

RESEARCH PAPER

Formulation and Apoptosis-Mediated Cytotoxicity of a Multicomponent Nanocomposite Hydrogel Containing Doxorubicin, AgNPs, and Clove Extract in MCF-7 Cells

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ARTICLE INFO

Article History:

Received 28 June 2026

Accepted 17 September 2026

Published 01 October 2026

Keywords:

Alginate nanocomposite hydrogel

Ag NPs

Apoptosis-related genes

BAX/BCL2 ratio

Clove extract

Doxorubicin

ABSTRACT

Breast cancer treatment remains challenged by the systemic toxicity and limited therapeutic selectivity of conventional chemotherapy. This study developed and evaluated a multicomponent nanocomposite alginate hydrogel incorporating doxorubicin (DOX), green-synthesized silver nanoparticles (AgNPs), and clove (*Syzygium aromaticum*) extract as an experimental therapeutic platform against MCF-7 breast cancer cells. AgNPs were synthesized using clove extract as a reducing and capping agent and characterized by X-ray diffraction (XRD) and energy-dispersive X-ray spectroscopy (EDX). XRD analysis indicated crystalline face-centered cubic metallic silver, with an estimated crystallite size of 12.9 nm based on the Scherrer equation. Following calcium-mediated crosslinking, field-emission scanning electron microscopy (FE-SEM) revealed a more heterogeneous and fibrillar morphology in the triple-component formulation compared with unloaded alginate, while Fourier-transform infrared spectroscopy (FTIR) indicated changes in the chemical environment of functional groups following incorporation of the active components. The cytotoxicity of single-, dual-, and triple-component formulations was evaluated in MCF-7 cells after 24, 48, and 72 h using the MTT assay, complemented by microscopic assessment of cellular morphology and RT-qPCR analysis of BAX and BCL2 expression. The triple-component nanocomposite hydrogel produced the most pronounced concentration- and time-dependent reduction in cell viability, reaching approximately 33% viability at 4 µg/mL after 72 h. This effect was accompanied by marked loss of cell adhesion, cellular rounding, and a reduction in monolayer confluence from approximately 90–95% in controls to 10–15% following treatment. RT-qPCR analysis indicated increased BAX and decreased BCL2 expression following treatment with the triple-component nanocomposite alginate hydrogel, resulting in an increased BAX/BCL2 expression ratio and a molecular profile consistent with an apoptosis-associated response. Overall, the triple-component nanocomposite hydrogel exhibited substantial *in vitro* activity against MCF-7 cells and provides a basis for further investigation of the component-specific contributions, therapeutic selectivity, and underlying mechanisms of cell death.

How to cite this article

Barzegar A., Al-Hadidi A., Khalaj-kondori M. Formulation and Apoptosis-Mediated Cytotoxicity of a Multicomponent Nanocomposite Hydrogel Containing Doxorubicin, AgNPs, and Clove Extract in MCF-7 Cells *J Nanostruct*, 2026; 16(4):5549-5566.
DOI: 10.22052/JNS.2026.04.086

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INTRODUCTION

Breast cancer is a major global health burden and remains one of the most frequently diagnosed malignancies worldwide. According to the GLOBOCAN 2022 estimates, breast cancer accounted for approximately 2.3 million new cases and 666,000 deaths globally in 2022, underscoring the continuing need for effective therapeutic strategies [1]. Although advances in systemic therapy have substantially improved outcomes for many patients, conventional anticancer drugs can be associated with dose-limiting toxicity, nonspecific exposure of healthy tissues, and therapeutic resistance. These limitations have stimulated continued interest in drug-delivery systems capable of modifying drug disposition, protecting therapeutic agents, and regulating their release [2, 3].

Hydrogels are particularly attractive for drug delivery because their 3D, water-rich polymer networks can accommodate a range of therapeutic molecules and provide a matrix in which diffusion and release can be modulated by polymer composition, crosslinking, swelling, and environmental conditions [4]. Among naturally derived polymers, alginate is an anionic polysaccharide composed primarily of mannuronic- and guluronic-acid residues and is widely investigated for biomedical applications because it can undergo mild ionic gelation in the presence of divalent cations such as Ca^{2+} [5-7]. Alginate-based hydrogels have consequently been explored as carriers for small molecules, biological agents, and nanomaterials, with their physicochemical properties and crosslinking characteristics influencing encapsulation and release behavior [5-8]. These features provide a rational basis for investigating alginate as a matrix for the incorporation of multiple anticancer agents.

Silver nanoparticles (AgNPs) have attracted considerable interest in cancer nanomedicine because experimental studies have demonstrated their ability to affect cancer-cell viability through several physicochemical and cellular processes, including oxidative stress, mitochondrial dysfunction, DNA damage, and activation of cell-death pathways [9, 10]. However, the biological effects of AgNPs are strongly dependent on particle size, surface chemistry, aggregation state, dose, and exposure conditions [9-11]. Incorporation of AgNPs into a biopolymeric

hydrogel therefore offers a potential strategy for integrating nanoparticle-associated biological activity with the physicochemical advantages of a polymeric matrix [12, 13].

Clove (*Syzygium aromaticum*) is a plant-derived source of biologically active phytochemicals, including the phenolic compound eugenol. Experimental studies have reported cytotoxic and antiproliferative effects of clove extract against cancer cells, including MCF-7 breast cancer cells [14, 15]. Accordingly, eugenol itself has also been reported to induce growth inhibition and apoptotic features in MCF-7 cells [16]. The combination of clove-derived bioactive compounds with iron oxide nanoparticles [17] or with doxorubicin (DOX) has been investigated as a potential strategy to enhance anticancer activity and chemosensitivity in breast cancer cells [18]. DOX remains an important chemotherapeutic agent in the treatment of breast cancer. However, its clinical use is constrained by adverse effects, including potentially dose-limiting cardiotoxicity [19, 20]. These limitations have motivated extensive investigation of drug-delivery approaches designed to modify the exposure and release of doxorubicin. Previous studies have demonstrated the feasibility of alginate-containing hydrogel systems for doxorubicin delivery and evaluation against breast-cancer models, including MCF-7 cells [21, 22]. In parallel, alginate-based systems containing AgNPs have been investigated in breast-cancer models, demonstrating the broader feasibility of combining metallic nanoparticles with alginate matrices for anticancer applications [12]. Thus, integrating AgNPs and clove extract with doxorubicin within an alginate hydrogel provides a rational experimental platform for investigating the combined physicochemical and biological properties of these components.

Taken together, existing studies support the individual relevance of alginate hydrogels, AgNPs, clove-derived phytochemicals, and doxorubicin for experimental cancer drug-delivery research. However, comparatively limited information is available regarding a multicomponent nanocomposite alginate hydrogel incorporating these components within a single formulation and evaluating their physicochemical characteristics and *in-vitro* activity against MCF-7 cells. The present study therefore aimed to prepare triple-component nanocomposite hydrogel incorporating AgNPs, clove extract, and

doxorubicin, characterize the resulting materials using spectroscopic, diffraction, microscopic, and elemental analyses, and investigate the cytotoxic effects of the resulting formulations against MCF-7 breast cancer cells.

MATERIALS AND METHODS

Materials

Sodium alginate, silver nitrate (AgNO_3), calcium chloride (CaCl_2), ethanol, doxorubicin hydrochloride, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), MTT reagent, were provided from Sigma-Aldrich (St. Louis, MO, USA). MCF-7 were achieved by the Tabriz Faculty of Medical Sciences. The acquisition of clove buds for this study was conducted through a purchase from the local market of Kohna Bazar, Tabriz. RPMI-1640 culture medium, fetal bovine serum (FBS), Trypsin-EDTA and Penicillin/Streptomycin solution were purchased from ThermoFisher Scientific. Sterile tissue-culture plastics and glassware were obtained from Disposable Products. Trizol reagent (Maxcell), cDNA synthesis kits and SYBR Green Master Mix were purchased from SMOBIO (Taiwan).

Deionized water was used throughout the experimental procedures. Clove (*Syzygium aromaticum*) flower buds were provided from traditional market.

