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Construction of Gold Nanoparticle-Loaded Bromelain with Potential Anti-Tumor and *in vitro* Wound Healing Activities

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Abstract

The most significant challenges in the advancement of cancer therapies including the adverse effects of chemotherapy, the emergence of drug resistance, and tumor metastasis, represent the major factors in therapies. In recent years, nanoparticles (NPs) have emerged as a promising paradigm in drug delivery technology. This study utilised the human breast cancer cell line MCF7 to evaluate the anticancer efficacy of gold nanoparticles (AuNPs) conjugated with bromelain (AuNPs-Br) and bromelain (Br). The AuNPs were synthesised using the Turkevich method, followed by the loading of bromelain onto these nanoparticles. Characterisation of the gold nanoparticles was conducted using various analytical techniques, including UV-Vis spectrophotometry, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), transmission electron microscopy (TEM), and scanning electron microscopy (SEM). The anticancer activities were assessed through MTT cytotoxicity assays, *in vitro* migration assays (wound healing assays), and apoptosis detection via flow cytometry in the MCF-7 cell line and normal REF cell line. The growth inhibition of the cell line exhibited a time and dose-dependent manner. Notably, AuNPs-Br demonstrated significant cytotoxicity against MCF-7 cells, which also exhibited apoptotic events. These findings suggest potential synergistic therapeutic benefits for cancer treatment and targeted delivery strategies in human breast cancer. It's important to consider that while these findings are promising, further research and clinical evaluations are necessary to fully understand the mechanisms, effectiveness, and safety of these treatments

Keywords: Gold- nanoparticles, bromelain, cytotoxicity, migration assay, Apoptosis, *in vitro* wound healing.

بناء وتوليف جسيمات الذهب النانوية المحملة بالبرملين و تطبقاتها المحتملة المضادة للاورام والتئام الجروح خارج الجسم الحي

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الخلاصة

تمثل التحديات الاكثر اهمية في تقدم علاجات السرطان بما في ذلك الاثار الجانبية الناتجة من العلاج الكيميائي ومقاومة الادوية المعالجة وانتشار الخلايا السرطانية من الصعوبات الرئيسية في العلاجات. في السنوات الاخيرة اظهرت الجسيمات النانوية نموذجا واعدا في انظمة توصيل الاودية. استخدمت الدراسة الحالية خلايا الثدي السرطانية نوع MCF7 لدراسة و تقيم فعالية جسيمات الذهب النانوية المقترنة بالبروملين او كلا على حده. صنعت الدقائق النانوبة بالطريقة الكيميائية باستخدام حامض السترات وشخصت باستخدام تقنيات مختلفة تضمنت XRD, TEM, FTIR. كما وتمت تحديد السمية الخلوية للمواد المصنعة باستخدام اختبار ال MTT. و تجربة معالجة الجروح (او هجرة الخلايا) خارج الجسم و تحديد مرحلة الوت الخلوي المبرمج للخلايا السرطانية مع مقارنتها بخط خلوي طبيعي REF. أظهر تثبيط نمو سلالة الخلايا طريقة تعتمد على الوقت والجرعة. والجدير بالذكر أن جزيئات AuNPs–Br أظهرت سمية خلوية كبيرة ضد خلايا MCF–7، والتي أظهرت أيضًا أحداث موت الخلايا المبرمج. تشير هذه النتائج إلى فوائد علاجية تآزرية محتملة لعلاج السرطان واستراتيجيات التوصيل المستهدفة في سرطان الثدي البشري.

1. Introduction

Globally, breast cancer is one of the biggest health issues affecting women. It is the second most prevalent cause of mortality for women in developed nations, accounting for 29% of all cancers in women [1].

Breast cancer is treated after diagnosis using several traditional methods, including surgery, immunotherapy, chemotherapy, and radiation. Chemotherapy and surgery are the first treatment options for cancer patients [2]. Due to the damage and side effects of chemotherapy and radiation and their effects on healthy cells, the development of drug resistance, and treatment deficiencies, there is an urgent need to develop new methods and techniques from natural sources that have anticancer efficacy as an alternative to traditional treatments [3,4].

Recently, the importance of nanotechnology has increased [5,6]. This technology includes the manufacture and applications of materials that have dimensions in the range of 1-100 nanometers and has been integrated with other sciences, especially pharmaceuticals, which has led to its entry into the field of medical applications, the most important of which is cancer treatment, as nanoparticles are used in diagnosis and treatment as antioxidants [7-9].

