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ADDITIVE EFFECTS OF *HELIX ASPERSA* MUCUS FRACTIONS WITH CHEMOTHERAPY DRUGS IN BREAST CANCER: EXPLORING ANTI-PROLIFERATIVE POTENTIAL

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Abstract

This study investigated the anti-proliferative effects of biologically active substances (BAS) derived from the mucus of the garden snail *Helix aspersa* on breast cancer cell lines (MCF-7 and MDA-MB-231) and a non-cancerous cell line (MCF-10A). Two mucus fractions with different molecular weights (Mw) were examined, and their interactions with the chemotherapy drugs cisplatin and tamoxifen were evaluated.

The results showed that the mucus fractions exhibited a slight anti-proliferative effect on breast cancer cells, with the fraction having Mw above 50 kDa demonstrating stronger activity compared to the fraction with Mw above 20 kDa. Furthermore, the combination of the mucus fractions with cisplatin or tamoxifen resulted in significant reductions in the half effective dose, indicating possible synergistic effects which were more pronounced with the fraction having Mw above 20 kDa. Dose-response curves and microscopic images supported the presence of such effects, revealing lower IC50 values and significant cell death

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when the cancer cells were treated with the combination of the mucus fractions and chemotherapy drugs. However, the analysis using the (highest single agent) HSA model indicated mostly additive interactions between the mucus fractions and chemotherapy drugs.

These findings suggest the potential of biologically active substances derived from *Helix aspersa* mucus in enhancing the anti-proliferative effects of chemotherapy drugs in breast cancer treatment. The identification of additive interactions provides insights into the development of combination therapies with reduced drug dosage. Overall, this study contributes to the understanding of the anti-proliferative effects of *Helix aspersa* mucus fractions and their interactions with chemotherapy drugs, highlighting their potential as novel therapeutic approaches in breast cancer treatment.

Key words: *Helix aspersa* mucus, breast cancer, anti-proliferative effect, HSA model

Introduction. Breast cancer ranks as the most prevalent form of cancer in women and stands as the second most frequently diagnosed cancer worldwide. In addition to surgical procedures, radiation therapy, and immunotherapy, chemotherapy is widely employed as a primary treatment option for cancer. This method employs various cytotoxic substances to induce cell death specifically in cancerous tissues [1]. Over the past few years, there has been a significant focus on exploring natural products derived from plants and animals as promising reservoirs for innovative anti-cancer agents.

Mollusks have long been acknowledged as a rich source of bioactive compounds with diverse pharmacological properties. In recent years, researchers have turned their attention towards exploring the potential anti-cancer effects of mollusk-derived compounds. These compounds are promising because they are naturally synthesized by mollusks to defend against predators or combat infections. By elucidating their mechanisms of action and evaluating their efficacy, mollusk-derived compounds may provide valuable insights and contribute to the development of novel anti-cancer therapies. Some good examples in the field are: Prialt (Ziconotide) [2], derived from the venom of the cone snail (*Conus magus*), Prialt has gained FDA approval for the management of severe chronic pain. Although not directly targeting cancer cells, Prialt is an example of a mollusk-derived compound successfully utilized in clinical settings. Isolated from the sea hare (*Dolabella auricularia*), dolastatins [3] have shown substantial potential as anticancer agents. Notably, Dolastatin 10 has demonstrated activity against various cancer types. Its mechanism of action involves disrupting microtubule formation, leading to cell cycle arrest and apoptosis. Discodermolide [4], obtained from a marine sponge (*Discodermia dissoluta*), functions as a microtubule stabilizing agent similar to Taxol. It exhibits promising anti-cancer activity, particularly in overcoming drug resistance. Our previous studies have demonstrated that isoforms and functional units of haemocyanins from *Helix lucorum* (HlH) (previously called

Helix vulgaris (HvH)) and hemolymph fraction isolated from *Rapana venosa* with molecular weight 50–100 kDa have anti-tumour activity and immunological properties [5,6]. In particular, HlH and *Helix aspersa* (HaH) haemocyanins have been shown to have an anti-proliferative effect on CAL-29 and T-24 bladder cancer cell lines. Recently in our laboratory we have shown that in vitro bioactive substances obtained from the hemolymph of the garden snail *Helix aspersa* have anti-tumour efficacy on various human cancer cell lines representing different types of cancer. The results demonstrate that certain hemocyanin subunits from *H. aspersa* exhibit cytotoxic activity comparable to cisplatin, a widely used chemotherapy drug.

