. Effects of Doxorubicin (DOX) and DOX+Propolis (Bee Gum, BG) combinations on MDA-MB-231 cell migration. Statistical significance between treatment groups is indicated: * $p < 0.05$, ** $p < 0.01$.

The cytotoxic and migratory effects of Doxorubicin (DOX) on the MDA-MB-231 cell line were evaluated. Additionally, cells were treated with DOX in combination with 40 µg/mL propolis. Cytotoxicity analysis determined the IC50 value of DOX to be 5 µM. As shown in Figures 10–13, the combination of DOX and propolis significantly inhibited cell proliferation and migration ($p < 0.001$). The study by Rouibah et al. (2021) also demonstrated that the combination of DOX and propolis suppressed cell proliferation and arrested the cell cycle in the S phase (Rouibah et al., 2021). The study by Caner et al. (2021) also reported that the combination of bee bread and DOX suppressed metastatic spread (Caner, Onal, & Silici, 2021). Our findings support that propolis, when combined with DOX, can enhance chemotherapy efficacy.

3.2. Findings from 3D Cell Culture

Due to its ability to better mimic the tumor microenvironment, a three-dimensional (3D) cell culture system was employed. Initially, the morphology and proliferation of untreated MDA-MB-231 cells were assessed over time. Subsequently, the combination of propolis and chemotherapeutic agents was found to disrupt spheroid integrity, which may contribute to the inhibition of migration and invasion processes within the tumor microenvironment (Figures 14–26).

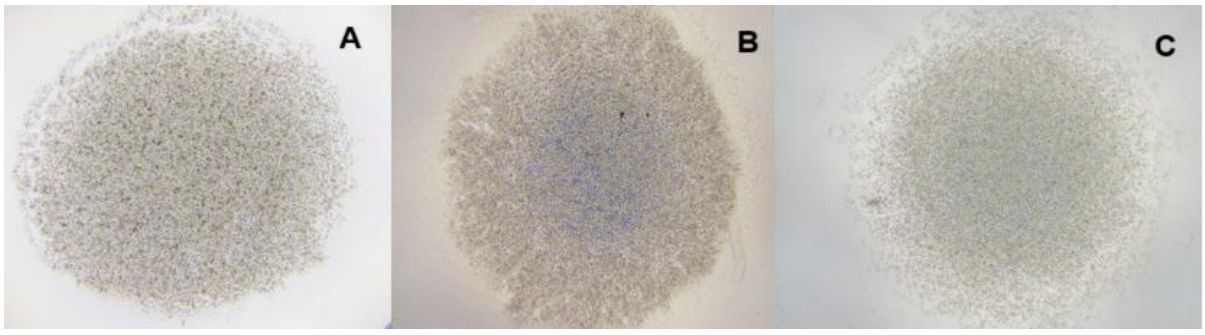

Figure 14. Cell morphology and proliferation of MDA-MB-231 cells in the control group during 24 (A), 48 (B), and 72 hours (C) of incubation (10X, light microscope). Cells were cultured without any treatment, and images were captured using a light microscope at 10X magnification.