Bromelain is a proteolytic enzyme found in pineapple stems and juice. Interesting pharmacological and possible medical uses have been demonstrated by bromelain reduction of metastasis: By modulating the expression of genes involved in cell migration and invasion, bromelain could help prevent the spread of cancer cells to other parts of the body [10]. Certain hypotheses state that bromelain has the ability to stop tumor cells from growth and dissemination in addition to inducing cancer cell death due to its proteolytic and immunomodulatory properties. Lowering inflammation may contribute to a less conducive environment for tumor growth [11]. Researchers reported the cytotoxic role of bromelain in various human breast cancer cells, and it has been found that bromelain reduced MCF-7 cells proliferation by 72.6% and 66.5% at 24 hours, so the reduction response is dependent on a dose and time manner [10]. Additionally, bromelain was mobilised on the surface of the nano-capsules, which facilitated entry into the cell. The cytotoxic effect was also reported in PC3 prostate cancer cells, by which a 25% reduction in cell viability was observed in a dose-dependent manner[12]. The current study aims to investigate the methods of enhancing the properties of bromelain, specifically its anticancer potentials, through increasing its apoptotic activity and wound-healing potential. Also, the immobilisation technique may improve the stability, activity and bioavailability, making it more effective for therapeutic applications.

2. Materials and Methods

Chemicals and cell culture line

Tetracholroareic acid ($HAuCl_4$,$3H_2O$) was obtained from (Fluka,USA), and bromelain (EC:3,4,22,32) from Sigma Chemical (USA). Breast cancer cell line (MCF-7) and Rat Embryo Fibroblast (REF) were a kind gift from Al-Nahrain University, Biotechnology Research Center, Iraq. All tissue culture media (RPMI-1640), fetal bovine serum, antimicrobial solution (Penicillin and Streptomycin) and EDTA-Trypsin were purchased from (Gibco-UK).

Preparation of the AuNPs and Bromelain enzyme-immobilisation

Bromelain was loaded with gold nanoparticles that were synthesised using the standard Turkevich method, followed by characterisation as detailed in our previous work [13]. The nanoparticles produced with and without bromelain were characterised using UV-Vis, FTIR, XRD, TEM, and Zeta potential.

Growth Inhibition Measurement

The toxicity of AuNPs, AuNPs-Br and Br on breast cancer cells MCF-7 and REF cell line (served as a normal cell) was assessed using the MTT test. To create a flowing monolayer, 10^4 cells/well were grown for 24 hours. Following the addition of (13.5, 62.5, 125, 250, and 500 µg/ml) of AuNPs, AuNPs-Br and bromelain, respectively, for 24 hours, 10 µL of MTT dye was added, and the mixture was then incubated for four hours at 37 °C. After removing the dye, each well was filled with 100 µL of dimethyl sulfoxide (DMSO) for air bubble dissolving, and it was left to incubate for an additional five minutes. The absorbance was measured at 575 nm using a spectrophotometer [14].

Assay for Migration by Wound Healing Process.

An *in vitro* scratch experiment was utilised to investigate the effects of AuNPs, AuNPa-Br and Br at concentrations of (15 and 30 µg/ml) on breast cancer and normal cell lines for a period of 0, 24, 48 hours in 6 well plate cell culture grade. Scratching the monolayer cells surface was done with a sterile 200 µl pipette tip. Cells that had been moved into the wound's surface were photographed at 4 and 10× magnification using inverted microscopy, and Image J Software was used to analyse the images where the gap was used. Each photograph's open wound area's width (µm) was calculated [15].

Measurement of apoptosis by flow-cytometry

The MCF-7 cell line treated with AuNPs was used to determine apoptosis using the flow cytometry technique. Apoptotic cells can be detected following staining with Annexin V-FITC and propidium iodide (PI) using flow cytometry. In this method, Annexin V-FITC binds to phosphatidylserine, which translocates to the outer membrane of apoptotic cells, while PI stains necrotic cells that have lost membrane integrity. This combination identifies early apoptotic cells (Annexin V-positive and PI-negative) and late apoptotic or necrotic cells (Annexin V-positive and PI-positive). The concentration and the proportion of cells with a nucleus fragment were measured. After 24 h culture, the MCF-7 cells were treated with AuNPs 30 µg/ml and then suspended in FACS buffer. Following staining with Annexin V-FITC and PI aye, apoptotic cells were detected using a flow cytometry experiment.