Materials and methods. ***Cell cultures.*** MDA-MB-231 (ATCC, LGC STANDARDS) is a triple-negative breast cancer cell line which is characterized by lack of expression of estrogen (ER), progesterone (PR) and human epidermal growth factor receptors (HER2). The cells expressed mutated TP53 gene and functional inactive p53 protein. The complete medium for the cells is Leibovitz's L-15 Medium (Thermo Fisher Scientific) with 10% fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin (Thermo Fisher Scientific). The MDA-MB-231 cells were cultivated in 5% CO_2 at 37 °C.

MCF-7 (ATCC, LGC STANDARDS) is a breast cancer cell line with functional p53 gene and estrogen receptor (ER) expression. MCF-7 cells were cultivated in Eagle's Minimum Essential Medium (Thermo Fisher Scientific) with 0.01 mg/ml human recombinant insulin (Sigma-Aldrich), 10% fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin (Thermo Fisher Scientific) in 5% CO_2 at 37 °C.

MCF-10A cell line (ATCC, LGC STANDARDS) is a non-tumourigenic epithelial cell line. The cells were grown in DMEM-F12 (Thermo Fisher) media supplemented with hydrocortisone 0.5 mg/ml (Sigma-Aldrich), insulin 10 mg/ml (Sigma-Aldrich), hEGF 20 ng/ml (Sigma-Aldrich) and 10% (v/v) FBS (Thermo Fisher) in a 37 °C incubator equipped with 5% CO_2.

Isolation of biologically active compounds from garden snail H. aspersa. The mucus was collected from the foot of *H. aspersa* snails, grown in Bulgarian eco-farms. For mucus production, each snail was stimulated by gently prodding the extended foot muscles with a glass rod. After a several step purification and homogenization process, subject of patent, including filtration and centrifugation for removal of rough particles, the crude mucus extract was obtained [7]. The crude mucus extract was separated by ultrafiltration using Centrifugal Filter Units with different pore size (Amicon® Ultra-15 Centrifugal Filter Units 50K MWCO and Thermo Scientific™ Pierce™ Protein Concentrator PES, 20K MWCO) into two fractions containing compounds with Mw above 20 kDa (with concentration 1.5 mg/ml protein) and with Mw above 50 kDa (with concentration 1.7 mg/ml protein). The fractions were characterised by SDS-electrophoresis and mass spectrometry as already described [8,9].

Cytotoxic assay. The cytotoxicity of biologically active compounds alone or in combination with the chemotherapy drug cisplatin (cis-Pt) (Sigma-Aldrich) or with the hormone therapy drug tamoxifen (Tam) (Sigma-Aldrich) was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT-dye reduction assay (MTT test) [10]. One thousand and five hundred cells per well in 96-well plate (Corning Costar Flat Bottom Cell Culture Plate) from each cancer cell line were seeded. The cells from each cell line were treated with serial dilutions of the biologically active compound alone in concentration from 16 to 512 µM or with a combination of a constant background concentration of the biologically active compound (corresponding to IC25 in µg/ml) and serial dilutions of cisplatin or tamoxifen ranging from 16 to 512 µM. Cisplatin and tamoxifen were dissolved in DMSO with a final concentration 2.5%. The medium was changed to a phenol red free medium containing MTT (Invitrogen) in a final concentration of 0.5 mg/ml after a 72 h incubation in 5% CO_2 at 37 °C. The cells were then incubated for 2 h at the same conditions. The formed formazan crystals were then dissolved in DMSO and absorbance was measured with a microplate reader VarioScan Lux (Thermo Scientific). Results were analyzed with GraphPad Prism software v.7.

Synergy scores determination. To analyze the cytotoxicity of combination treatments, we carried out MTT-dye reduction assay (as described above), applying a non-constant ratio of the compounds. We used a fixed concentration of the hemolymph fractions corresponding to the IC25 value for each cancer cell line and concentrations of tamoxifen or cisplatin ranging from 1.5 to 190 µg/mL and 1.2 to 154 µg/mL, equivalent to the concentrations in µM, respectively. The dose-response data and synergy scores for each pair of drug combinations used to treat the panel of cells were calculated using the Bioconductor package SynergyFinderPlus [11] using the HSA model in RStudio.