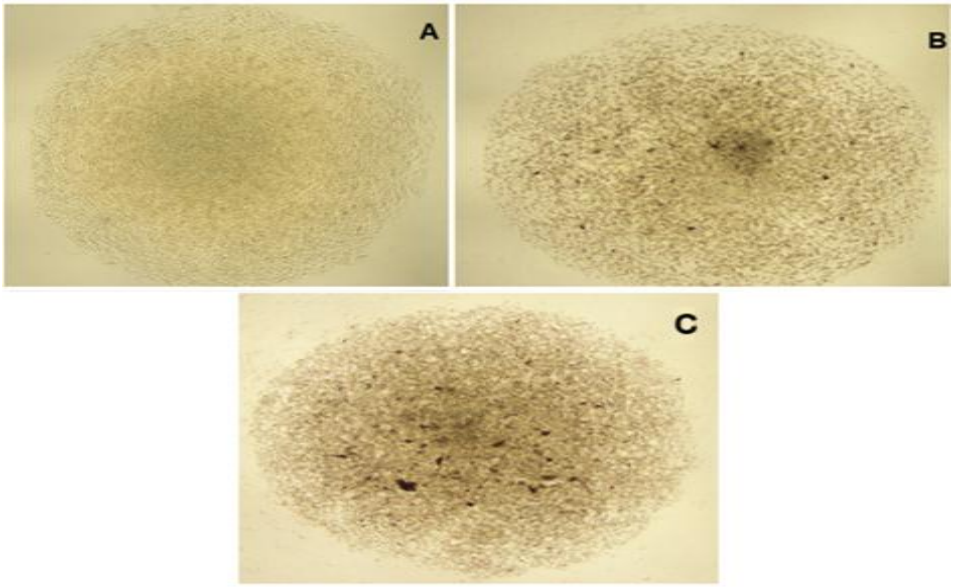

Figure 15. Effects of TAM and TAM+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (24 hours). (A) 0.5 µM TAM, (B) 0.5 µM TAM+40 µg/mL BG, (C) 0.5 µM TAM+80 mg/mL BG (2X) (10X, light microscope).

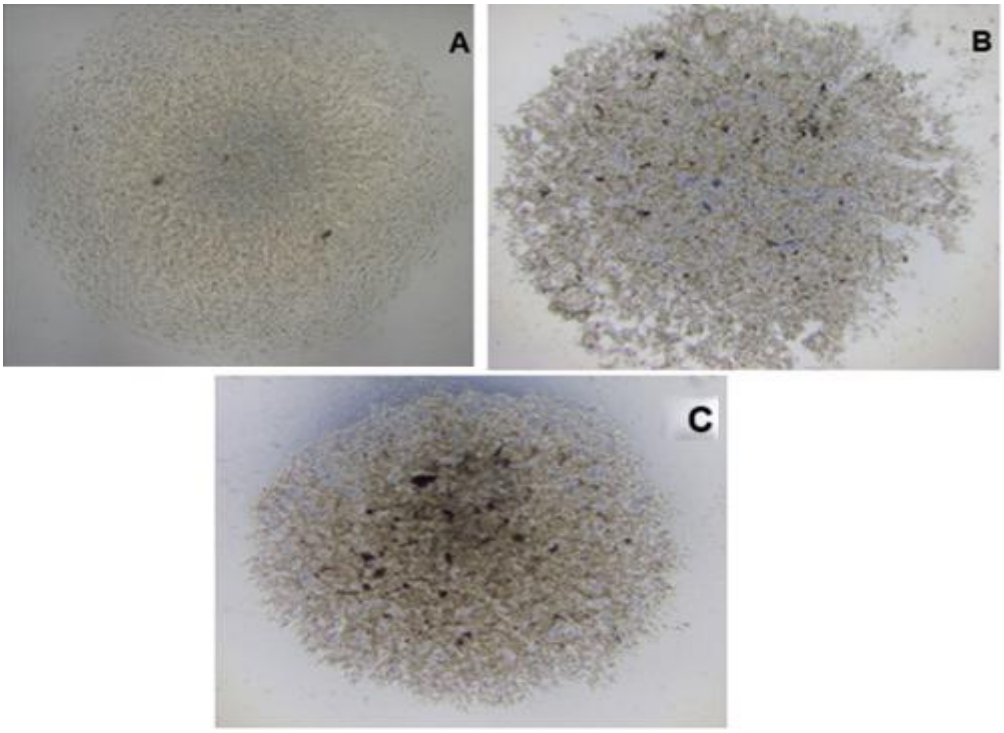

Figure 16. Effects of TAM and TAM+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (48 hours). (A) 0.5 µM TAM, (B) 0.5 µM TAM+40 µg/mL BG, (C) 0.5 µM TAM+80 mg/mL BG (2X) (10X, light microscope).