Preparation of clove bud extract

Clove (*Syzygium aromaticum*) flower buds were thoroughly washed several times with distilled water and subsequently dried in the dark for 24 h. The dried buds were finely powdered using a mixer grinder. A 25-g portion of the resulting powder was mixed with 1000 mL of 50% (v/v) ethanol and maintained at room temperature for 96 h under continuous shaking. The use of a hydroethanolic extraction procedure is suitable for recovering bioactive constituents from clove and that approximately 50% ethanol can provide favorable extraction performance [23]. Therefore the hydroalcoholic extract mixture was subsequently filtered using Whatman No. 5 filter paper (pore size, 2.5 μm). The filtrate was concentrated at 40 °C using a vacuum rotary evaporator (IKA RV 10 Digital, Germany). The resulting dark-brown extract was further dried in a vacuum desiccator to remove residual moisture and stored at -20 °C until further use.

Green synthesis of silver nanoparticles using clove extract

Silver nanoparticles (AgNPs) were synthesized using clove extract as the plant-derived reducing and stabilizing component. Plant-mediated synthesis of AgNPs is based on the ability of phytochemicals present in plant extracts to participate in the reduction of Ag^+ ions and to contribute to stabilization/capping of the resulting nanoparticles [11, 24]. Clove-AgNPs were produced by using the prepared clove extract as a stabilizing and reducing agent. Briefly, 0.05 g of the dried clove extract was dispersed in 20 mL of deionized water. Separately, 0.05 g of AgNO_3 was dissolved in 10 mL of deionized water. The AgNO_3 solution was then added to the clove-extract solution, and the resulting mixture was magnetically stirred for 40 min. A visible color change from yellow to dark brown was observed during the reaction and was used as a preliminary visual indication of Ag^+ reduction and formation of AgNPs. The resulting precipitate was collected, washed once with deionized water, and dried at 70 °C for 5 h. The synthesized AgNPs were subsequently characterized by X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), and scanning electron microscopy (SEM).

Preparation of calcium-crosslinked alginate hydrogels

For preparation of the alginate hydrogel matrix, 1.5% alginate was dissolved in 40 mL of deionized water. Calcium chloride solution (4%) was subsequently used as the crosslinking solution to form the calcium-crosslinked alginate hydrogel. Alginate crosslinking by divalent cations such as Ca^{2+} is an established ionic-gelation approach for preparing alginate-based hydrogels [5-7]. Following gel formation, the samples were frozen and subsequently freeze-dried after 24 h.

Preparation of multicomponent nanocomposite alginate hydrogel formulations

A series of alginate-based hydrogel formulations containing doxorubicin (DOX), silver nanoparticles (AgNPs), and clove extract were prepared to evaluate the effects of individual and combined components. The formulations were categorized into single-, dual-, and triple-component groups, with the triple-component formulation referred

to as the multicomponent nanocomposite. The single-component formulations consisted of alginate hydrogels containing DOX, AgNPs, or clove extract individually. The dual-component formulations contained either DOX and AgNPs, AgNPs and clove extract, or DOX and clove extract. The triple-component formulation incorporated DOX, AgNPs, and clove extract within the alginate hydrogel matrix. The formulations were prepared at the predetermined concentrations described below and were subsequently subjected to physicochemical characterization, including X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and scanning electron microscopy (SEM). The formulations were also evaluated for their *in vitro* effects on MCF-7 breast cancer cells using the experimental assays described in the following sections.

Physicochemical characterization

The crystalline structure of the synthesized nanoparticles (NPs) and multicomponent alginate-based nanocomposite formulations was investigated by X-ray diffraction (XRD) over an angular range of 20–90° (2 θ). Fourier-transform infrared (FTIR) spectroscopy was employed to characterize the functional groups of the synthesized alginate-based hydrogel formulations and to compare their spectral features with those of the individual components. FTIR spectra were recorded over the range of 400–4000 cm⁻¹, and the observed spectral features were interpreted to identify changes in functional-group environments associated with the incorporation and interaction of the formulation components. The morphology and surface characteristics of the synthesized AgNPs and multicomponent alginate-based hydrogel formulations were examined using field-

emission scanning electron microscopy (FE-SEM). Energy-dispersive X-ray spectroscopy (EDX/EDS), together with elemental mapping, was performed on selected samples to assess their elemental composition and spatial distribution.

In-vitro cytotoxicity assay

The cytotoxicity of free DOX, AgNPs, clove extract, and the corresponding alginate-based formulations was evaluated against MCF-7 breast cancer cells using the MTT assay. MCF-7 cells were cultured as a monolayer in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin and maintained at 37 °C in a humidified incubator containing 5% CO₂. When the cells reached approximately 80% confluence, they were transferred to 96-well plates. The cells were exposed to different concentrations of free DOX, AgNPs, clove extract, and the corresponding multicomponent nanocomposite hydrogel formulations for 24, 48, and 72 h.

Following each treatment period, the culture medium was removed and the wells were washed once with PBS. Subsequently, 100 μ L of culture medium and 100 μ L of MTT solution (5 mg/mL) were added to each well. The plates were incubated for 3 h at 37 °C in the dark. The resulting solution was then removed, and 200 μ L of DMSO was added to each well to dissolve the formed formazan crystals. Absorbance was measured at 570 nm using an ELISA plate reader. Cell viability was calculated relative to untreated control cells.

The experimental groups comprised free DOX, drug-free alginate hydrogel (blank hydrogel), and triple-component nanohydrogel formulations. For comparative evaluation, the concentrations of the active agents in the hydrogel formulations were matched to those used for the corresponding free

Table 1. Primer sequences used in RT-PCR analysis of BAX and BCL2 gene expression.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
BAX	AGGGTTTCATCCAGGATCGAGCAG	ATCTTCTCCAGATGGTGAGCGAG
BCL2	GTGAACTGGGGGAGGATTGT	GGAGAAATCAAACAGAGGCC
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

agents.

Quantitative RT-PCR analysis of BAX and BCL2 gene expression

Total RNA was extracted from MCF-7 cells following treatment with the triple-component nanohydrogel formulation at concentrations of 3 and 4 $\mu\text{g/mL}$ for 48 h. RNA was extracted using TRIzol reagent and subsequently used for cDNA synthesis according to the manufacturer's instructions using a commercial reverse-transcription kit. Quantitative real-time PCR (RT-qPCR) was performed on an ABI StepOnePlus Real-Time PCR System using SYBR Green Master Mix to evaluate the expression of the apoptosis-related genes *BAX* and *BCL2*. *GAPDH* was used as the reference gene for normalization, and untreated cells were used as the calibrator group. Specific primers provided in Table 1 [25].

Relative gene expression was calculated using the comparative $2^{-\Delta\Delta\text{Ct}}$ method [24]. The ΔCt value for each sample was calculated by subtracting the Ct value of *GAPDH* from that of the target gene. The $\Delta\Delta\text{Ct}$ value was then determined relative to the untreated control group, and relative gene expression was calculated as $2^{-\Delta\Delta\text{Ct}}$. The expression levels of BAX and BCL2 were evaluated to investigate treatment-associated changes in the balance between pro-apoptotic and anti-

apoptotic gene expression. In addition, the BAX/BCL2 expression ratio was calculated from the corresponding relative expression values as an indicator of changes in the balance between pro-apoptotic and anti-apoptotic signaling.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10. Data are presented as mean $\pm$ standard deviation (SD) from three independent biological experiments, with three technical replicates per condition. Statistical comparisons were performed using one-way/two-way analysis of variance (ANOVA), followed by Tukey's multiple-comparisons test. A p-value < 0.05 was considered statistically significant. The assumptions of the statistical tests, including normality and homogeneity of variance, were assessed before analysis.