Results and discussion. In this study, we investigated the anti-proliferative effects of biologically active substances (BAS) extracted from the mucus of the garden snail *Helix aspersa*. Our focus was on two breast cancer cell lines MCF-7 and MDA-MB-231, as well as a non-cancerous cell line MCF-10A. Specifically, we examined two fractions isolated from the snail's mucus, one with a molecular weight (Mw) above 20 kDa and another with Mw above 50 kDa.

To evaluate the potential synergistic effects, we combined these biologically active substances with two commonly used chemotherapy drugs, cisplatin (cis-Pt) and tamoxifen (Tam), and tested their impact on both the cancerous and non-cancerous cell lines.

In the preliminary experiments, cisplatin and tamoxifen were used as positive controls to determine the expected anti-proliferative effect of the selected substances. To achieve this, we treated cells from all cell lines with the chemotherapy drugs and the two different mucus fractions from *H. aspersa* for a duration of 72 h. The purpose was to determine the half effective dose IC50 of the drugs and biological substances. The results are presented in Fig. 1, panel E.

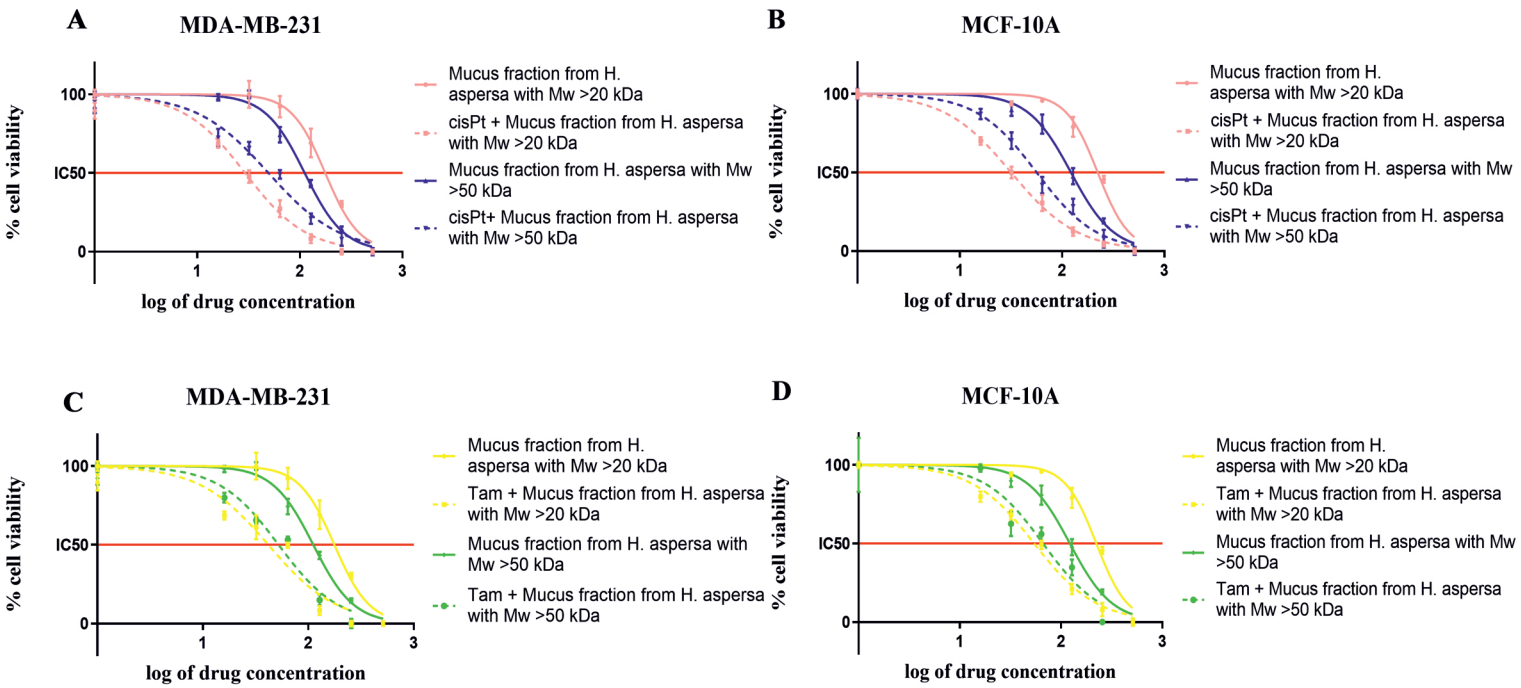


Fig. 1. Dose-response curves for mucus fractions with Mw over 20 and over 50 kDa alone and both substances in combination with cisplatin or tamoxifen. Logarithm of concentrations of the substances in µg/ml and combination of the substances with cisplatin or tamoxifen in µM are plotted against cell viability in %. MDA-MB-231 (A) and MCF-10A (B) cells treated with mucus fractions with Mw over 20 or over 50 kDa alone and each one of them in combination with cisplatin (cis-Pt). MDA-MB-231 (C) and MCF-10A (D) cells treated with mucus fractions with Mw over 20 or over 50 kDa alone and each one of them in combination with tamoxifen (Tam). (E) IC50 of biologically active substances (BAS), isolated from *Helix aspersa* snails, alone and in combination with cisplatin or with tamoxifen tested on breast cancer and non-cancerous cell lines (see the next page)