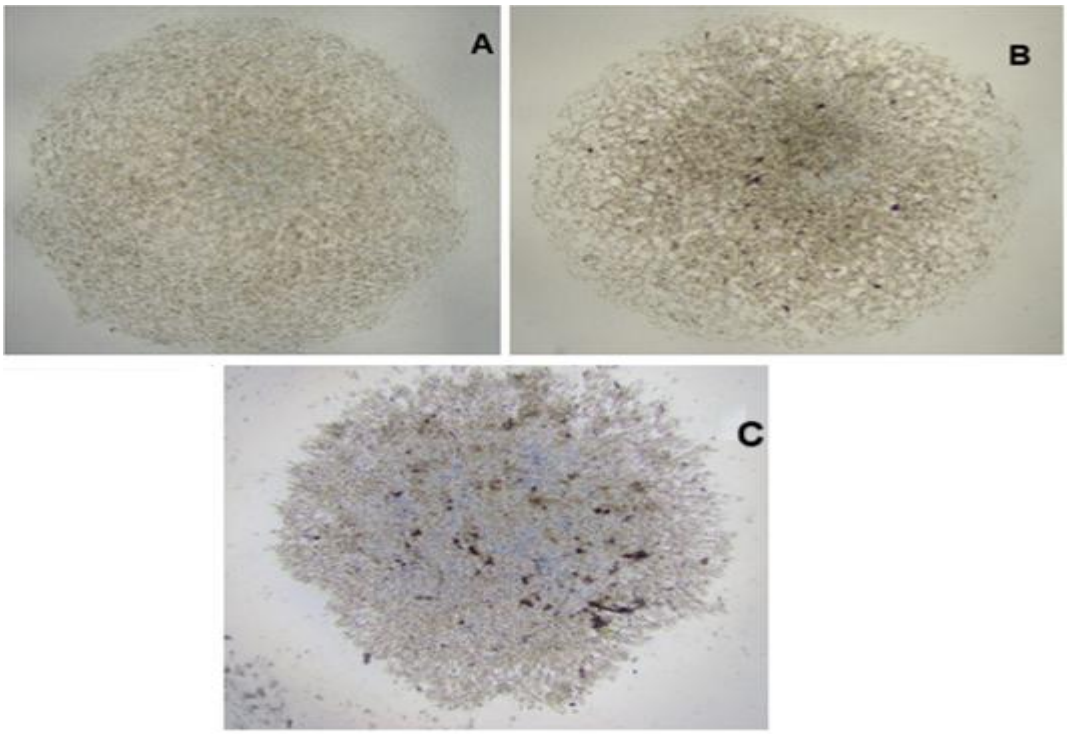

Figure 17. Effects of TAM and TAM+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (72 hours). (A) 0.5 µM TAM, (B) 0.5 µM TAM+40 µg/mL BG, (C) 0.5 µM TAM+80 mg/mL BG (2X) (10X, light microscope).

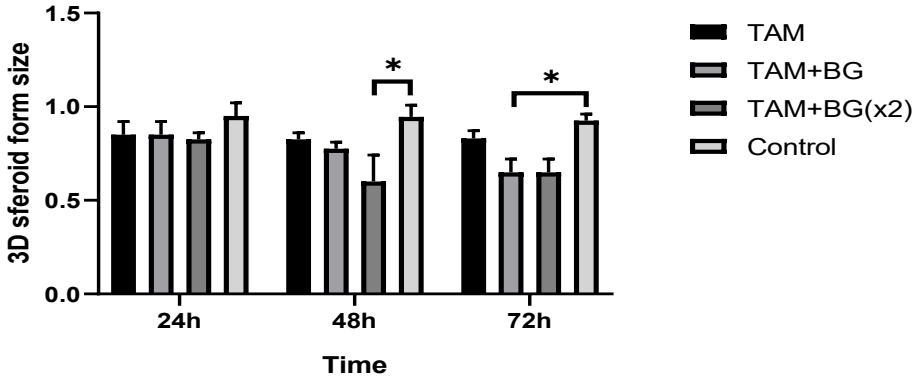

Figure 18. Effects of TAM, TAM+Propolis (Bee Gum, BG), and TAM+Propolis (2X) on 3D MDA-MB-231 spheroids at 24, 48, and 72 hours. Statistical significance between treatment groups is indicated: * $p < 0.05$, ** $p < 0.01$.

In our study, the effects of Tamoxifen (TAM), TAM + Propolis, and TAM + Propolis (2X) on the 3D spheroid structures of the MDA-MB-231 cell line were evaluated. After 48 hours of incubation, a statistically significant alteration in spheroid structure was observed in the TAM + Propolis (2X) group compared to the control group ($p < 0.05$, Figures 15–18). These findings align with the study by Rosales et al. (2018), which reported that TAM suppressed cell

growth in a 3D spheroid environment and that drug effects became more pronounced in models that better mimic the tumor microenvironment compared to 2D cell cultures (Rosales et al., 2018). In contrast, a study on TAM-resistant breast cancer cells demonstrated that HOXB5 enhances 3D spheroid formation, promoting tumor aggression and progression (Kim et al., 2021). This suggests that the resistance mechanisms that may develop against TAM and the potential role of propolis in this process should be further investigated in detail.

