Acta Pharm. Sci. Vol 63:(2), 2025
Original article
DOI: 10.23893/1307-2080.APS6324

Investigation the selective anticancer activities of montmorillonite and curcumin on the pancreatic and breast cancer

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ABSTRACT

Breast and pancreatic cancers are among the leading causes of death worldwide. In this study, the anticancer activities of natural-based montmorillonite and modified curcumin are examined in pancreatic and breast cancer. MRC-5, PANC-1, and MDA-MB-231 cell lines are used as healthy fibroblast cell lines (control), pancreatic and breast cancer respectively. 10-1000 μg/mL doses applied to each cell line. As a result, we obtained that montmorillonite has no significant reducing effects on cell viability in the MRC-5 cell line. IC_{50} values were 1456 μg/mL for MDA-MB-231 and 1166 μg/mL for PANC-1 cells. When modified curcumin therapy doses were applied, IC_{50} values were 215 μg/mL for MRC-5, 56.45 μg/mL for MDA-MB-231, and 72.34 μg/mL for PANC-1 cells. In conclusion, this study demonstrates that these two natural compounds have antitumoral effects on pancreatic and breast cancer. These compounds may be useful in the development of natural-based treatments for breast and pancreatic cancer.

Keywords: Bentonite, montmorillonite, curcumin, pancreatic neoplasms, breast neoplasms

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(Received 22 Mar 2024, Accepted 27 Jun 2024)

INTRODUCTION

Breast cancer (BC) and pancreatic cancer (PC) are two of the most lethal cancers worldwide, and effective treatment options for these diseases are urgently needed. Montmorillonite, a natural mineral, and curcumin, a natural compound derived from the spice turmeric, have shown potential anticancer effects *in vitro* and *in vivo*. In this study, we examined the anticancer activities on pancreatic and breast cancer by using natural compounds of montmorillonite and modified curcumin.

Pancreatic cancer

PC ranks 12th among the most common types of cancer worldwide and 7th in terms of cancer-related mortality[1]. Some risk factors for PC can be altered, like smoking, obesity, diabetes, chronic pancreatitis, and exposure to specific workplace chemicals. However, there are immutable risk factors such as age, gender, race, family history, inherited genetic syndromes, and chronic pancreatitis stemming from a gene mutation[2,3]. Surgical removal is a potential treatment option for localized PC tumors, but early detection is challenging due to the lack of accurate and reliable detection methods. As a result, most patients with PC are diagnosed at advanced stages, and only 10-20% of them have tumors that are eligible for surgical removal. Adjuvant therapies, such as chemotherapy and radiotherapy, are commonly used after surgery to improve the survival rate of patients with PC[4]. Unfortunately, PC is known for its aggressive nature and fast spread, which can lead to fatal outcomes. The 5-year survival rate for localized PC tumors is 42%, while for regional metastasized tumors, it is 14%, and for distal metastasized tumors, it is only 3%[5]. Despite advancements in medical technology, there is still no effective medical treatment for metastasized PC.

Breast cancer

BC is the most common cancer and 5th in terms of cancer-related mortality[1]. Several BC risk factors can be changed, including alcohol consumption, being overweight or obese, physical inactivity, nulliparity, not breastfeeding, and the use of birth control or menopausal hormone therapy. Conversely, there are also BC risk factors that cannot be changed, such as being female, increasing age, inheriting specific gene mutations, having a family history of BC, having a personal history of BC, ethnicity, height, dense breast tissue, specific benign breast conditions, early onset of menstruation, late onset of menopause, exposure to radiation to the chest, and exposure to diethylstilboestrol[6]. The 5-year survival rate of BC tumors is 90%[7]. Due to the presence of numerous muta-

tions, the development of resistance to treatments, and individual variations in treatment response in BC, there is a focus on alternative therapies. In addition to traditional treatments such as surgery, chemotherapy, and radiotherapy in BC treatment, receptor inhibitors and nanotechnological approaches are among the current therapies[8-10].