Figure 1, Panel E

BAS/Cells	MCF-10A	MCF-7	MDA-MB-231
Cisplatin [µM]	28	35	24
Tamoxifen [µM]	26	18	21
Mucus from *H. aspersa* Mw > 20 kDa [µg/ml]	222	186	176
Cisplatin [µM] + Mucus from *H. aspersa* Mw > 20 kDa [µg/ml]	32	30	29
Tamoxifen [µM] + Mucus from *H. aspersa* Mw > 20 kDa [µg/ml]	54	48	51
Mucus from *H. aspersa* Mw > 50 kDa [µg/ml]	122	96	107
Cisplatin [µM]+ Mucus from *H. aspersa* Mw > 50 kDa [µg/ml]	57	60	53
Tamoxifen [µM] + Mucus from *H. aspersa* Mw > 50 kDa [µg/ml]	67	62	64

As shown in Fig. 1, panel E, the IC50 values of the biological substances were higher than those of cisplatin or tamoxifen, measured in µg/ml. However, the mucus fraction with Mw above 50 kDa exhibited significantly lower IC50 values compared to the fraction with Mw above 20 kDa. This suggests a slight antiproliferative effect on breast cancer cell lines. Notably, the IC50 values for most tested drugs and natural products on MCF-10A cells were higher than those for the breast cancer cells, indicating some selectivity towards cancerous cells.

Furthermore, we investigated the synergistic effects between chemotherapy drugs (cisplatin or tamoxifen) and biologically active substances isolated from *H. aspersa*. All cell lines were treated for 72 h with a combination of constant background concentration of the tested biological substances (corresponding to IC25) and serial dilutions ranging from 16 to 512 µM of cisplatin or tamoxifen. The results are summarized in Fig. 1, panel E. We observed a significant reduction in the half effective dose when the chemotherapy drugs were combined with the mucus fraction with Mw over 50 kDa, approximately two-fold. A more pronounced reduction was observed with the combinations of cisplatin and tamoxifen with the mucus fraction with Mw over 20 kDa, approximately six-fold and 3.5-fold, respectively. These results might indicate a synergistic effect between the chemotherapeutics and the biological products, with a more pronounced effect observed with the fraction having Mw over 20 kDa.