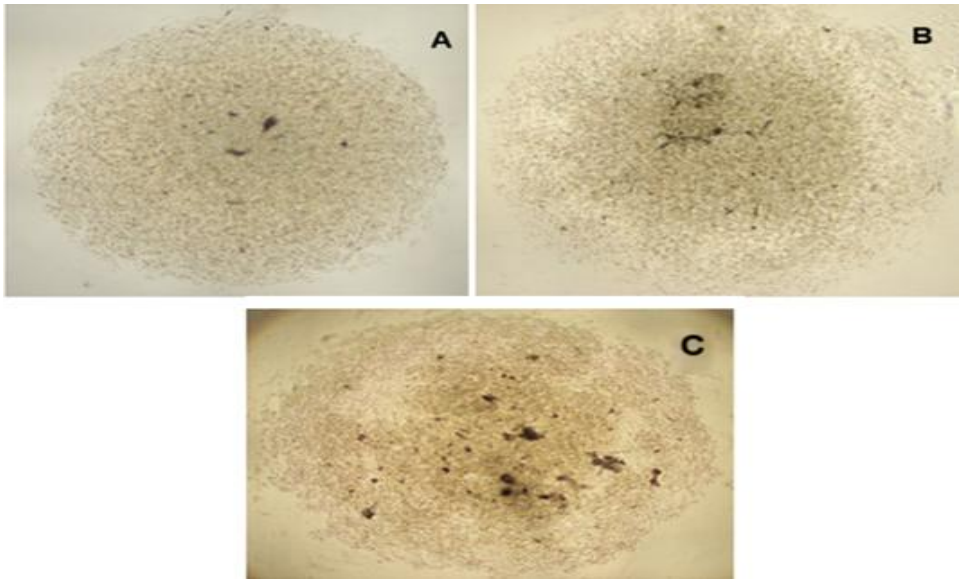

Figure 19. Effects of CLB and CLB+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (24 hours). (A) 10 µM CLB, (B) 10 µM CLB+40 µg/mL BG, (C) 10 µM CLB+80 mg/mL BG (2X) (10X, light microscope).

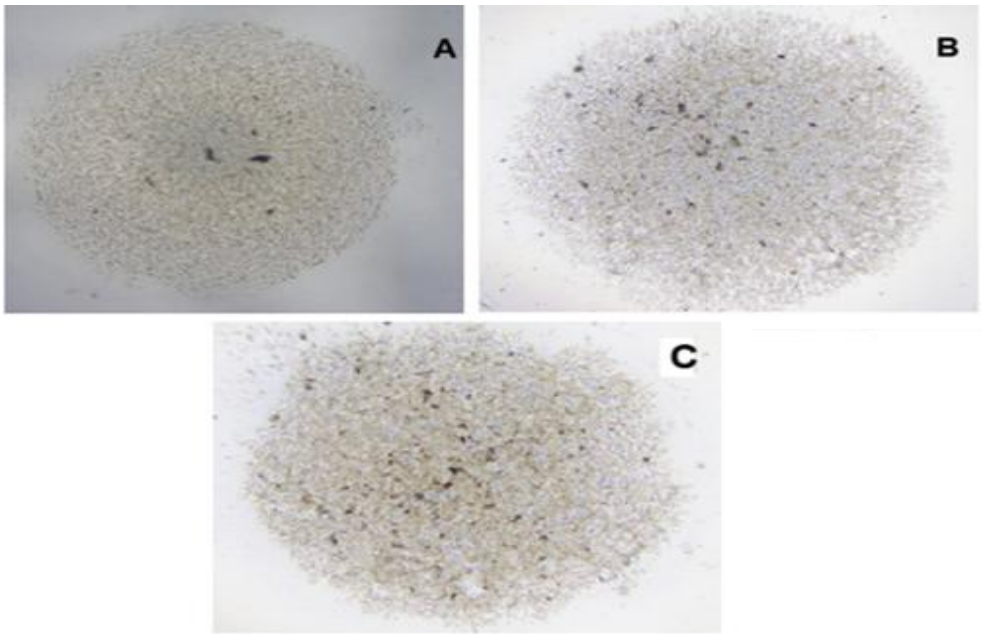

Figure 20. Effects of CLB and CLB+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (48 hours). (A) 10 µM CLB, (B) 10 µM CLB+40 µg/mL BG, (C) 10 µM CLB+80 mg/mL BG (2X) (10X, light microscope).