Effects of montmorillonite on breast and pancreatic cancer

Montmorillonite (MMT) is a natural nanomolecular clay[11] that has been used for treatment for over 100 years and has been observed to have no side effects in phase 1 and phase 2 studies[12,13]. According to the WHO, MMT is mainly composed of highly colloidal and plastic clays that belong to the Smectite Group and are predominantly made up of the clay mineral MMT. In the healthcare sector today, 99% of the clays used are from the Smectite Group. The most common subgroup among the Smectite Group is MMT (Bentonite). Its chemical formula is (Na, Ca)(Al, Mg)(Si_4O_{10})(OH)[14]. MMT has a large surface area of 750 m^2/gram, possesses high water absorption and swelling properties (2-20 times its volume). Additionally, in suspension form, it has the ability to release a significant amount of negative (-) ions into the environment[15,16]. Most of the substances that threaten human health, from the air we breathe to the water we drink, such as heavy metals, toxins, viruses, bacteria, and radiation, are positively charged (+) ions. MMT has a nano-particle structure, and its particles are layered. Its particle size ratio is around 1000:1. It is not harmed by stomach acid and can easily pass through the intestines, skin, and blood-brain barrier[17]. It is excreted from the body without being metabolized, through faces, urine, sweat, and tears. MMT is not a chemically synthesized compound, but rather a natural substance that nature has provided us. MMT is classified as SCOGS Type 1 by the FDA, meaning that "there is no evidence to demonstrate or suggest reasonable grounds to suspect, a hazard to the public when they are used at levels that are currently in use or might reasonably be expected in the future"[18]. Due to its properties, MMT can be used in detoxification, immune system disorders, gastrointestinal system disorders, metabolic syndrome, skin diseases, radiation protection, and reducing the adverse effects of chemotherapy and radiation therapy in cancer patients[19-26]. Preclinical and clinical studies on MMT have shown that it is antibacterial, antiviral, antifungal, antitumor, and radioprotective. Additionally, MMT is a good drug carrier. MMT has been modified with drugs used in cancer treatment such as Docetaxel, Irinotecan, 5-fluorouracil, and Tamoxifen, and has been shown to increase their effects and reduce their side effects[27]. Recent studies on MMT have observed that it has antitumor properties[25-28]. A study by Cervini-Silva et al. in 2016 showed

that MMT inhibits the development of high-grade gliomas[28]. MMT has an antiproliferative effect that is affected by both cell type and protein levels. In a 2020 study conducted by Sabzevari et al., MMT was found to induce G0/G1 phase arrest in MRC-5 and HT-29 cell cultures by modulating the expression of the P21, P27, and Cyclin D1 genes. Additionally, MMT induced S phase arrest in HepG2 cell cultures by regulating the expression of the mTOR gene. As a result of this study, it has been demonstrated that MMT induces apoptosis in cells by modulating pro/anti-apoptotic genes[25]. In an *in vitro* study conducted by Abduljauwad et al., MMT exhibited a significant reduction in melanoma cell viability and proliferation in a dose-dependent manner. In the same study's *in vivo* tumor model, treatment with MMT significantly decreased tumor mass and reduced cell mitosis[26]. Based on these studies, it has been proven that MMT has different effects on each tumor. There are no studies on the effect of MMT on pancreatic and breast tumors.

Effects of curcumin on breast and pancreatic cancer

Turmeric's primary active compound, curcumin, has a long history of use in traditional medicine spanning centuries and has recently gained attention for its potential health benefits. The vibrant yellow compound, extracted from the *Curcuma longa* plant, is a member of the ginger family and exhibits powerful anti-cancer, antibacterial, anti-inflammatory, and antioxidant characteristics[29]. Research findings indicate that curcumin exhibits tumor-suppressing properties across a range of cancer types, encompassing breast cancer, leukemia, lymphoma, neurological cancers, gastrointestinal cancers, ovarian cancer, lung cancer, head and neck squamous cell carcinoma, melanoma, and genitourinary cancers[30]. Research has shown that curcumin may be beneficial in the treatment of various conditions, including arthritis, diabetes, Alzheimer's disease, and cancer[31-33]. Studies have demonstrated its ability to inhibit tumor formation in different cancer types by affecting multiple signaling pathways.

In PC cells, curcumin has been shown to inhibit KRAS expression and stabilize p53 to prevent mutations[34]. It also exhibits anti-angiogenic effects by inhibiting the expression of VEGF and VEGFR1 genes and increases apoptosis by inhibiting COX-2 and EGFR[35]. Furthermore, it prevents metastasis by regulating miRNAs and blocking CAF-mediated EMT[36]. Curcumin also affects signaling pathways such as EGF-Akt, EGF-ERK, Hedgehog, Wnt-β-catenin, PI3K-Akt, and ATM-Chk1, thereby demonstrating anti-tumoral effects[37]. Additionally, it regulates pro-inflammatory cytokines (IL-8↓, IL-8R↑) and inhibits transcription factors such as NF-κB, Sp1, Sp3, and Sp4, thereby exerting anti-tumoural effects[38] (Table 1).