The data for MDA-MB-231 and MCF-10A cells treated with the tested biological substances, alone or in combination with cisplatin or tamoxifen, are presented in Fig. 1; graphs are generated using GraphPad Prism. Figure 1A and 1C shows the dose-response curves obtained after treating the MDA-MB-231 cancer cells with the studied fractions from *H. aspersa* alone and in combination with cisplatin or tamoxifen, respectively. The curves indicate lower IC50 values for the mucus fraction with Mw over 50 kDa compared to the fraction with Mw over

20 kDa for both cell lines. Notably, a significant decrease in IC50 values is observed when the cells are treated with cisplatin in combination with the mucus fraction with Mw over 20 kDa, and to a lesser extent, with the fraction with Mw over 50 kDa (Fig. 1A). A similar trend is observed when treating the cells with tamoxifen in combination with the tested fractions (Fig. 1C). The lower logarithm of concentration values suggests a synergistic anti-proliferative action between the mucus fractions and widely used chemotherapeutic agents. The results for the non-cancerous cell line MCF-10A, representing the treatment with the two different fractions alone and in combination with cisplatin (Fig. 1B) or tamoxifen (Fig. 1D), are also shown in Fig. 1. The obtained curves depict higher IC50 values compared to those of MDA-MB-231.

Furthermore, we focused on two cell lines representing both molecular subtypes of breast cancer – the triple-negative MDA-MB-231 and the hormone-dependent MCF-7. Microscopic images of MDA-MB-231 and MCF-7 control cells, cells from both cell lines treated with an IC50 concentration of mucus fraction with a molecular weight (Mw) over 20 kDa alone, and in combination with an IC50 concentration of cisplatin, as well as cells treated with an IC50 concentration of cisplatin alone, are presented in Fig. 2. After 72 h of treatment with the mucus fraction, a slight delay in cell division was observed (Fig. 2, panel B). In contrast, treatment with the combination of the mucus fraction and cisplatin resulted in a disturbed cell monolayer and changes in cell morphology for both cancer cell lines (Fig. 2, panel C). The treatment with the chemotherapeutic agent caused significant cell death (Fig. 2, panel D) compared to the cells from both cancer lines treated with the combination of mucus fraction with Mw over 20 kDa and cisplatin. These results suggest the potential use of a lower dose of the chemotherapeutic agent in therapy.

Next, we performed experiments in which the chemotherapeutics (cisplatin or tamoxifen) were mixed with the mucus fractions with Mw above 20 kDa or with Mw above 50 kDa and were applied in serial dilutions to the MDA-MB-231 (triple-negative) and MCF-7 (hormone-dependent) cell lines. The investigation of synergistic effects, wherein the combined effect of multiple substances or factors surpasses the sum of their individual effects, is of paramount importance in various scientific disciplines including pharmacology and toxicology. In our study we analyzed synergistic effects utilizing the HSA model implemented in RStudio. The HSA model, a widely employed method, enables the quantification and prediction of synergistic or antagonistic interactions between different substances. The analysis carried out with the SynergyFinderPlus Bioconductor package [11] (Fig. 3, 4) indicated mostly additive interaction between both mucus fractions with Mw above 20 kDa and Mw above 50 kDa and cisplatin and tamoxifen in both tested cell lines, the effect being most pronounced in MCF-7. The additive effect refers to a phenomenon where the combined effect of two or more substances or factors is equal to the sum of their individual effects. In other words, when two or more