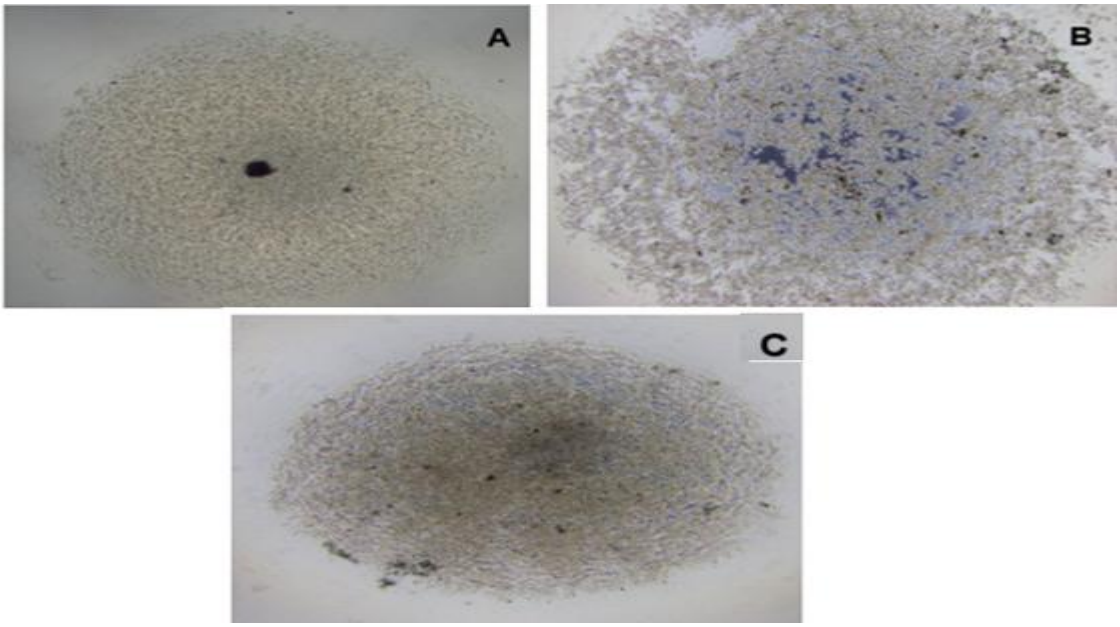

Figure 21. Effects of CLB and CLB+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (72 hours). (A) 10 µM CLB, (B) 10 µM CLB+40 µg/mL BG, (C) 10 µM CLB+80 mg/mL BG (2X) (10X, light microscope).

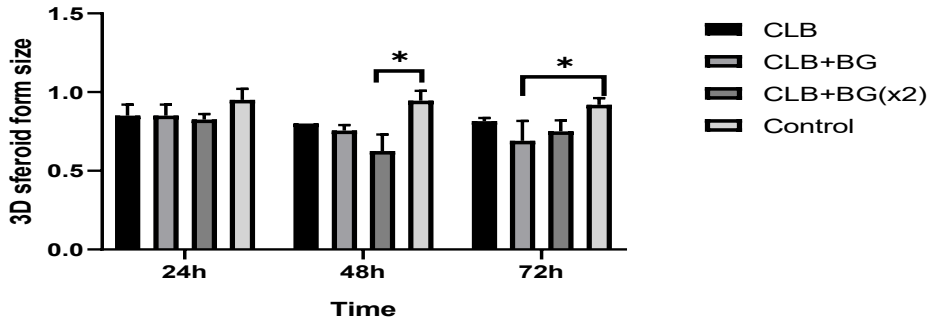

Figure 22. Effects of CLB, CLB+Propolis (Bee Gum, BG), and CLB+Propolis (2X) on 3D MDA-MB-231 spheroids at 24, 48, and 72 hours. Statistical significance between treatment groups is indicated: * $p < 0.05$, ** $p < 0.01$.