Statistical Analysis

Data were analysed using the SPSS programme (V. 23), where analysis of variance (ANOVA) was used to assess significant differences, followed by the LSD test. Values were demonstrated as mean ± SD, and the difference was accepted at P≤0.05.

3. Results and Discussion

Characterisation of gold nanoparticles

Some results presented herein were previously reported in Ausaj *et al*., [13] and content is included for the sake of comprehensiveness and to enhance understanding as it is a key methodology. These results include the initial evidence that nanoparticles (NPs) are forming, such as a change in color, UV-Spectrometer, FTIR, TEM and XRD. In brief, UV-Vis spectroscopy detected Gold nanoparticles (AuNPs) with peaks at 520 nm, 534 nm for AuNPs-Br, and 278 nm for Br. FTIR analysis in the XRD analysis showed peaks at (311,220,200 and 111cm^{-1}). TEM electron microscopy AuNPs were formed at sizes between 35 and 44 nm, and the AuNP-Br showed sizes between 95-100 nm. The zeta potential of AuNPs at 25 °C was observed as 0.7 mV, and the Zeta potential measurement of the AuNPs - Br was -32.2 mV (Figure 1).

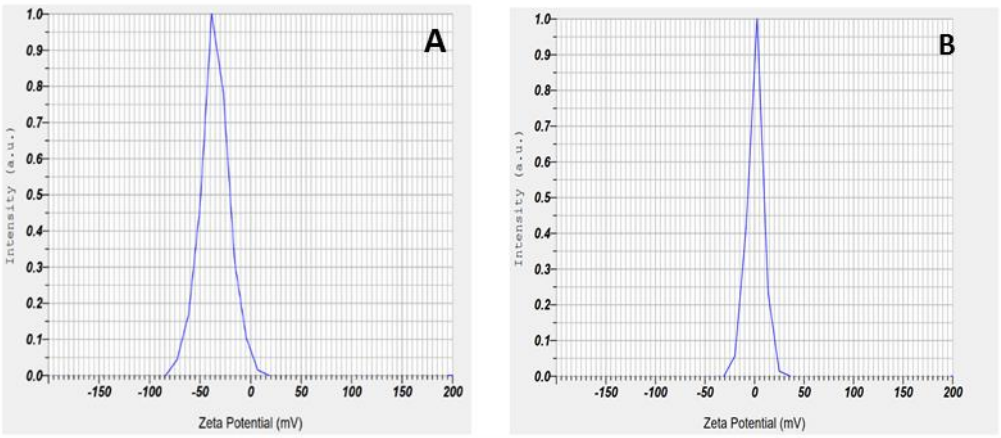

Figure 1: Zeta potential measurement of (A)AuNPs,(B) AuNP-Br

Cell cytotoxicity

The cytotoxic effect was evaluated using the MTT method, and various concentrations as 31.5, 62.5, 125, 250, and 500 µg ml^{-1} of AuNPs solution, AuNPs-Br and Br were determined. The results indicated a dose-dependent effect on cancer cell lines with no significant effects on normal cells. At 31.5, 62.5 µg ml$^{-1,}$ the results showed a significant decrease in growth inhibition percentage. However, there was no toxic effect on normal REF cells (Tables 1 and 2).

Gold nanoparticles may be an effective tool in the fight against cancer. In order to specifically target the target area with biological payloads and anticancer medications, gold nanoparticles were widely employed as drug delivery systems. Gold nanoparticles represent a great option for a drug delivery system because of their small size, spherical shape, and capacity to combine and functionalise different ranges of molecules. These properties allowed AuNPs to easily penetrate cancer cells [16]. Their small size, which allows them to easily enter cells through transport mechanisms like phagocytosis and pinocytosis alternatively by building up in the basal plasma extracellular membrane space, AuNPs are

also believed to be a very useful source of potent cytotoxic and anti-proliferative agents for a variety of cell lines [17].
Bromelain's ability to inhibit tumor cell growth and spread as well as cause cancer cell death, may be due to its immunomodulatory and proteolytic properties. AuNPs can cause protein and lipid oxidation, severely impaired mitochondrial function, and ultimately cell death [18].