Table 1. Effect of curcumin on pancreatic cancer

Prevent mutations	**Inhibit KRAS expression and stabilize p53**
Anti-angiogenic effects	Inhibiting the expression of VEGF and VEGFR1 genes
Increases apoptosis	Inhibiting COX-2 and EGFR
Signaling pathways	EGF/ERK↓, EGF/Akt↓, Hedgehog↓, PI3K/Akt↓, ATM/Chk1↑, and Wnt/b-catenin↓
Pro-inflammatory cytokines	IL-8↓, IL-8R↑
Transcription factors	Inhibits NF-κB and AP-1

In BC, curcumin has been found to inhibit the activity of ornithine decarboxylase, which is an enzyme that plays a role in cell proliferation[39]. Moreover, curcumin has been demonstrated to suppress the expression of various genes and enzymes in breast cancer cells, including the aromatic hydrocarbon receptor, cytochrome P450 1A1, COX-1, and COX-2 enzymes, and VEGF and b-FGF growth factors. Curcumin also induces the expression of p-53-dependent Bax, which can lead to apoptosis (cell death) in cancer cells. Furthermore, curcumin has been found to reduce the expression of MMP-2 and increase the expression of TIMP-1, which can inhibit cancer cell invasion and metastasis. Ultimately, curcumin has demonstrated its ability to inhibit the activation of several transcription factors, including AP-1 and NF-κB, both of which are involved in the proliferation and growth of cancer cells[40].

Unfortunately, due to the breakdown of curcumin in the stomach, low absorption in the intestines, and rapid metabolism in the body, its efficacy in clinical application was limited[41]. As a result, enhancing the drug delivery system becomes a crucial strategy to augment the bioavailability of curcumin and enhance its effectiveness. Several drug delivery systems, including micelles, liposomes, nanoparticles, and cyclodextrins, have been devised to enhance the solubility and stability of curcumin, thus improving its delivery to target tissues[42]. MMT, a type of clay, has a high surface area and adsorption capacity, which allows it to bind to curcumin and protect it from degradation in the gastrointestinal tract. This, in turn, enhances the absorption of curcumin from the gastrointestinal tract into the bloodstream and increases its effectiveness in the body. In a study conducted by Karataş et al., it was shown that modified curcumin with montmorillonite passes through the intestine 2000 times more than PLGA and significantly increases the retention time in the body[43]. In preclinical studies, employing MMT as a carrier for curcumin has yielded promising results. Therefore, the modification of curcumin with MMT has been shown to improve its bioavailability and retention time in the body.

As mentioned above, in the past decade, much research has focused on the effect of curcumin on cancer cells. However, there is no study in the literature about MMT and curcumin with enhanced bioavailability using MMT (as a drug carrier) on pancreatic and breast tumors. Hence, this study endeavors to examine the anticancer effects of modified curcumin with MMT and MMT alone on breast and pancreatic cancer cells, thereby offering novel insights to the existing literature.

METHODOLOGY

Cell culture and cell growth

Human pancreatic cancer cell line (PANC-1, CRL-1469, ATCC, Rockville, MD, USA), human breast cancer cell line (MDA-MB-231, HTB-26, ATCC, Rockville, MD, USA), and healthy human fibroblast lung cells as control (MRC-5, CCL-171, ATCC, Rockville, MD, USA) were utilized in the study. All cells were cultured in high glucose DMEM (Pan Biotech, Aidenbach, Germany) supplemented with 10 % heat-inactivated FBS (Gibco Company, Grand Island, NY, USA), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Pan Biotech, Aidenbach, Germany) in a humidified atmosphere at 37°C with 5% CO_2. Upon reaching over 80% confluency, cells were harvested using 0.25% trypsin-EDTA (Gibco Company, Grand Island, NY, USA).

Preparation of montmorillonite and montmorillonite (as a drug carrier) along with modified solutions

MMT (MediClay) and modified curcumin solutions were taken from Alya Mineral Company, Ordu, Türkiye. MMT solution was a liquid form and consisted of water and montmorillonite (5.4 g montmorillonite / 60 mL). Modified curcumin solution consisted of 0.5 mg/ml *Rosa canina* L., 4 mg/ml *Curcuma longa*, 1 mg/ml *Rosmarinus officinalis* extracts, and 70 mg/ml MMT (To enhance the bioavailability of curcumin). Experiments were done for MMT and modified curcumin solutions separately. MMT dilutions were made with sterilized distilled water for 10, 25, 50, and 100 μg/mL doses. Following doses which were 500, 750, and 1000 μg/mL were made with DMEM. Preparing MMT along with modified curcumin solutions was mentioned in Figure 1. All of the modified curcumin testing doses were made in DMEM.