In this study, the effects of Chlorambucil (CLB), CLB+Propolis, and CLB+Propolis (2X) on 3D spheroid structures in the MDA-MB-231 cell line were examined. A statistically significant alteration was observed in the CLB+Propolis (2X) group at 48 hours, and in the CLB+Propolis group at 72 hours, compared to the control group ($p < 0.05$, Figures 19–22). Our findings are consistent with the study by Mengji et al. (2024), which reported that CLB enhances tumor regression in a 3D breast cancer spheroid model (Mengji et al., 2024). On the other hand, another study reported that CLB alone has a limited effect on 3D colorectal cancer spheroids, but its efficacy can be enhanced through combination therapies (Montagner et al., 2018). In conclusion, the combination of CLB and propolis exhibits an anti-proliferative effect in 3D spheroid models.

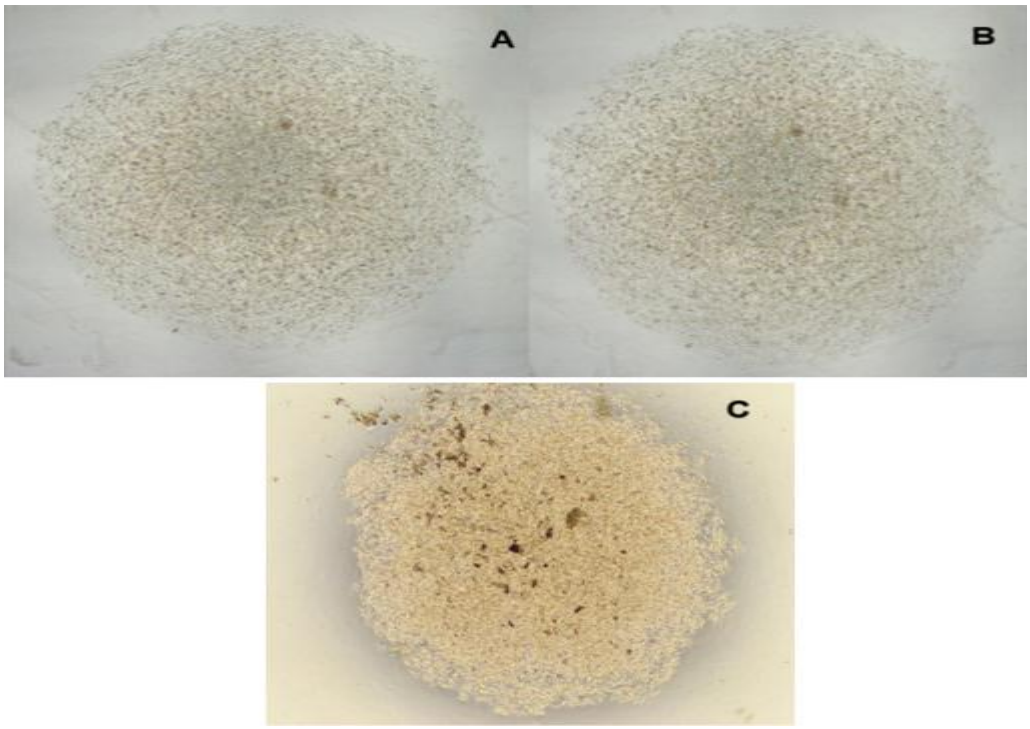

Figure 23. Effects of DOX and DOX+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (24 hours). (A) 5 µM DOX, (B) 5 µM DOX+40 µg/mL BG, (C) 5 µM DOX+80 mg/mL BG (2X) (10X, light microscope).