RESULTS AND DISCUSSION

X-ray diffraction analysis

X-ray diffraction (XRD) analysis was performed to investigate the crystalline structure of the green-synthesized silver nanoparticles (AgNPs). As shown in Fig. 1, the diffraction pattern recorded over a 2θ range of $20\text{--}90^\circ$ exhibited characteristic reflections at approximately 38.1° , 44.3° , 64.4° , and $77.5\text{--}77.8^\circ$, which can be indexed to the (111),

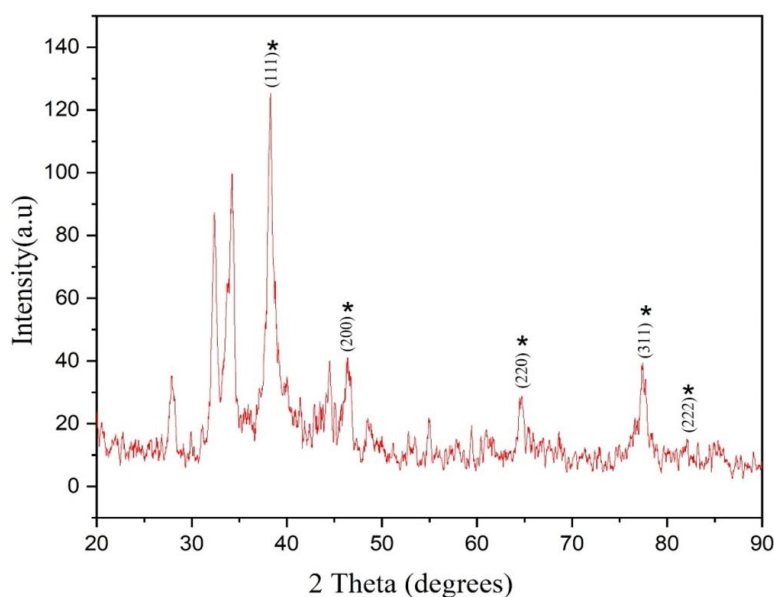


Fig. 1. X-ray diffraction pattern of green-synthesized silver nanoparticles using *Syzygium aromaticum* (clove) extract.

(200), (220), and (311) crystallographic planes, respectively, of face-centered cubic (FCC) metallic silver. A further reflection at approximately 81.5° is consistent with the (222) plane of FCC silver. Standard Ag data and published XRD studies support the (111), (200), (220), (311), and (222) assignments. These reflections are in close agreement with the standard diffraction pattern of crystalline metallic Ag (JCPDS/ICDD No. 04-0783) and with previously reported XRD patterns of green-synthesized AgNPs, including AgNPs prepared using *Syzygium aromaticum* (clove) extract [26]. The predominance of the (111) reflection indicates that this plane contributes the strongest diffraction intensity in the synthesized Ag phase. Several non-indexed features, particularly in approximately the 30–37° and 45–60° regions, are also visible in the diffractogram. Because their origin cannot be established from the XRD pattern alone, they were not assigned to specific crystalline phases. These features could arise from background contributions and/or residual organic material associated with the clove-mediated synthesis. This possibility is relevant to plant-mediated nanoparticle synthesis because phytochemical constituents from the extract may remain associated with the nanoparticle preparation. Overall, the characteristic Ag reflections support the formation of crystalline metallic Ag with an FCC structure.

The crystallite size of the AgNPs was additionally estimated using the Scherrer equation:

$$D = \frac{K\lambda}{\beta \cos \theta} \quad (1)$$

where D is the mean crystallite size, K is the Scherrer shape factor ~ 0.9 , λ is the Cu $K\alpha$ radiation wavelength (0.15406 nm), β is the full width at half maximum (FWHM) of the diffraction peak expressed in radians, and θ is the corresponding Bragg angle. For the most prominent Ag(111) reflection at approximately ($2\theta = 38.1^\circ$), the Scherrer equation yielded an estimated mean crystallite size of approximately 12.9 nm. This value represents the size of the coherently diffracting crystalline domains of particle size.

FTIR spectroscopy analysis

Fourier-transform infrared (FTIR) spectroscopy was used to characterize the major functional groups present in the AgNPs, doxorubicin,

clove extract, and final triple-component nanocomposite alginate hydrogel. The individual spectra provide reference signatures for the components, whereas the spectrum of the final formulation allows assessment of the preservation and possible changes in these functional groups following incorporation into the alginate hydrogel matrix.

The FTIR spectrum of the green-synthesized AgNPs exhibited several bands associated with oxygenated and organic functional groups derived from the clove extract. The broad bands at approximately 3413 and 3165 cm^{-1} are consistent with O–H/N–H stretching vibrations, while the bands in the ~ 2950 – 2850 cm^{-1} region can be attributed mainly to aliphatic C–H stretching. Additional bands were observed at approximately 1713, 1582, 1448, and 1368 cm^{-1} , together with bands in the fingerprint region around 1058, 980, 887, 702, and 663 cm^{-1} . Because clove phytochemicals contain abundant phenolic and other oxygen-containing functional groups, these spectral features are consistent with the presence of plant-derived constituents associated with the AgNP preparation. Previous studies of clove-mediated AgNP synthesis have similarly demonstrated the association of phytochemical constituents with the nanoparticle surface and have proposed a role for eugenol and other extract constituents in the reduction and stabilization of AgNPs [26, 27].

The clove extract spectrum showed a broad O–H stretching band centered at approximately 3442 cm^{-1} , together with bands at approximately 2923 cm^{-1} , 1653 cm^{-1} , 1484 cm^{-1} , and 1383 cm^{-1} , followed by several bands in the fingerprint region. These features are consistent with the presence of hydroxyl-containing phenolic compounds and other oxygenated organic constituents in the extract. The strong broad O–H band is particularly compatible with the phenolic character of clove constituents, while the bands in the C–H stretching and fingerprint regions reflect the complex composition of the plant extract.

The doxorubicin spectrum exhibited a broad absorption band centered at approximately 3413 cm^{-1} , bands around 2924 cm^{-1} , and several characteristic absorptions in the ~ 1700 – 1000 cm^{-1} region, including bands near 1719, 1619, 1441, and 1028 cm^{-1} . These bands are consistent with the diverse oxygen-containing and aromatic functional groups of doxorubicin. FTIR spectroscopy

has previously been used to characterize the vibrational features of doxorubicin and provides a molecular fingerprint that can be compared with the spectrum of a drug-containing formulation [28].

The most important spectrum for the present study is that of the multicomponent nanocomposite alginate hydrogel, containing alginate together with doxorubicin, AgNPs, and clove extract. The final formulation exhibited a broad and intense band centered at approximately 3387 cm^{-1} , accompanied by bands at 2939 and 2885 cm^{-1} . The broad high-wavenumber band is attributable primarily to O–H stretching associated with the hydrated polysaccharide network and hydroxyl-containing components, whereas the bands near 2939 and 2885 cm^{-1} are consistent mainly with aliphatic C–H stretching vibrations. Several important bands were

observed in the fingerprint region. The absorption at approximately 1647 cm^{-1} can be associated with O–H bending of absorbed water and/or overlapping contributions from functional groups of the incorporated components. The band near 1420 cm^{-1} is particularly relevant to alginate and is consistent with the symmetric stretching vibration of carboxylate groups (COO^-). Alginate commonly exhibits asymmetric and symmetric COO^- stretching bands in the approximately 1600 and 1410 cm^{-1} regions, respectively, and changes in these bands can accompany ionic interactions and Ca^{2+} -mediated alginate crosslinking. The bands at approximately 1218 , 1110 , and 1044 cm^{-1} are located within the characteristic carbohydrate/polysaccharide fingerprint region and are compatible with C–O and C–O–C vibrations of the alginate network, with possible overlapping contributions from the incorporated clove and