Figure 1. Montmorillonite and montmorillonite along with modified curcumin solutions. The left one is the montmorillonite clay solution which is composed of montmorillonite and water (a). Right one is modified curcumin (Bentonizer) solution which is composed of *Curcuma longa, Rosmarinus officinalis* and *Rosa canina* L. (b). 100 mL of concentrated bentonizer was poured into a 400 mL montmorillonite bottle. It was shaken for 2-3 minutes. After 20 hours in the refrigerator at +4°C, it became ready for use.

Cytotoxicity assay

A cytotoxicity assay was conducted using an MTT solution. PANC-1 and MDA MB-231 cells were seeded in 96-well plates at a density of $8x10^3$ cells per well. MRC-5 cells were seeded at a density of $1x10^4$ cells per well due to their slower doubling time compared to the cancer cell lines. The cell lines were then incubated for 24 hours. After incubation, the medium was removed, and the appropriate doses were applied to each well for an additional 24 hours. Following this incubation period, the supernatant was carefully removed, and MTT solution (5 mg/mL in 100 µL medium) was added to each well. The plates were then incubated for 2.5 hours. Afterward, the supernatant was removed, and 200 µL of DMSO was added to each well. The absorbance was measured at 570 nm using a spectrophotometer (Thermo Scientific, USA). The experiment was performed in triplicate for each condition.

RESULTS and DISCUSSION

The anticancer activities of natural-based MMT and MMT along with modified curcumin were measured depending on cell viability for MRC-5, PANC-1, and MDA-MB 231 cell lines by using an MTT assay. MRC-5 cells, PANC-1 cells, and MDA-MB-231 cells belong to healthy cell control, pancreatic cancer, and breast cancer respectively. The MTT assay is based on the metabolic activity that reduction of tetrazolium salts to formazan crystals. The results of MMT and modified curcumin are presented in concentration-dependent cell viability graphs. The cell viability percentages for 10-1000 µg/mL doses of MRC-5, MDA MB-231, and PANC-1 cells of the MMT. The effect of MMT on MRC-5

cells showed that increased in 10, 50, and 100 µg/mL. The cell viability exhibited a decrease below 100% specifically in response to the administration of doses at 750 and 1000 µg/µl. However, within the given dose ranges, no cytotoxic effect was observed, and the viability did not drop below 90%. In MDA MB-231 (human breast cancer cell line) cells, MMT demonstrated a consistent decrease across the administered doses. A 40% decrease in viability was observed with the application of 1000 µg/µl of MMT. The cell viability decreased to below 80% when the smallest doses of 10 ug/ml were given to the PANC1-1 cell line. MMT has shown a more effective result at lower doses in PANC-1 cells compared to MDA-MB-231 cells. IC_{50} values of MDA MB-231 and PANC-1 cells were approximately 1456 and 1166 µg/mL for MMT (Figure 2).

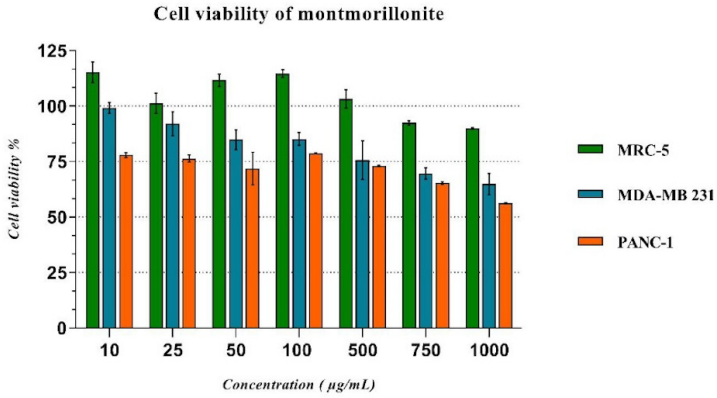

Figure 2. Percentage cell viability graph according to the doses of montmorillonite applied to MRC-5, MDA-MB-231, and PANC-1 cells. 10-1000 µg/mL doses were applied to each cell line. Error bars show SEM.