Table 1: Cell growth inhibition (%) in human breast cancerMCF-7 cell line induced by AuNPs, AuNPs-Br and bromelain.

Concentration (µg ml^{-1})	MCF-7 growth inhibition (%) (Mean±S.E.)			P value
	AuNPs	AuNPs-Br	Bromelain	
31.5	12.81±1.71	21.39±2.49	13.93±3.39	0.1 NS
62.5	28.75±2.41	41.14±4.13	22.49±5.89	0.6 NS
125	79.94±1.05c	81.98±3.2c	64.10±4.5c	0.01*
250	83.47±0.33a	86.91±2.2b	73.95±2.42ba	0.007**
500	87.97±3.65	92.15±1.28	84.84±0.77	0.15 NS
P value	<0.001**	<0.001**	<0.001**	-

a vs. AuNPs, b vs. AuNPs-Br, c vs. Br, NS= Non-Significant

Table 2 : Cell growth inhibition (%) in REF cell line induced by AuNPs, AuNPs-Br and bromelain.

Concentration (µg/mL)	RIF growth inhibition (%) (Mean±S.E.)			P value
	AuNPs	AuNPs-Br	bromelain	
31.5	95.56±0.56	96.27±0.09	95.61±0.29	0.37 NS
62.5	91.86±1.68	94.97±0.26	96.0±0.63	0.07 NS
125	93.23±0.25	91.12±1.50	93.41±1.52	0.4 NS
250	90.69±1.20	90.3±1.24	91.22±0.45	0.82 NS
500	89.87±1.15	89.2±1.47	89.96±1.54	0.9 NS
P value	0.03*	0.004**	0.008**	-

NS= Non-Significant

Migration assay (scratch assay)

Using the scratch/wound healing assay, the effects of AuNPs, AuNPs-Br, and Br on the migration of MCF-7 and REF cell lines were assessed. All of the findings are included in (Figures 2, 3, 4 and 5). The treatment with AuNPs, AuNPs-Br, and Br considerably decreased the migration of the MCF-7 cell line, with a higher rate in the AuNP-Br group. A larger migration rate was seen in the control group.
One of the most serious clinical processes is metastasis, which is the migration of cancer cells from their source to anatomically distant organs, according to Liu *et al.,* and Lai *et al.,* [19-20]. E-cadherin is a well-known tumor suppressor protein that prevents metastasis and invasion. Reduced expression of the E-cadherin gene has been observed in a variety of malignancies, most notably breast cancer [21]. Adhesion proteins, such as vimentin protein, E-cadherin, N-cadherin, Snail, Slug, Twist, MMP-2, and MMP-9, are important in cell migration [22,23],

The study indicates that treatment with AuNPs, AuNPs-Br, and bromalin can inhibit the migration of MCF-7 breast cancer cells, likely due to the up-regulation of E-cadherin, which acts as a tumor suppressor by preventing invasion and metastasis. Gold nanoparticles can

influence the regulation of E-cadherin, a crucial protein for maintaining cell adhesion. While studies indicate that the suppression of E-cadherin is associated with increased invasiveness and metastasis of tumors, the role of gold nanoparticles appears to vary. Specifically, unmodified gold nanoparticles have been reported to inhibit the proliferation and metastasis of certain cancer cells, suggesting a complex interaction where their effects may depend on the cellular context and modifications of the nanoparticles [24,25],

Additionally, matrix metalloproteinase-9 (MMP-9) is upregulated by snails, which helps the extracellular matrix (ECM) invasion. MMPs break down structural elements of the ECM, permitting tumor invasion and metastasis. According to Ridley *et al*., [26], MMP-9 and MMP-2 are essential for invasive metastasis and angiogenesis in cancer cells. Therefore, when AuNPs are used to treat the MCF-7 breast cancer cell line, AuNPs-Br and bromelain may inhibit the activity of MMP-9 and MMP-2 by down-regulating Snail protein, which may aid in the inhibition of MCF-7 breast cancer cell line migration [27].