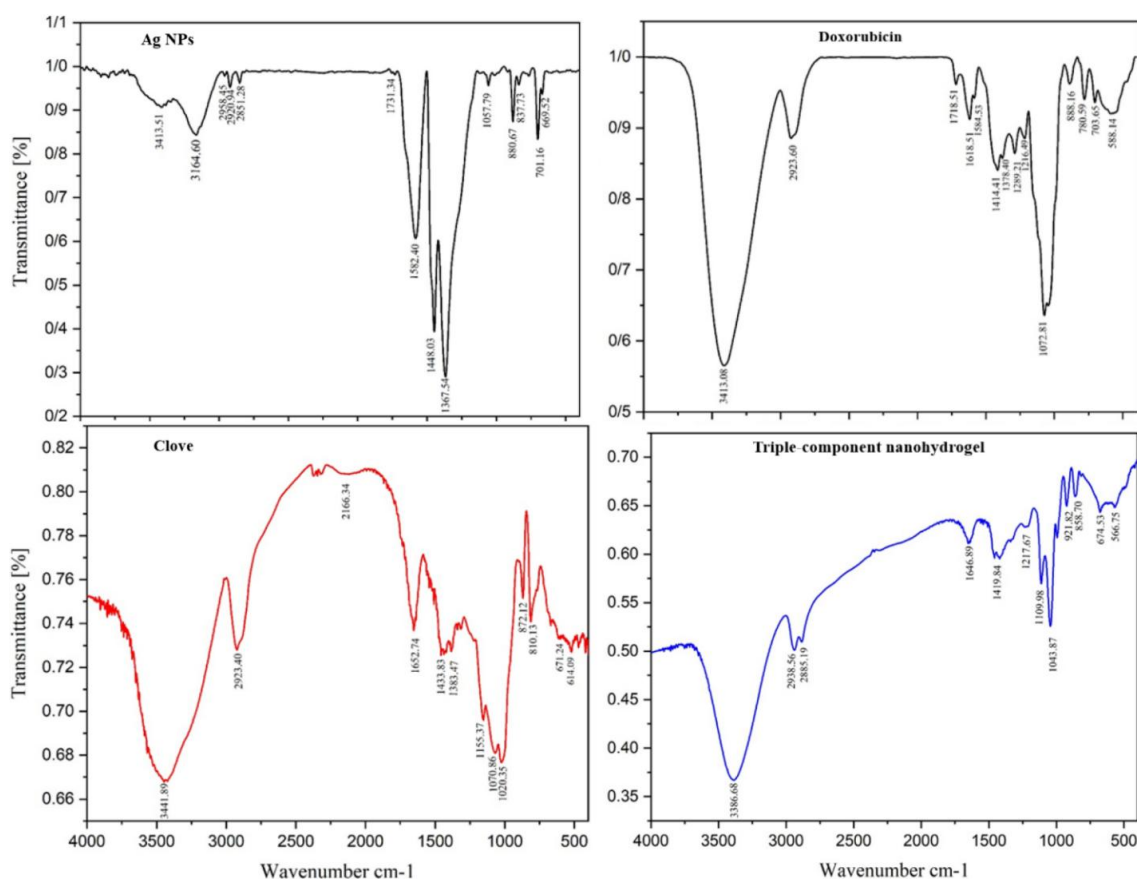


Fig. 2. FTIR spectra of the individual components and the final triple-component nanocomposite alginate hydrogel. FTIR spectra of green-synthesized AgNPs, doxorubicin, clove (*Syzygium aromaticum*) extract, and the triple-component nanocomposite hydrogel comprising alginate, doxorubicin, AgNPs, and clove extract.

doxorubicin components. Additional bands at approximately 922 and 859 cm^{-1} occur in the lower-wavenumber fingerprint region of polysaccharides and may reflect structural vibrations associated with the alginate backbone.

Comparison of the final nanohydrogel spectrum with the spectra of the individual components shows that several characteristic spectral regions of the starting materials are retained, although their positions and relative intensities are altered or partially overlapped in the final formulation. In particular, the broad O–H band, C–H stretching bands, carboxylate-associated region around 1420 cm^{-1} , and carbohydrate fingerprint region remain evident in the triple-component material. The increased spectral complexity and changes in band intensity are consistent with the coexistence of alginate and the incorporated organic components within the final formulation. The spectra demonstrate the presence and altered spectral environment of functional groups after formulation, which is consistent with intermolecular interactions and/or ionic, hydrogen-bonding, or surface-associated interactions within the nanohydrogel. Such an interpretation is also consistent with the known ability of alginate carboxylate groups to participate in ionic crosslinking and interactions with incorporated molecules and nanoparticles

[29].

Overall, the FTIR results support the successful incorporation of the major organic components into the alginate-based formulation and demonstrate that characteristic functional-group signatures are retained in the final triple-component nanohydrogel.

FE-SEM Analysis of Green-Synthesized AgNPs

The morphology of the green-synthesized AgNPs was examined by FE-SEM at different magnifications (Fig. 3). The micrographs revealed a population of predominantly spherical to near-spherical particles with some degree of morphological heterogeneity and local particle aggregation. The nanoparticles were distributed throughout the examined fields, although individual particles were not uniformly separated in all regions. Based on the particle-size assessment reported for the FE-SEM images, the synthesized AgNPs had an approximate particle size of 32.5 nm. The observed nanoscale morphology is consistent with the formation of silver-containing nanostructures following the green synthesis procedure. The variation in particle appearance and the presence of some clustered regions indicate that the preparation was not completely monodisperse.

Overall, the FE-SEM observations provide

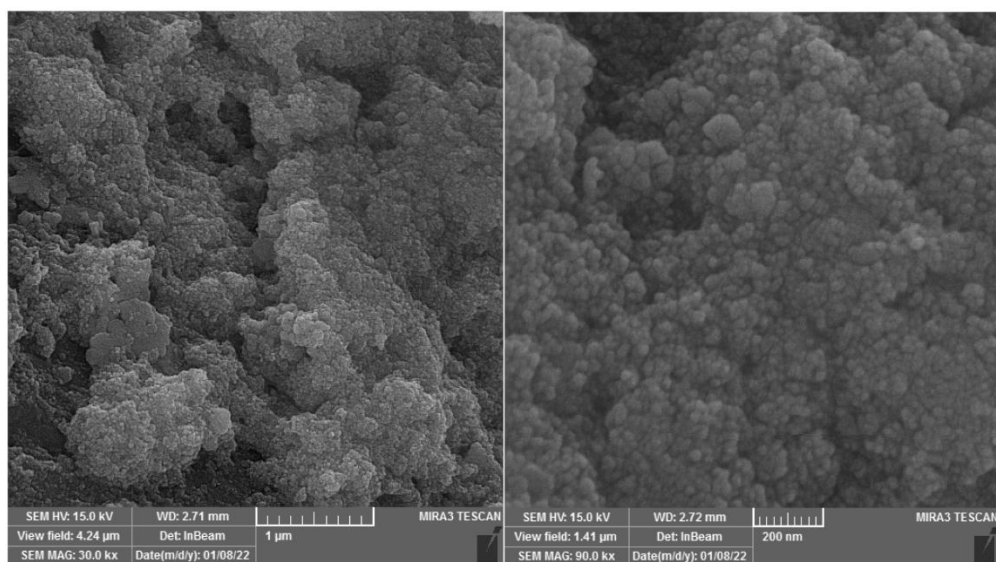


Fig. 3. FE-SEM images of the green-synthesized silver nanoparticles (AgNPs) at two different magnifications. The micrographs show predominantly spherical to near-spherical nanoparticles with some degree of particle aggregation and morphological heterogeneity.

morphological evidence for the formation of nanoscale particulate structures in the green-synthesized sample. However, FE-SEM morphology alone does not establish the crystalline phase or elemental identity of the particles; these characteristics are addressed by the XRD and EDX analyses, respectively.

EDX Elemental Composition and Mapping of Green-Synthesized AgNPs

Energy-dispersive X-ray spectroscopy (EDX) was performed to determine the elemental composition of the green-synthesized AgNP sample (Fig. 4). The EDX spectrum was dominated by characteristic silver signals, with a prominent Ag signal in the approximately 3 keV region, confirming the presence of silver as the major detectable element in the analyzed sample. In addition to Ag, weaker signals corresponding to C, O, and N were detected. The elemental maps

further demonstrated the spatial distribution of Ag, C, N, and O throughout the analyzed field. The Ag map showed a widespread distribution of silver-associated signal across the examined area, while the C, N, and O signals were also distributed throughout the field and were visible in the composite elemental map.