The anticancer effects of modified curcumin for 10-1000 µg/mL doses of MRC-5, MDA MB-231, and PANC-1 cells. The cell viability of the MRC-5 cell line reduced as of 25 µg/mL and sharply below 30% in 500 µg/mL. However, in MDA-MB-231 cells, a decrease in viability was observed at the lowest dose (10 µg/mL), indicating cell viability decreased to 50% when a 50 µg/mL dose was applied. In PANC-1 cells, similar to MDA-MB-231 cells, a decrease in viability was also observed at the lowest dose of 10 µg/mL, with viability dropping to approximately 75%. Viability dropped below 50% when a dose of 100 µg/mL was applied. IC_{50} values of modified curcumin for MRC-5, MDA-MB-231, and PANC-1 cells were about 215, 56.45, and 72.34 µg/mL respectively (Figure 3). Looking at both graphs, it is evident that when MMT is applied alone, it promotes an increase in viability in healthy cell lines. In cancer cell lines, however, viability decreased by approximately 50% with the application of the highest

dose. However, when MMT (as a drug carrier) is applied in combination with modified curcumin, decreases in viability in cancer cells have been observed even at low doses. Within the dose ranges that kill 50% of cancer cells, viability in healthy cells remains high. These results indicate that modified curcumin has been successful in killing cancer cells and MMT has been found to effectively kill cancer cells without causing harm to healthy cells.

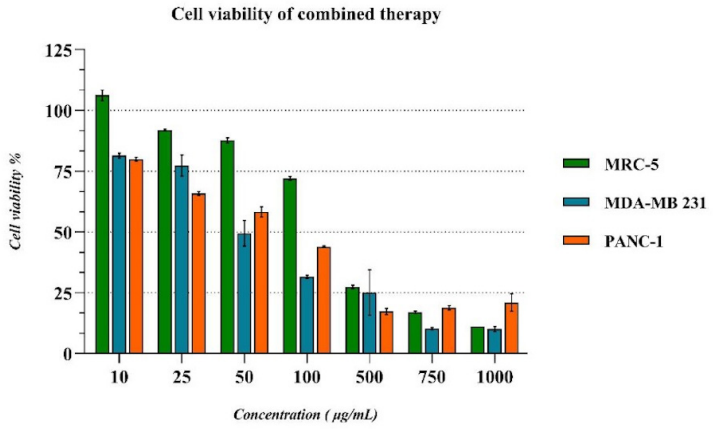

Figure 3. Percentage cell viability graph based on the doses of modified curcumin applied to MRC-5, MDA-MB-231, and PANC-1 cells. 10-1000 µg/mL doses were applied to each cell line. Error bars show SEM.

Based on the results of our study, it is evident that both MMT and combined therapy have cytotoxic effects on breast and pancreatic cell lines. It has been proven that MMT has no cytotoxic effects on healthy fibroblast cells, and in fact, it has been observed to increase cell viability at doses of 10, 50, and 100 µg/mL. However, in the breast cancer cell line, MMT exhibited cytotoxic effects at a dose of 25 µg/mL and above, with an IC_{50} of 1456 µg/mL. Similarly, the pancreatic cancer cell line, exhibited cytotoxic effects at a dose of 10 µg/mL and above, with an IC_{50} of 1166 µg/mL. These findings indicate that the effects of MMT vary in different cell lines, and it even suggests that MMT is capable of distinguishing between healthy and cancer cells.

In a study conducted by Sabzevari et al., MMT was found to have a cytotoxic effect of around 40% on MRC5 healthy lung fibroblast cells at a dose of 1000 µg/mL, whereas, in our study, cell viability was in the range of 98% at the same dose[25]. This study also showed that MMT increased cell viability at low doses, but then decreased it. In this study, it is hypothesized that the low cell growth observed at low doses is attributed to the interactions between MMT and the cell surface. The IC_{50} of MMT for MRC-5, HT-29, and HepG2 cells was approximate-

ly 1000, 880, and 625 μg/mL, respectively. Thus, it can be inferred that cancerous HT-29 and HepG2 cells exhibit greater susceptibility to MMT compared to normal MRC-5 cells. In a study by Javier Cervini-Silva et al., it was observed that low-dose MMT induced growth inhibition in the presence of U251 cells, while promoting growth in the presence of SKLU-1 cells[28]. Hence, it is clear that the interactions between MMT and cell surfaces are highly specific. Similar to our study, these findings also illustrate that MMT exerts varied effects on different cell lines at different dosage levels.