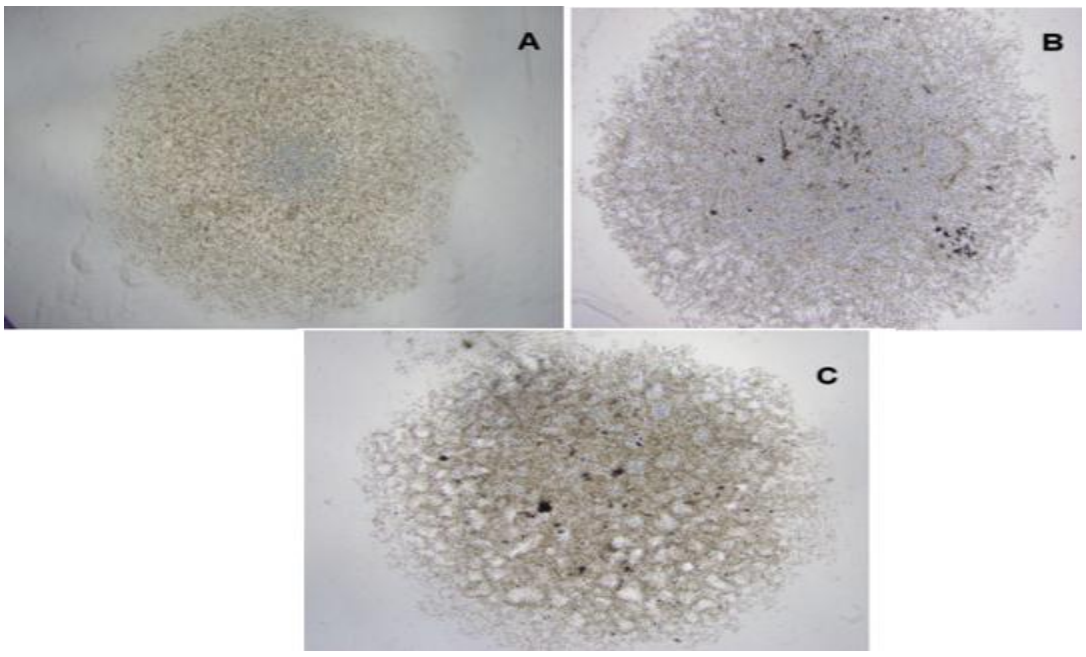

Figure 24. Effects of DOX and DOX+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (48 hours). (A) 5 µM DOX, (B) 5 µM DOX+40 µg/mL BG, (C) 5 µM DOX+80 mg/mL BG (2X) (10X, light microscope).

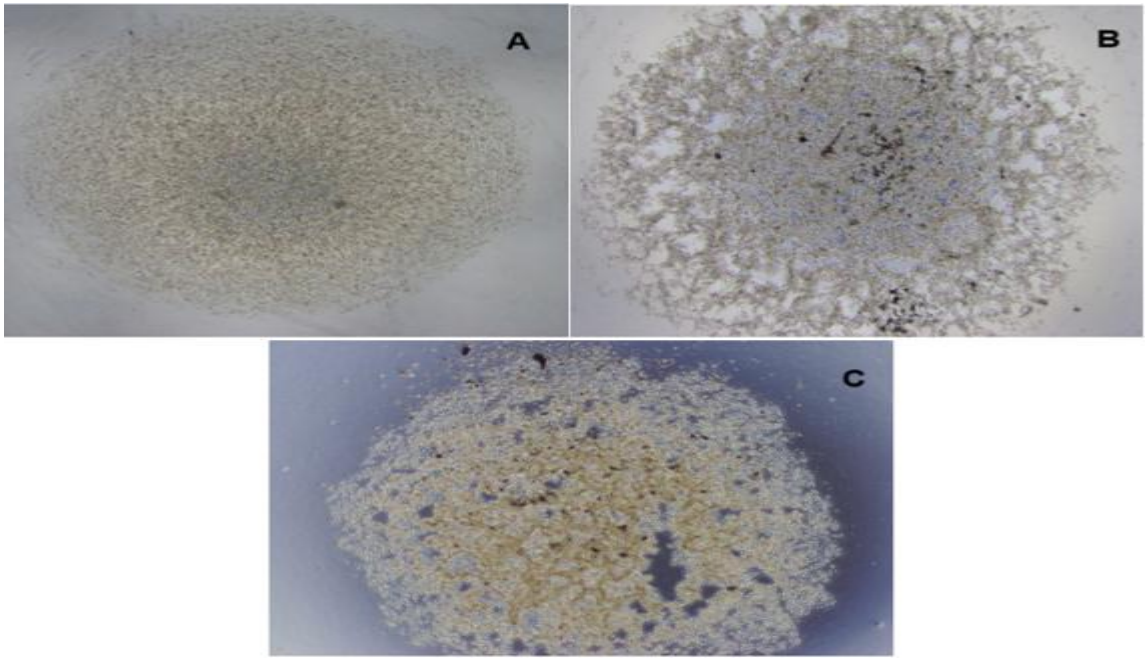

Figure 25. Effects of DOX and DOX+Propolis (Bee Gum, BG) on 3D MDA-MB-231 cells (72 hours). (A) 5 µM DOX, (B) 5 µM DOX+40 µg/mL BG, (C) 5 µM DOX+80 mg/mL BG (2X) (10X, light microscope).