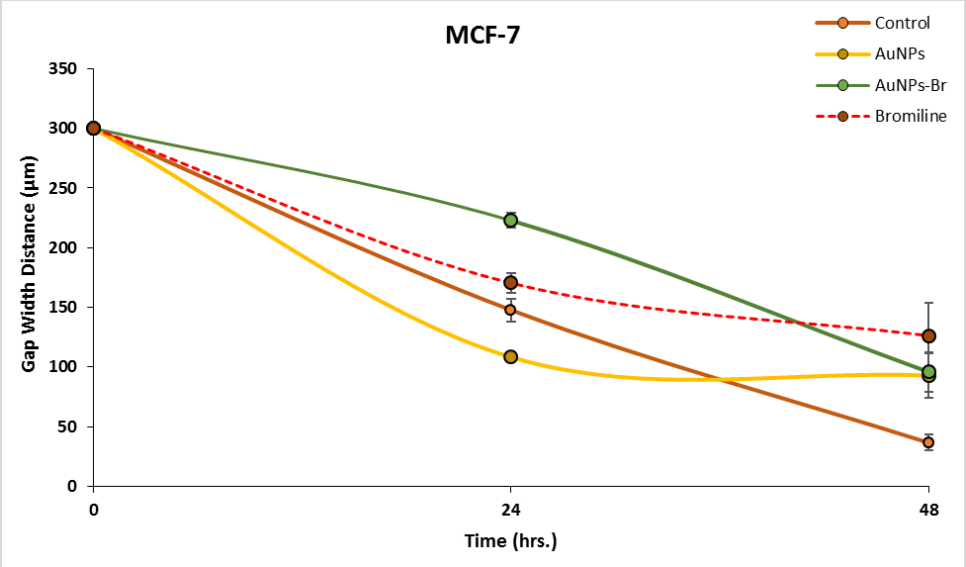

Figure 2: Effects of AuNPs, AuNPs-Br, and bromelain on breast cancer MCF-7 cell line migration using scratch assay.

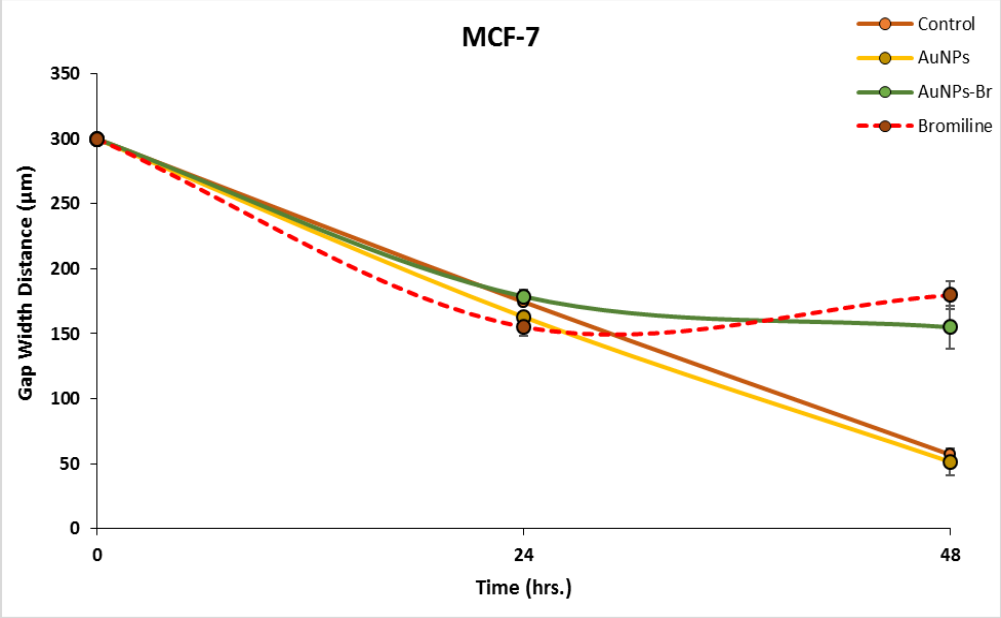

Figure 3: Effects of AuNPs, AuNPs-Br, and bromelain on REF cell line migration using scratch assay