Several studies have explored the efficacy of curcumin against breast cancer using MDA-MB-231 cell lines in the literature. For instance, a study by Bimonte et al. revealed that curcumin, at doses of 50 μM, effectively eliminated half of the breast cancer cells within 48 hours, while enhancing the apoptotic effect at a concentration of 10 μM[44]. In 2018, Li et al. discovered an IC_{50} value of 37 μg/mL for free curcumin in MDA-MB-231 cell lines[45]. Following a 24-hour treatment with 15-100 μM curcumin, the viability of MDA-MB-231 cell cultures decreased by up to 25%[46]. Treatment with 50 μg/mL curcumin resulted in a 55.2% decrease in the viability of MDA-MB-231 colonies. Additionally, other studies have reported apoptosis in MDA-MB-231 cells treated with curcumin concentrations ranging from 10 to 50 μM for durations of 24 and 48 hours[47-49]. It seems that in our study, modified curcumin was also effective in the MDA-MB-231 cell line, exhibiting an IC_{50} value of 56 μg/mL. These findings align with other studies in the literature and endorse the potential utility of modified curcumin as a cytotoxic agent against breast cancer cells.

It was first demonstrated by Li et al., that curcumin exhibits anti-tumour activity in pancreatic tumors[50]. It is known in the literature that curcumin induces apoptosis and significantly reduces proliferation, invasion, metastasis, viability, migration, and colony formation in the PANC-1 cell line[51]. Guo et al. showed that the IC_{50} of curcumin in the PANC-1 cell line is 68 μM[52]. Another study found the IC_{50} of curcumin in the same cell line to be 73 μM[53]. Consistent with the literature, our study found the IC_{50} of curcumin in the PANC-1 cell line to be 72 μg/mL.

It is known that curcumin exhibits antitumor effects when used alone or in combination with other treatment methods *in vitro* studies of breast and pancreatic tumors[54]. However, due to its low bioavailability, curcumin is broken down and does not show these effects in *in vitro* and clinical studies. Therefore, studies to increase the bioavailability of curcumin are ongoing. In this study, modified curcumin with increased bioavailability was used, and its effect on breast and pancreatic tumors was proven *in vitro*. Our study is the first in the literature to show the anti-tumor effect of MMT in pancreatic tumors. Further *in vivo* and clinical studies should be conducted to investigate the effectiveness of MMT and modified curcumin.

In this study, we aimed to study the anticancer activity of MMT and MMT (as a drug carrier) along with modified curcumin in MDA MB-231 and PANC-1 cancer cells and MRC-5 healthy cells. MDA MB-231 cell line was used for breast cancer studies, the PANC-1 cell line was used for pancreatic cancer studies and the MRC-5 cell line was used for healthy control fibroblastic cells. In conclusion, the findings of this study clearly show that MMT supports cell viability in MRC-5 healthy cell lines and has a dose-dependent reduction effect on MDA-MB-231 and PANC-1 cancer cell lines. In addition, IC_{50} values of MMT for MDA-MB-231 cell was 1456 μg/mL and for PANC-1 cell was 1166 μg/mL. Whereas modified curcumin showed a reducing effect on cell viability depending on the given doses for MDA MB-231 and PANC-1 cancer cell lines. Our data showed that after the modified curcumin was applied, the intervals in which the viability of the cancerous cells began to decrease, and the viability of the MRC-5 cell line used as the control did not affect the viability in the same way. For the MRC-5 cells, the viability remained high. In addition, IC_{50} values of modified curcumin for the MRC-5 cell were 215 μg/mL, for the MDA MB-231 cell was 56.45 μg/mL, and for the PANC-1 cell was 72.34 μg/mL. Contrary to heavy drugs and chemotherapies in cancer treatments, it is necessary to discover methods that use chemicals as little as possible and adverse cause side effects in the human body. Therefore, regarding all data, our study presents that the MMT and modified curcumin have the potential to reduce pancreatic and breast cancer cells, which could be studied for future alternative therapies to treat other tumor cells using natural substances. Furthermore, the effects of MMT vary in different cell lines, and it even suggests that MMT is capable of distinguishing between healthy and cancer cells.

STATEMENT OF ETHICS

Ethical approval was not required to perform this study.

CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the work.

FUNDING SOURCES

This research received no external funding.

ACKNOWLEDGMENTS

Not applicable.

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