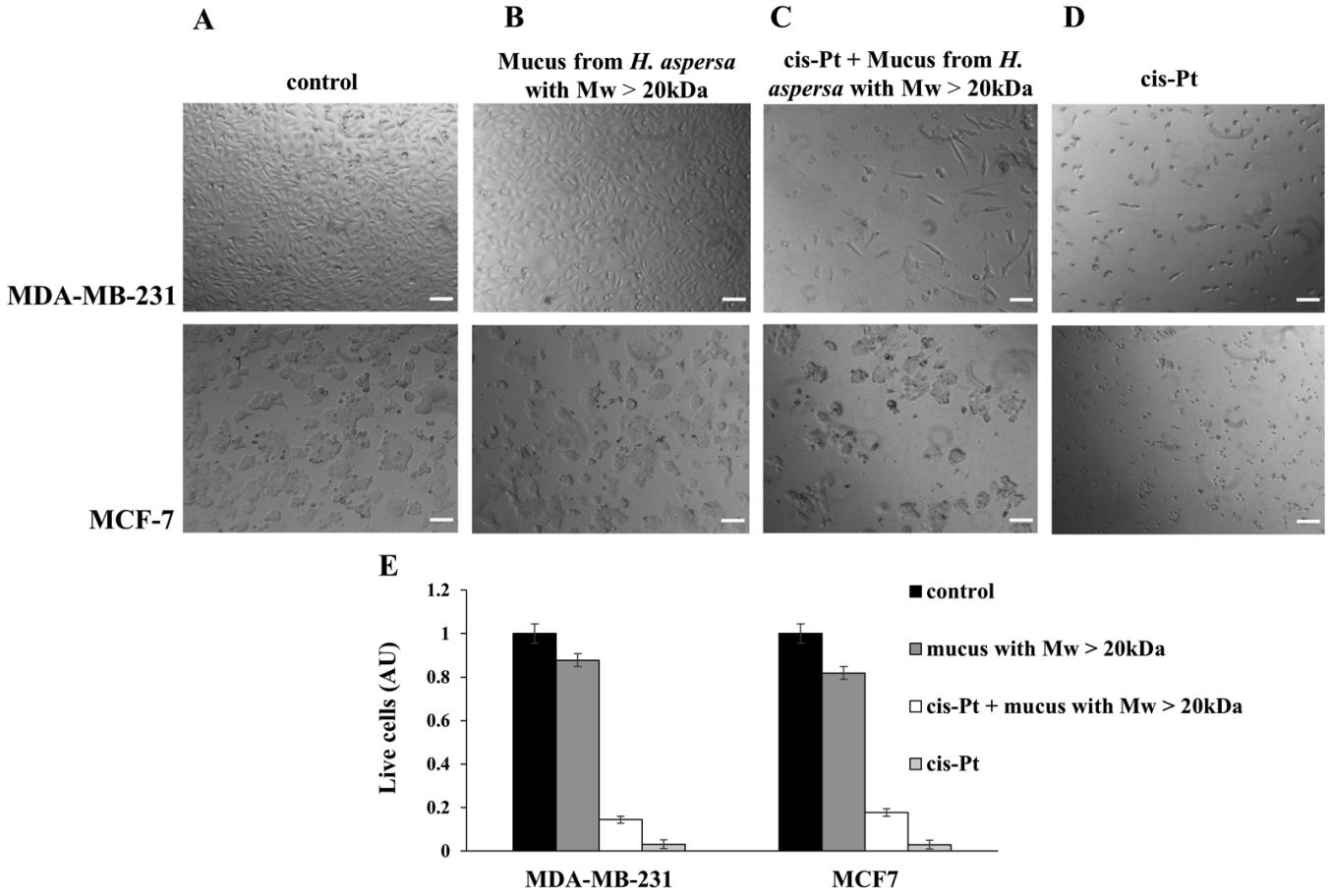


Fig. 2. Microscope images of breast cancer cell lines MDA-MB-231 and MCF-7. A) control cells; B) cells treated with IC50 of mucus fraction with Mw over 20 kDa; C) cells treated with IC50 of mucus fraction with Mw over 50 kDa; D) cells treated with IC50 of cisplatin; E) Quantification of live cells determined after Trypan Blue staining, calculated as arbitrary units (AU)