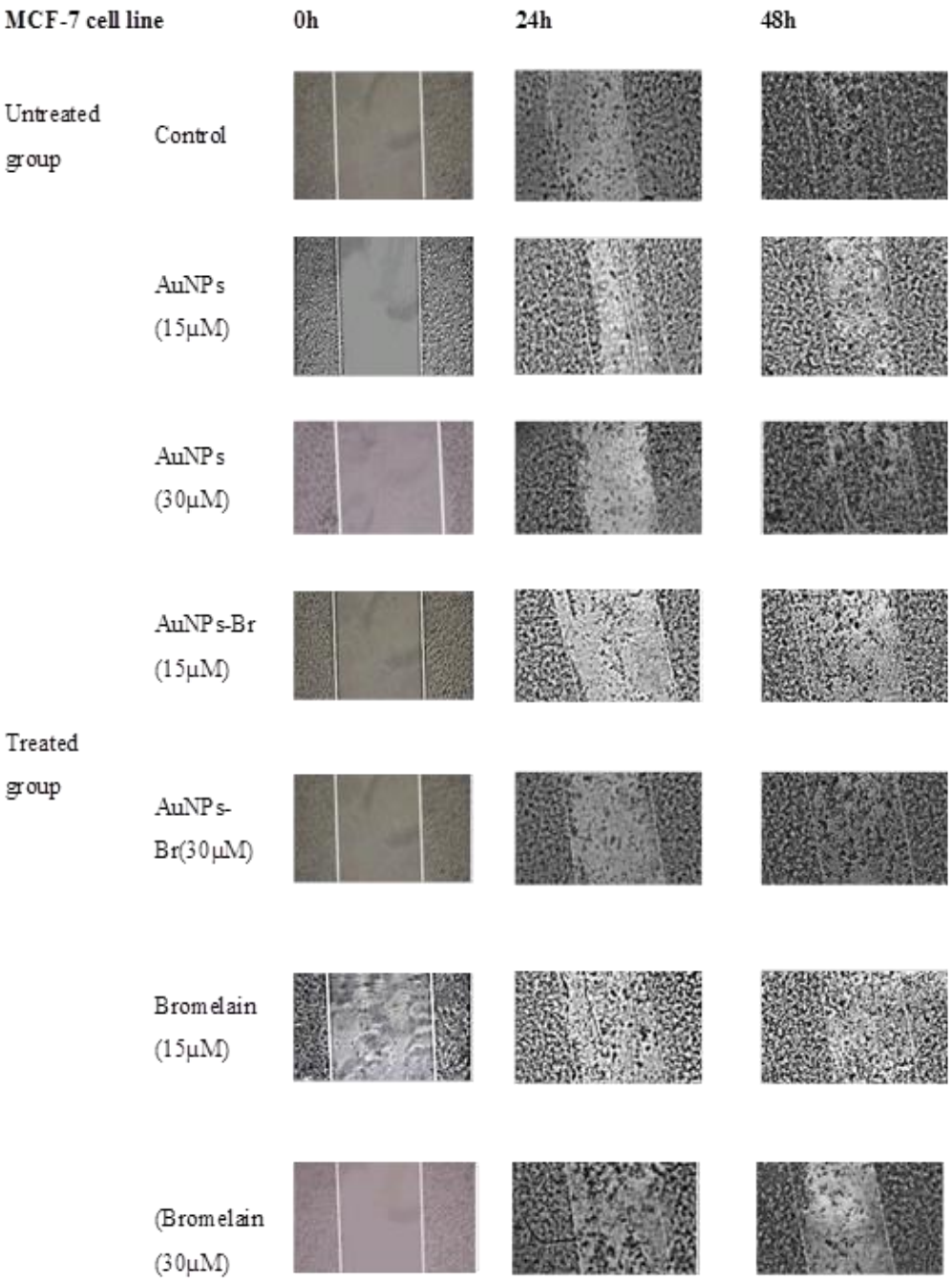

Figure 4: The breast cancer MCF-7cells were treated with vehicle control DMSO (0.01%) as control and with AuNPs, AuNPs-Br and bromelain