The detection of C, O, and N is compatible with the presence of organic constituents associated with the plant-mediated synthesis process. Because clove extract was used during the green synthesis of AgNPs, these elements may be associated with residual or surface-associated plant-derived organic compounds. Hence, EDX elemental analysis is as evidence of phytochemicals that constitute a capping layer of Ag NPs. The EDX results therefore complement the FE-SEM observations by confirming a high Ag contribution in the analyzed material and demonstrating the presence and spatial distribution of minor C-, O-,

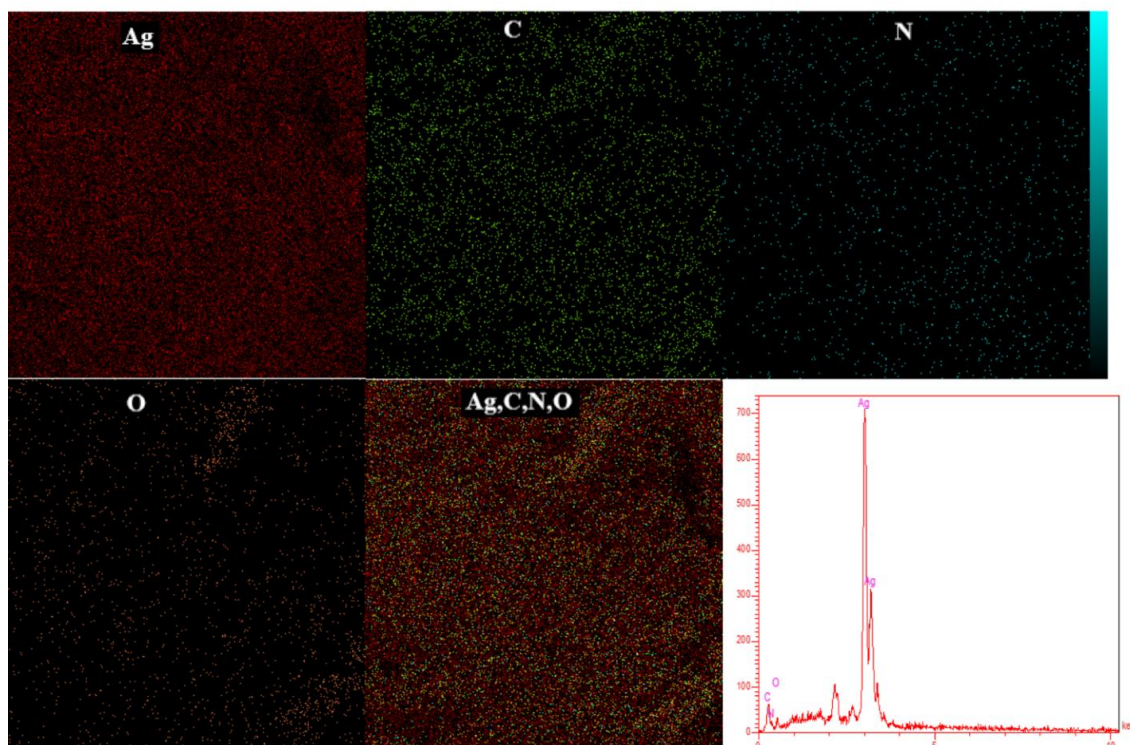


Fig. 4. EDX spectrum and elemental mapping of the green-synthesized silver nanoparticles (AgNPs). The Figure includes elemental maps for Ag, C, N, and O, a composite overlay of the detected elements, and the corresponding EDX spectrum. The prominent Ag signal indicates that silver is the major detectable element in the analyzed sample, while weaker C, O, and N signals indicate the presence of additional organic/oxygen- and nitrogen-containing components associated with the sample.

and N-containing components.

Morphology of triple-component nanocomposite hydrogel

The surface morphology of the alginate hydrogel and the multicomponent nanocomposite alginate hydrogel was examined by FE-SEM. As shown in Fig. 5, the two formulations exhibited clearly different microstructural characteristics. The unloaded alginate hydrogel displayed a highly porous and irregular architecture characterized by relatively large, interconnected voids separated by continuous polymeric walls or lamellar structures. The presence of interconnected pores indicates the formation of a three-dimensional hydrogel network with substantial internal void space.

In contrast, incorporation of doxorubicin, AgNPs, and clove extract produced a visibly more heterogeneous and structurally complex morphology. The triple-component hydrogel formulation exhibited a more irregular, fibrillar network with numerous interconnecting strands and smaller, more tortuous void spaces distributed throughout the observed field. Compared with the relatively open architecture of the unloaded alginate hydrogel, the loaded formulation

appeared more compact and heterogeneous, with increased complexity of the polymeric network. These morphological differences indicate that incorporation of the three components was accompanied by a substantial alteration of the observed microstructure of the alginate matrix.

The observed change in morphology is consistent with the physicochemical characterization presented above, particularly the FTIR results showing retention and alteration of characteristic functional-group bands following formulation. The altered architecture may reflect changes in polymer–component interactions, ionic crosslinking and/or the physical distribution of the incorporated materials within the alginate network. Therefore, the FE-SEM results are interpreted as evidence of a changed and more heterogeneous hydrogel microarchitecture following incorporation of doxorubicin, AgNPs, and clove extract rather than as direct proof of specific molecular interactions or uniform nanoparticle distribution. Overall, the FE-SEM images demonstrate that incorporation of the three active components substantially modified the morphology of the alginate hydrogel, resulting in a more heterogeneous and fibrillar porous

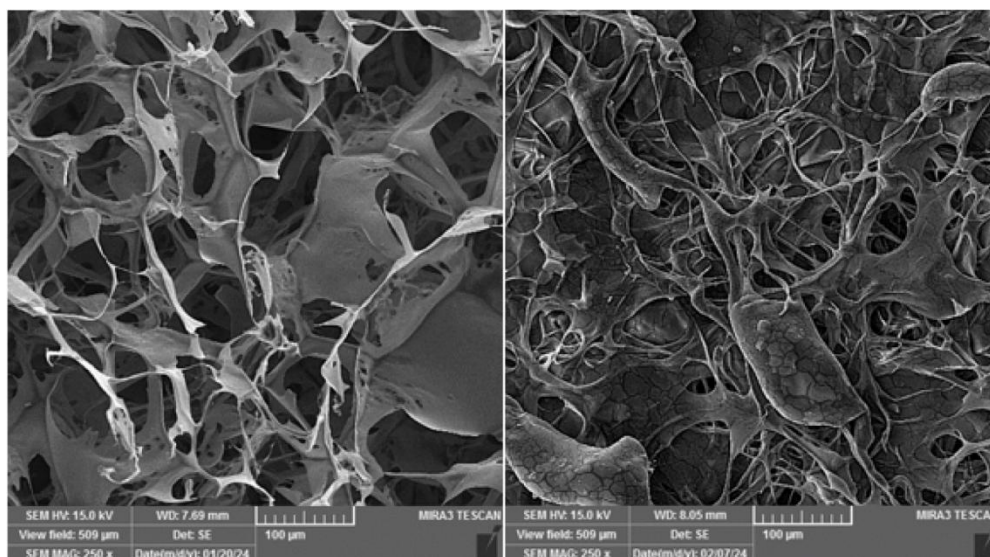


Fig. 5. FE-SEM morphology of the alginate hydrogel (left) and triple-component nanocomposite alginate hydrogel incorporating doxorubicin, silver nanoparticles, and clove extract (right). The unloaded alginate hydrogel exhibits a relatively open, irregular, interconnected porous architecture, whereas the triple-component formulation displays a more heterogeneous and fibrillar network with increased structural complexity.

architecture in the final formulation.

In vitro cytotoxicity of single-, dual-, and triple-component nanocomposite against MCF-7 cells

MTT assay was used to evaluate the effects of the alginate-based formulations on the viability of MCF-7 cells following 24, 48, and 72 h of exposure. Cell viability was determined relative to the untreated control, which was set at approximately 100%. The tested formulations comprised single-, dual-, and triple-component nanocomposite alginate hydrogel formulations. The MTT assay was performed according to the experimental procedure described in the Methods, with absorbance measured at 570 nm and cell viability calculated relative to untreated cells.

As shown in Fig. 6, the tested formulations produced concentration- and time-associated reductions in MCF-7 cell viability, although the magnitude of the response varied according to formulation composition. After 24 h, the effects were generally moderate across the tested concentration range. At the highest tested concentration of 4 $\mu\text{g/mL}$, the single-component formulations produced different levels of viability reduction, with Alginate@Dox showing approximately 71% viability, whereas Alginate@Ag and Alginate@Clove maintained approximately 85% and 89% viability, respectively. The dual-component formulations also showed variable effects, with viability of approximately 69%, 74%,

and 64% for Alginate@Dox@Ag, Alginate@Dox@Clove, and Alginate@Ag@Clove, respectively. The triple-component formulation resulted in approximately 64% viability at this concentration.

After 48 h of exposure, the overall reduction in cell viability became more apparent for several formulations. At 4 $\mu\text{g/mL}$, viability was approximately 70% for Alginate@Dox, 84% for Alginate@Ag, and 87% for Alginate@Clove. Among the dual-component formulations, Alginate@Dox@Ag, Alginate@Dox@Clove, and Alginate@Ag@Clove resulted in approximately 69%, 73%, and 64% viability, respectively. The triple-component formulation produced approximately 64% viability at the same concentration. Thus, the dual- and triple-component formulations containing combinations of the active agents generally produced greater reductions in viability than the corresponding formulations containing AgNPs or clove extract alone.