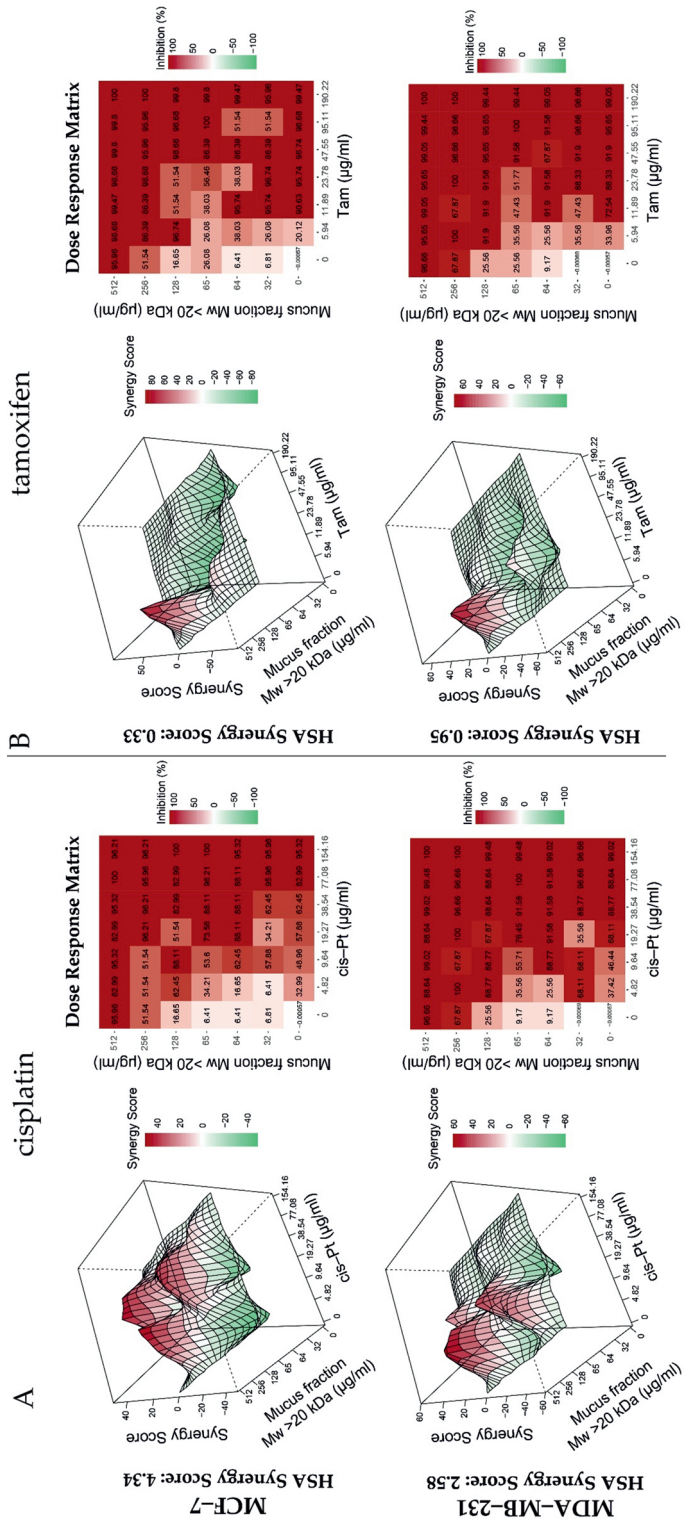


Fig. 3. Combination landscapes and dose-response matrices in MCF-7 and MDA-MB-231 cell lines treated with mucus fraction of *H. aspersa* with Mw above 20 kDa and cisplatin (A) or tamoxifen (B)