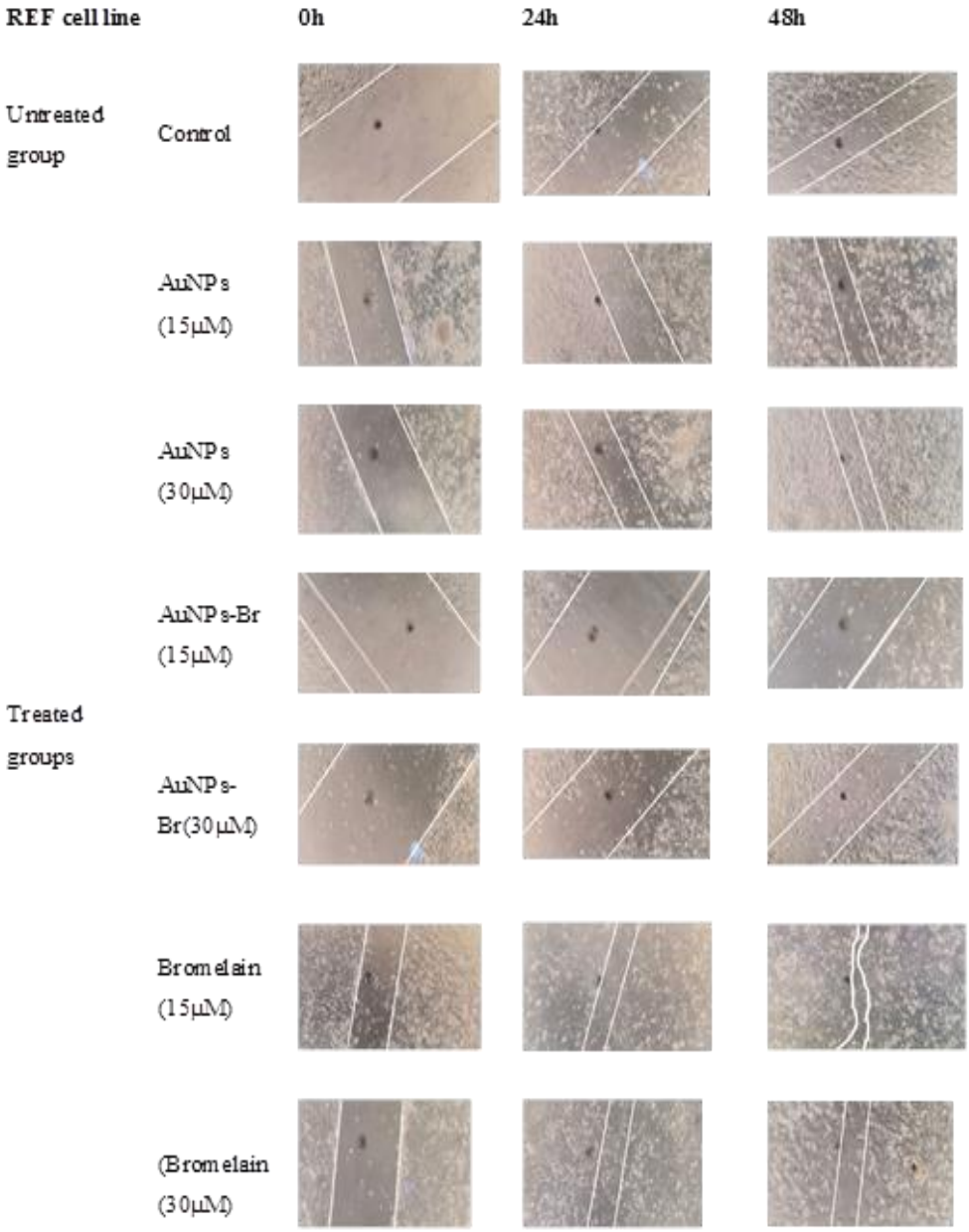

Figure 5: The REF cell line was treated with vehicle control DMSO (0.01%) as control and with AuNPs, AuNPs-Br and bromelain

Apoptosis analysis with flow cytometry

Flow cytometric data is typically represented as follows: cells in the lower left quadrant are regarded to be live cells (Annexin V-/PI-), whereas cells in the lower right quadrant are thought to be in the early apoptotic stage (Annexin V+/PI-). Cells in the necrotic stage are shown by the upper quadrant (Annexin V-/PI+), whereas cells in the late apoptotic stage are represented by the upper right quadrant (Annexin V+/PI+). The control untreated MCF-7 cell line has 92.54% living cells, 2.21% early apoptotic cells, 1.11% late apoptotic cells and 4.14% necrotic cells according to flow cytometric analysis as shown in Figure 9 (A). For the AuNPs-Br (at 30 µm/ml for 24 h), treatment of the MCF-7 cell line resulted in 4.53% living

cells, 89.1% apoptotic early cells, 5.14% apoptotic late cells and 1.23% necrotic cells (Figure 6).