A more pronounced cytotoxic response was observed after 72 h of exposure. Cell viability decreased progressively with increasing formulation concentration for most treatment groups. At 4 $\mu\text{g/mL}$, the viability of MCF-7 cells was approximately 45% following treatment with Alginate@Dox, compared with approximately 54% and 58% for Alginate@Ag and Alginate@Clove, respectively. The dual-component formulations produced approximately 38–42% viability, with Alginate@Dox@Ag showing approximately 38%,

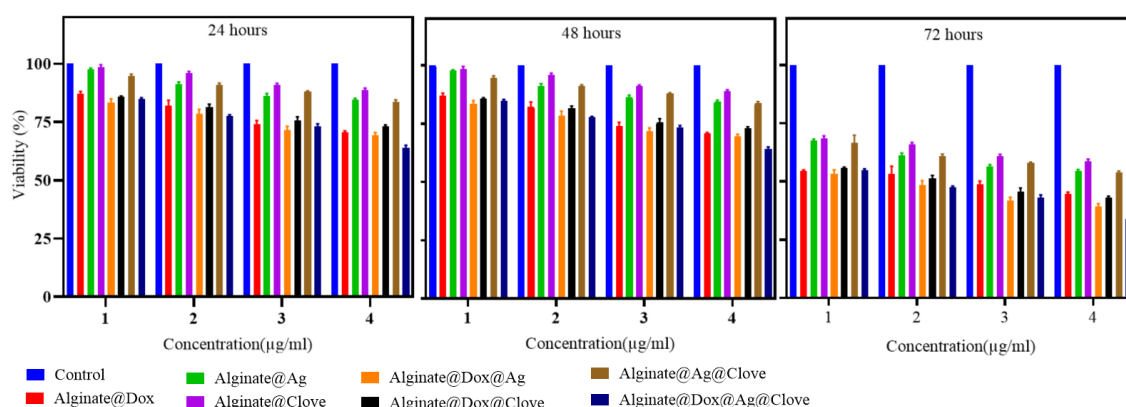


Fig. 6. In vitro cytotoxic effects of single-, dual-, and triple-component nanocomposite alginate hydrogel formulations on MCF-7 cell viability base on MTT assay. MCF-7 cells were exposed to the indicated concentrations (1–4 $\mu\text{g/mL}$) of single-component formulations containing doxorubicin (Alginate@Dox), silver nanoparticles (Alginate@Ag), or clove extract (Alginate@Clove); dual-component formulations containing doxorubicin and AgNPs (Alginate@Dox@Ag), doxorubicin and clove extract (Alginate@Dox@Clove), or AgNPs and clove extract (Alginate@Ag@Clove); and the triple-component formulation containing doxorubicin, AgNPs, and clove extract (Alginate@Dox@Ag@Clove) for 24, 48, and 72 h.

Alginate@Dox@Clove approximately 42%, and Alginate@Ag@Clove approximately 42% viability. The greatest reduction in viability was observed for the alginate triple-component nanocomposite alginate hydrogel formulation, for which cell viability decreased to approximately 33% at 4 $\mu\text{g}/\text{mL}$.

Comparison across the three exposure times indicates that the cytotoxic effects of the alginate formulations generally became more pronounced with prolonged incubation. This time-associated response was particularly evident for the triple-component formulation, in which viability at the highest concentration decreased from approximately 64% at 24 h to 64% at 48 h and approximately 33% at 72 h. The progressive reduction in viability with increasing exposure duration is consistent with an accumulation of treatment-associated effects over time.

The comparison between single-, dual-, and triple-component nanocomposite alginate hydrogel further indicates that incorporation of multiple active components into the alginate matrix was associated with a greater reduction in MCF-7 cell viability, particularly after 72 h. The triple-component formulation produced the lowest observed viability at the highest tested concentration, followed by several dual-component formulations. This finding is consistent with the overall rationale of the study, in which doxorubicin, AgNPs, and clove-derived constituents were incorporated into a common alginate matrix to generate a multifunctional formulation. The preceding physicochemical characterization demonstrated the crystalline Ag phase by XRD, retention of characteristic functional-group regions by FTIR, and a modified porous/fibrillar morphology of the final formulation by FE-SEM. Together, these findings establish the physicochemical characteristics of the formulation before its biological evaluation.

Nevertheless, the MTT results alone cannot establish whether the greater reduction in viability produced by the dual- or triple-component nanocomposite alginate hydrogel results from synergistic interactions among the incorporated agents. Such a conclusion requires a formal quantitative drug-interaction analysis using an appropriate experimental design and model. Accordingly, the present results are more appropriately interpreted as demonstrating enhanced cytotoxic effects or greater reductions in

MCF-7 cell viability associated with the combined formulations, rather than as direct evidence of synergy.

Overall, the MTT assay demonstrated that the single-, dual-, and triple-component alginate formulations exerted differential effects on MCF-7 cell viability, with generally greater reductions observed at higher formulation concentrations and longer exposure times. The alginate triple-component formulation produced the greatest reduction in viability at 72 h, indicating that this formulation warrants further investigation in subsequent quantitative analyses of cytotoxicity and component interactions.

Morphological assessment of MCF-7 cells treated with the triple-component formulation

The morphological response of MCF-7 cells to the alginate-based triple-component nanocomposite alginate hydrogel was evaluated by inverted phase-contrast microscopy after 48 h of exposure at Fig. 7. Untreated cells exhibited a dense and relatively homogeneous adherent monolayer, with predominantly polygonal and flattened morphology and extensive surface coverage. This appearance is consistent with a relatively healthy, proliferating adherent cell population. Treatment with the triple-component nanohydrogel containing doxorubicin, AgNPs, and clove extract produced evident morphological and density-dependent changes. At 3 $\mu\text{g}/\text{mL}$ (Fig. 7B), the cell monolayer was substantially less dense than that of the untreated control, with reduced surface coverage and a greater proportion of rounded and refractile cells. Several cells appeared detached or only weakly attached, while the remaining adherent cells showed changes in their spreading morphology. These observations indicate a pronounced treatment-associated alteration in cell attachment and morphology. At the higher concentration of 4 $\mu\text{g}/\text{mL}$ (Fig. 7C), the reduction in cell density was more pronounced. The previously continuous monolayer was largely disrupted, and the remaining cells were predominantly rounded, shrunken, and highly refractile, with substantial loss of normal cell spreading and adhesion. The greater morphological alteration observed at 4 $\mu\text{g}/\text{mL}$ compared with 3 $\mu\text{g}/\text{mL}$ is consistent with a concentration-associated increase in the cytotoxic response.

The microscopic findings are consistent with the MTT results presented in Fig. 6, where the triple-

component formulation produced a progressively greater reduction in MCF-7 cell viability with increasing concentration and exposure time. In particular, the marked reduction in cell density and disruption of the adherent monolayer observed after 48 h provide morphological evidence supporting the reduced metabolic viability detected by the MTT assay. The observations are also consistent with the preceding physicochemical characterization, in which incorporation of doxorubicin, AgNPs, and clove extract produced a distinct and heterogeneous alginate-based nanohydrogel structure.

The morphological changes observed in Figs. 7B and 7C, including cell rounding, shrinkage, increased refractility, and loss of adhesion, are compatible with cell-death-associated morphological alterations and may be consistent with apoptotic processes. Quantitative confluence values were obtained using ImageJ analysis, as presented in Table 2. Briefly, the images were converted to 8-bit grayscale, followed by

thresholding to segment the cell-covered regions. Confluence was then calculated as the ratio of the segmented cell area to the total image area. The resulting confluence values were used as an indirect measure of cell attachment and, consequently, inferred viability. For the triple-component nanocomposite hydrogel, approximately 35–40% confluence was observed at 3 $\mu\text{g/mL}$, whereas confluence decreased to approximately 10–15% at 4 $\mu\text{g/mL}$. However, these features are not specific enough to distinguish apoptosis from other forms of cell death. Confirmation of the underlying mode of cell death requires an appropriate biochemical or molecular gene expression analysis. For this aim, treatments with the triple-component nanohydrogel at both 3 $\mu\text{g/mL}$ and 4 $\mu\text{g/mL}$ conditions were selected for the subsequent RT-qPCR analysis. Thus, the morphological assessment provides a visual context for the molecular analysis that follows, while the gene-expression results provide complementary information concerning the cellular response to treatment.