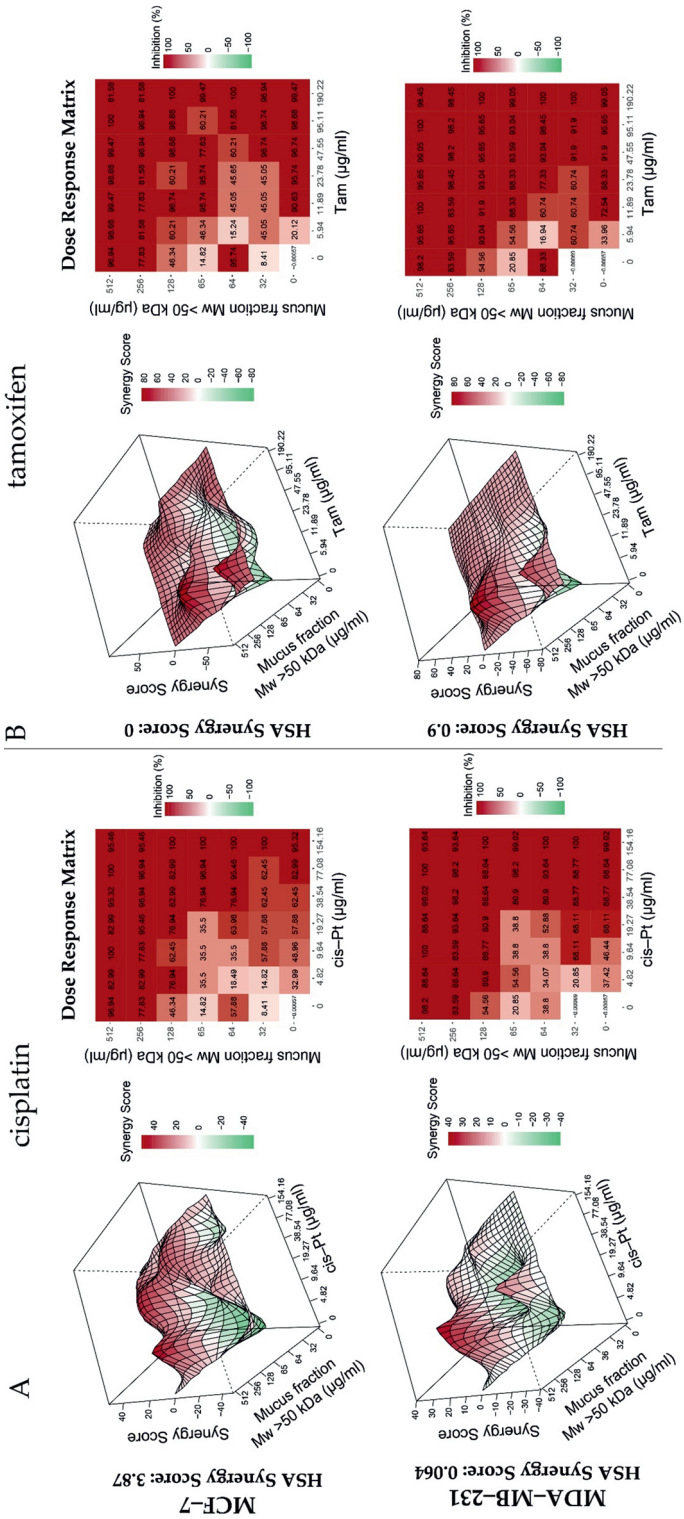


Fig. 4. Combination landscapes and dose-response matrices in MCF-7 and MDA-MB-231 cell lines treated with mucus fraction of *H. aspersa* with Mw above 50 kDa and cisplatin (A) or tamoxifen (B)

substances or factors work together, their effects add up in a linear manner without any enhancement or suppression. Here we found that both mucus fractions have additive effect in combination with the chemotherapeutics cisplatin or tamoxifen. The highest additive effect was observed in the combination of mucus fraction with Mw above 20 kDa and cisplatin towards MCF-7 cell line.

In conclusion, this study contributes to the understanding of the anti-proliferative effects of *Helix aspersa* mucus fractions and their interactions with chemotherapy drugs, offering prospects for improving breast cancer treatment strategies and promoting personalized medicine approaches in the future. The results demonstrated that the mucus fractions exhibited a slight anti-proliferative effect on breast cancer cell lines, as indicated by higher IC50 values compared to the chemotherapy drugs. However, a difference was observed between the two fractions, with the fraction with Mw above 50 kDa displaying stronger anti-proliferative activity compared to the fraction with Mw above 20 kDa.

Furthermore, the combination of the mucus fractions with cisplatin or tamoxifen revealed possible synergistic effects, as evidenced by a significant reduction in the half effective dose. The synergistic interactions were more pronounced with the mucus fraction with Mw above 20 kDa, indicating its potential in enhancing the anti-proliferative effects of the chemotherapy drugs. Dose-response curves and microscopic images supported the presence of synergistic effects, showing lower IC50 values and significant cell death when the cancer cell lines were treated with the combination of the mucus fractions and chemotherapy drugs.

However, the application of the HSA model in RStudio indicated mostly additive interactions between the mucus fractions and cisplatin or tamoxifen, emphasizing the combined effect being equal to the sum of their individual effects.

These findings highlight the potential of biologically active substances derived from *Helix aspersa* mucus as promising candidates for enhancing the efficacy of chemotherapy drugs in breast cancer treatment. The identification of additive interactions provides insights into the development of combination therapies that may lower the required dosage of chemotherapy drugs while maintaining or enhancing their anti-proliferative effects.

Further research is warranted to elucidate the underlying mechanisms of action and to explore the specific bioactive compounds present in the mucus fractions. Additionally, in vivo studies and clinical trials are necessary to validate the potential therapeutic applications of these substances and their synergistic interactions with chemotherapy drugs.

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