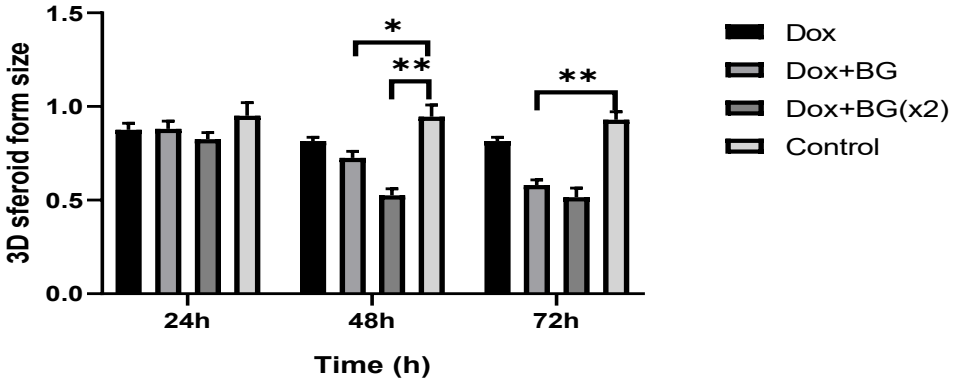

Figure 26. Effects of DOX, DOX+Propolis (Bee Gum, BG), and DOX+Propolis (2X) on 3D MDA-MB-231 spheroids at 24, 48, and 72 hours. Statistical significance between treatment groups is indicated: * $p < 0.05$, ** $p < 0.01$.

The effects of Doxorubicin (DOX), DOX+Propolis, and DOX+Propolis (2X) on 3D spheroid structures were evaluated. At the end of 48 and 72 hours of incubation, the DOX+Propolis (2X) group showed a statistically significant alteration in spheroid structure compared to the control group ($p < 0.001$, Figures 23–26). Additionally, the DOX+Propolis combination suppressed cell invasion in the 3D culture, indicating a potential role in limiting metastatic-related cellular behavior ($p < 0.001$). The study by Eralp et al. (2024) also reported that the combination of Abemaciclib and DOX exhibited a synergistic effect in MDA-MB-231 cells (Eralp, Sevinc, & Mansuroglu, 2024). Our findings suggest that propolis may similarly

enhance the effects of DOX on the tumor microenvironment. The cytotoxicity results showed that the IC50 value of propolis was found to be 40 µg/mL, demonstrating its significant inhibitory effect on cell proliferation. Propolis exhibited dose-dependent cytotoxicity in both 2D and 3D models, with the highest dose (80 µg/mL) showing the most pronounced effect in inhibiting cell viability (Duran, 2024).

4. CONCLUSION

This study demonstrates that propolis can enhance the effects of DOX, TAM, and CLB on cancer cells when applied individually, in combination, and at different doses. The findings reveal that when propolis is used alongside chemotherapeutic agents, it significantly suppresses cell proliferation and metastatic spread ($p < 0.001$). The IC50 value of DOX was determined to be 5 µM, and the DOX+Propolis combination was observed to increase cell death rates ($p < 0.001$). The IC50 value of CLB was 10 µM, and the CLB+Propolis combination significantly reduced cell viability and inhibited metastatic activity ($p < 0.001$). The IC50 value of TAM was 0.5 µM, and the TAM+Propolis combination was found to suppress proliferation and migration ($p < 0.001$).

In the 3D culture environment, the combination of propolis and chemotherapeutic agents disrupted spheroid integrity, which may contribute to the inhibition of migration and invasion processes within the tumor microenvironment ($p < 0.01$). These results suggest that the co-administration of propolis with chemotherapeutic agents may enhance treatment efficacy and could be considered a supportive agent in cancer therapy. However, further preclinical and clinical studies are necessary to evaluate the clinical efficacy and safety of these combinations.

ACKNOWLEDGMENTS

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DECLARATIONS

The authors declare that the research was conducted in an environment in which there was no commercial or financial relationship with any institution or person that could be construed as a potential conflict of interest.

AUTHOR CONTRUBITIONS

All authors contributed significantly to the study. Habibe Sema ARSLAN designed and supervised the study, performed experiments, and contributed to manuscript writing. Serap YALÇIN AZARKAN handled data collection and analysis. Gamze TURNA SALTOGLU critically reviewed the manuscript. All authors approved the final version. Raw data are available from the corresponding author upon reasonable request.

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