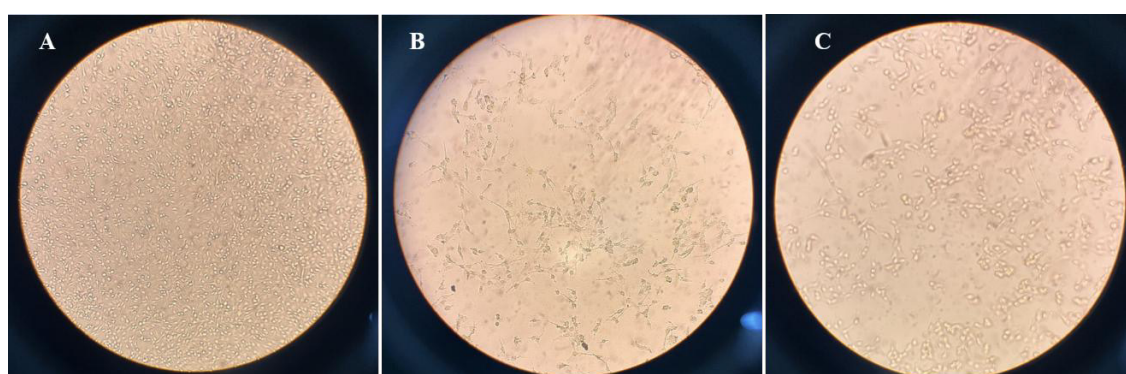


Fig. 7. Morphological changes in MCF-7 cells following 48 h exposure to the triple-component nanohydrogel. Representative inverted phase-contrast microscopic images of (A) untreated control MCF-7 cells, (B) cells treated with the alginate-based triple-component nanocomposite alginate hydrogel containing doxorubicin, silver nanoparticles (AgNPs), and clove extract at 3 $\mu\text{g/mL}$, and (C) cells treated with the same formulation at 4 $\mu\text{g/mL}$.

Table 2. Quantitative assessment of MCF-7 cell coverage/confluence and cellular morphology following 48 h exposure to the triple-component nanocomposite alginate hydrogel. Morphological observations were used to qualitatively characterize changes in cell attachment and monolayer integrity.

Condition	Approximate cell coverage/confluence	Morphological interpretation
Control	90–95%	Dense, relatively homogeneous adherent monolayer
Triple-component nanohydrogel, 3 $\mu\text{g/mL}$	35–40%	Marked reduction in coverage; rounding/shrinkage and loss of adhesion
Triple-component nanohydrogel, 4 $\mu\text{g/mL}$	10–15%	Severe disruption of monolayer and extensive loss of attached cells

Overall, the microscopy findings demonstrate a clear treatment-associated alteration in MCF-7 cell morphology, attachment, and apparent cell density after 48 h of exposure to the triple-component nanocomposite hydrogel, with more pronounced morphological disruption at 4 $\mu\text{g/mL}$ than at 3 $\mu\text{g/mL}$.

Gene expression analysis of apoptosis-related markers

To further investigate the molecular response of MCF-7 cells to the triple-component nanocomposite alginate hydrogel, the relative expression of the pro-apoptotic BAX and anti-apoptotic BCL2 genes was evaluated after 48 h of treatment.

Gene expression was normalized to GAPDH as the reference gene and expressed relative to untreated cells using the comparative $2^{-\Delta\Delta C_t}$ method, as described in detail in Supplementary Table S1. The resulting relative expression values and BAX/BCL2 ratios are presented in Table 3. Treatment with the triple-component nanohydrogel resulted in an increase in BAX expression at both tested concentrations. At 3 $\mu\text{g/mL}$, BAX expression increased to 1.85-fold relative to the untreated control, corresponding to an approximately 85% increase. At 4 $\mu\text{g/mL}$, BAX expression further increased to 1.95-fold, corresponding to an approximately 95% increase relative to control. Thus, both concentrations were associated with increased expression of the pro-apoptotic BAX gene, with the higher concentration producing a slightly greater relative increase. In contrast, BCL2 expression was reduced following treatment. At 3 $\mu\text{g/mL}$, BCL2 expression decreased to 0.24-fold of the untreated control, corresponding to an approximately 76% reduction. At 4 $\mu\text{g/mL}$, BCL2 expression was 0.55-fold of

control, corresponding to an approximately 45% reduction. Therefore, treatment with the triple-component nanocomposite hydrogel produced a marked reduction in the anti-apoptotic BCL2 transcript at both concentrations, with the greater reduction observed at 3 $\mu\text{g/mL}$.

The combined changes in BAX and BCL2 expression resulted in a pronounced increase in the BAX/BCL2 expression ratio, from 1.00 in untreated cells to 7.70 at 3 $\mu\text{g/mL}$ and 3.55 at 4 $\mu\text{g/mL}$. The approximately 7.7-fold increase in the BAX/BCL2 ratio at 3 $\mu\text{g/mL}$ reflects the combined increase in the pro-apoptotic BAX transcript and the substantial reduction in BCL2 expression. Although the ratio was lower at 4 $\mu\text{g/mL}$ than at 3 $\mu\text{g/mL}$, it remained approximately 3.6-fold higher than the untreated control, indicating a shift toward a more pro-apoptotic gene-expression profile at both treatment concentrations.

These molecular findings are consistent with the morphological observations described in Fig. 7. Treatment with the triple-component nanohydrogel produced reduced cell density, loss of normal spreading and adhesion, cell rounding, and increased morphological abnormalities after 48 h, with more pronounced changes at 4 $\mu\text{g/mL}$. The gene-expression data provide complementary molecular evidence that the treatment was associated with altered regulation of apoptosis-related genes. In particular, the increased BAX/BCL2 ratio is consistent with a shift in the balance between pro- and anti-apoptotic signaling toward an apoptosis-associated state. The molecular findings are also consistent with the MTT results presented in Fig. 6, in which the triple-component formulation produced a concentration- and time-associated reduction in MCF-7 cell viability. Together, the MTT, morphological, and gene-expression findings indicate that exposure to the

Table 3. Relative expression of BAX and BCL2 and the BAX/BCL2 ratio in MCF-7 cells following 48 h treatment with the triple-component nanocomposite alginate hydrogel.

Treatment	Relative BAX expression	Relative BCL2 expression	BAX/BCL2 ratio
Control	1.00	1.00	1.00
Triple-component nanohydrogel, 3 $\mu\text{g/mL}$	1.85	0.24	7.70
Triple-component nanohydrogel, 4 $\mu\text{g/mL}$	1.95	0.55	3.55

Values represent relative gene expression normalized to GAPDH and expressed relative to the untreated control (calibrator = 1.00), calculated using the comparative C_t ($2^{-\Delta\Delta C_t}$) method. The BAX/BCL2 ratio was calculated from the relative expression values of BAX and BCL2.

triple-component nanocomposite hydrogel was associated with reduced cellular viability and alterations in apoptosis-related gene expression.

Overall, the results demonstrate that the triple-component nanohydrogel altered the expression profile of two key apoptosis-associated genes in MCF-7 cells, characterized by increased BAX expression, decreased BCL2 expression, and an elevated BAX/BCL2 ratio at both tested concentrations. These findings, together with the reduction in MTT-derived viability and the treatment-associated morphological alterations, support the interpretation that apoptosis-associated signaling may contribute to the cellular response to the triple-component nanocomposite alginate hydrogel.

Characteristics of the triple-component nanocomposite hydrogel formulation

The present study successfully formulated and characterized a multicomponent nanocomposite alginate hydrogel incorporating doxorubicin, green-synthesized AgNPs, and clove extract, demonstrating enhanced *in vitro* cytotoxicity against MCF-7 breast cancer cells. Physicochemical analyses confirmed the structural and elemental integrity of the incorporated components. The incorporation of doxorubicin, green-synthesized AgNPs, and clove extract into the alginate matrix generated a structurally distinct triple-component nanohydrogel. The XRD profile was consistent with a face-centered cubic crystalline Ag phase, in agreement with previously reported AgNPs synthesized through plant-mediated approaches [9,10,23]. The predominance of the (111) reflection further supports the assignment of crystalline metallic silver. The crystallite size estimated using the Scherrer equation was 12.9 nm, providing an estimate of the coherent crystalline domain size of the Ag phase. Importantly, this value represents a crystallographic estimate derived from X-ray peak broadening rather than a direct measurement of the physical dimensions of individual nanoparticles.