A fundamental mechanism of cell death, apoptosis plays an important role in physiological processes by preserving cellular homeostasis. However, it can also be deleterious in pathological processes, such as the suppression of tumors. This cell death process can be responsible for blocking cancer cell spread, intervening in the inhibition of the metastasis process [28]. In LPS-stimulated macrophages, AuNPs increase the generation of reactive oxygen species (ROS) and cause apoptosis [29]. The increase in ROS is a sign of oxidative stress and is necessary to cause apoptosis [30]. One study confirmed that a change in gene expression of the proteins responsible for programmed death (caspases) in mitochondria-dependent pathways causes the death of tumor cells, as the presence of Zinc nanoparticles ions in the medium enables the cells to absorb them quickly and induce differentiation caspase to enter programmed death [31]. A study confirmed that treatment of the MCF-7 cell line with Ag ZnO compound led to increased expression of genes responsible for the production of Proteins of the programmed cell death, including caspase 3, which is one of its products caspase 9 and caspase 8, which in turn stimulate other proteins that contribute to cell death [32]. According to Yang *et. Al.,* [33], bromelain can promote cell death and apoptosis by increasing pro-apoptotic proteins and changing apoptotic pathways. Various phytochemicals can effectively treat neoplasms by inducing apoptotic pathways in human cancer cells. It has been reported that bromelain induced apoptosis and damaged the mitochondrial membrane by upregulating the expression of pro-apoptotic proteins, including p53, p21 and Bax, in breast cancer cell line due to ROS [34]. Also, bromelain suppresses NF-kB and initiates apoptosis by inducing cancer cells to arrest G2/M phase [35]. By initiating the mitochondrial pathway and inhibiting the production of COX-2 and NF-kB, bromelain triggered apoptosis in skin tumors and caused cells to undergo apoptosis [36]. The results of the current study revealed that the synergistic effect of AuNPs-Br causes apoptosis of the MCF-7 cell line. the current findings emphasises the potential benefits of gold nanoparticles with bromelain in future anticancer multi-modal therapy. By interfering with the inhibition of the metastasis process, this cell death mechanism may prevent the spread of cancer cells [37].

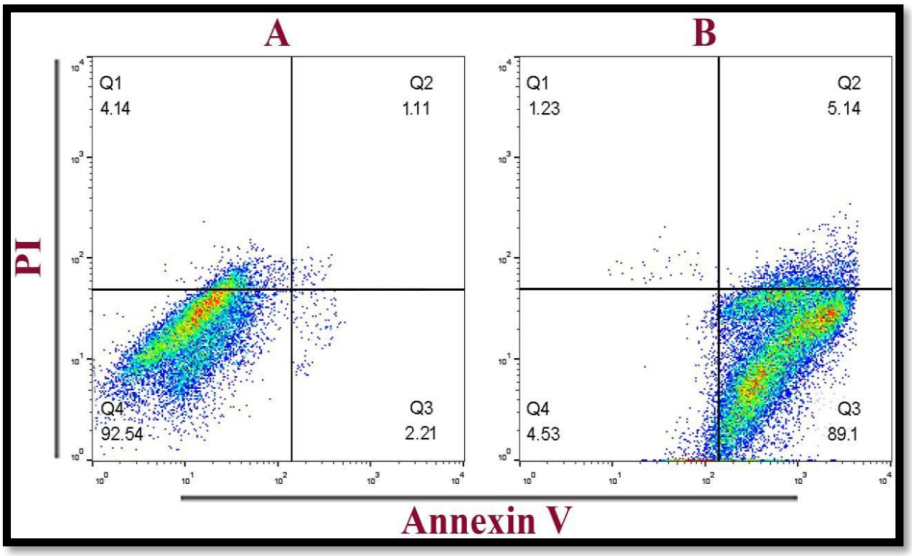

Figure 9 : Flow cytometric data analysis of apoptosis of MCF-7 cell line. A. control untreated MCF-7 cell line. B. MCF-7 cell line treated with AuNPs-Br.

4. Conclusions

The purpose of this work is to develop effective delivery methods using gold nanoparticles to deliver sufficient amounts of bromelain to the tumor site. The results showed that the synergistic combination of bromelain and gold nanoparticles successfully inhibited the growth of cancer cells and suppressed the migration of cancer cells along with induced apoptosis in the MCF-7 cell line. While these properties are promising, more studies are necessary to fully understand bromelain alone or in combination with god-nanoparticles role in cancer therapy and to establish effective dosing and treatment regimens.

Conflicts of interest:

All authors report no conflicts of interest

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