The observed FTIR spectral changes, including shifts within the carbohydrate fingerprint region and changes associated with COO⁻ symmetric stretching, are compatible with interactions among the alginate backbone, doxorubicin, AgNPs, and oxygen-rich constituents of the clove extract. These changes may reflect ionic interactions, hydrogen bonding, and other non-covalent

associations within the polymeric network, as commonly observed in polysaccharide-based hydrogel systems containing drugs and nanoparticles [5–8, 12]. The presence of oxygen-containing phytochemicals may also contribute to the observed changes in the local chemical environment of the alginate matrix. These physicochemical characteristics provide a plausible structural basis for the subsequent biological behavior of the formulation.

The morphological analysis provided further evidence of substantial structural modification of the alginate matrix following incorporation of the active components. Whereas the native alginate hydrogel exhibited a highly porous morphology, the triple-component nanocomposite alginate hydrogel displayed a more heterogeneous and fibrillar network. This transition suggests that incorporation and crosslinking of the therapeutic components altered the organization and microstructure of the polymeric matrix. Such morphological modifications are consistent with previous observations in alginate-based systems containing nanoparticles or anticancer agents [12, 21, 22]. The altered morphology may provide a physical framework for accommodating the incorporated therapeutic components.

Biological assay of the triple-component nanocomposite hydrogel formulation

The biological evaluation demonstrated a pronounced concentration- and time-dependent reduction in MCF-7 cell viability following treatment with the alginate-based formulations. Notably, the triple-component nanocomposite alginate hydrogel produced a greater reduction in viability than the corresponding single- and dual-component formulations under the tested conditions, suggesting that the combined formulation provided a stronger overall cytotoxic response. At 4 µg/mL after 72 h of exposure, cell viability was reduced to approximately 33%. This quantitative reduction was accompanied by marked morphological deterioration, including loss of cell adhesion, reduced cellular spreading, and a substantial decrease in monolayer confluence from approximately 90–95% in untreated controls to 10–15% following treatment. The close correspondence between the MTT results and the observed morphological alterations provides complementary evidence for a substantial impairment of MCF-7 cell growth and viability.

The enhanced activity of the triple-component formulation may be related to the simultaneous presence of doxorubicin, AgNPs, and clove-derived constituents within the alginate matrix. AgNPs have been reported to exert anticancer effects through mechanisms involving oxidative stress, mitochondrial dysfunction, DNA damage, and activation of cell-death pathways [9, 10]. Clove extract contains several bioactive phytochemicals, including eugenol as a major constituent, and previous studies have described antiproliferative and apoptosis-associated effects of clove-derived preparations and eugenol in breast-cancer models [14–18]. Doxorubicin, in contrast, has well-established DNA-associated cytotoxic mechanisms, including topoisomerase II inhibition and induction of DNA damage [19, 20]. The co-localization of these components within an alginate-based matrix therefore provides a plausible basis for the enhanced biological response observed with the triple-component formulation.

Importantly, the greater cytotoxicity observed with the combined formulation should not be interpreted as definitive evidence of pharmacological synergy. A greater reduction in cell viability following combination treatment may result from additive effects, independent or partially overlapping mechanisms, altered cellular uptake, or other formulation-related effects. Accordingly, the present findings support enhanced cytotoxic activity of the triple-component nanocomposite hydrogel formulation under the tested conditions rather than a definitive synergistic interaction.

The molecular findings provide additional evidence that apoptosis-associated signaling contributes to the observed cytotoxic response. RT-qPCR analysis demonstrated upregulation of the pro-apoptotic BAX transcript together with downregulation of the anti-apoptotic BCL2 transcript. At 3 $\mu\text{g/mL}$, these changes resulted in a BAX/BCL2 expression ratio of 7.70, indicating a pronounced shift in the relative expression of these apoptosis-associated genes toward a pro-apoptotic profile. Because the balance between pro- and anti-apoptotic BCL2-family proteins is an important regulator of mitochondrial apoptotic signaling, the observed transcriptional changes are consistent with involvement of the intrinsic apoptotic pathway. These molecular findings are also compatible with the substantial

morphological deterioration and reduction in cell viability observed following treatment.

Interestingly, the gene-expression response did not increase monotonically with concentration. At 4 $\mu\text{g/mL}$, BCL2 expression was reduced to 0.55-fold, compared with 0.24-fold at 3 $\mu\text{g/mL}$, resulting in a lower BAX/BCL2 ratio of 3.55 despite the greater overall loss of cellular viability. This apparent divergence between cytotoxicity and the relative expression of apoptosis-associated genes warrants careful interpretation. One possible explanation is that, under conditions of severe cellular injury, transcriptional responses may no longer correlate linearly with the extent of cell death. At the higher concentration, extensive cellular damage may compromise transcriptional activity and RNA integrity or alter the relative contribution of apoptosis and other cell-death processes. AgNP-associated oxidative stress and doxorubicin-induced cellular damage could potentially contribute to such a response. Alternatively, the observed pattern may reflect the presence of heterogeneous cellular subpopulations with different responses to treatment.

However, the available data do not permit definitive attribution of the reduced BAX/BCL2 ratio at 4 $\mu\text{g/mL}$ to a switch from apoptosis toward necrosis or to a specific alternative cell-death mechanism. In particular, the present RT-qPCR data measure transcriptional changes rather than apoptosis or necrosis directly. Confirmation of such a mechanistic transition would require complementary assays capable of distinguishing apoptotic, necrotic, and other cell-death pathways, such as Annexin V/PI analysis, caspase activation assays, mitochondrial membrane-potential assessment, or other validated mechanistic approaches. Thus, the observed biphasic gene-expression pattern should be regarded as an important finding that warrants further investigation rather than as direct evidence of a change in the dominant cell-death pathway.

The microscopic observations further support the biological relevance of these findings. The marked reduction in cell density, loss of adhesion, altered cellular morphology, and diminished monolayer confluence observed after treatment are consistent with the quantitative viability data and indicate substantial cellular stress and growth inhibition. When considered together with the BAX/BCL2 expression profile, these findings suggest that apoptosis-associated

signaling contributes to the cytotoxic response induced by the triple-component formulation, while the precise contribution of other cell-death mechanisms remains to be established.

Overall, the physicochemical and biological findings demonstrate the feasibility of using alginate as a matrix for the co-formulation of doxorubicin, green-synthesized AgNPs, and clove extract. The resulting triple-component nanohydrogel exhibited substantial cytotoxic activity against MCF-7 cells, accompanied by concentration- and time-dependent loss of viability, pronounced morphological deterioration, and an apoptosis-associated BAX/BCL2 expression pattern. The stronger response observed with the multi-component nanocomposite hydrogel formulation supports its potential as a platform for combined delivery of multiple therapeutic modalities. Nevertheless, the current findings establish biological activity rather than definitive pharmacological synergy or a fully resolved mechanism of cell death.

CONCLUSION

The present study demonstrated the feasibility of developing an alginate-based multi-component hydrogel incorporating doxorubicin, green-synthesized AgNPs, and clove extract and evaluating its physicochemical characteristics and in-vitro anticancer activity against MCF-7 breast cancer cells. XRD analysis supported the formation of crystalline metallic Ag with an FCC structure, while FTIR analysis demonstrated spectral changes associated with incorporation of the formulation components. FE-SEM revealed a porous alginate structure and a morphologically distinct triple-component nanocomposite alginate hydrogel, and EDX confirmed the presence of Ag within the analyzed material.

The biological experiments demonstrated concentration- and time-dependent reductions in MCF-7 cell viability following exposure to the tested alginate-based formulations. The triple-component formulation produced a pronounced cytotoxic response under the tested conditions and was accompanied by changes in cell morphology consistent with cellular injury. The reported BAX/BCL2 expression pattern further provided molecular evidence consistent with activation of apoptosis-associated pathways. These findings support the experimental potential of the formulation as an alginate-based multi-

component anticancer platform, but they should not be interpreted as evidence of pharmacological synergy, selective tumor targeting, controlled release, or therapeutic superiority.

ACKNOWLEDGEMENTS

The authors would like to thank University of Tabriz for the financial supports.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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