Preparation and controlled-release studies of a protocatechuic acid-magnesium/aluminum-layered double hydroxide nanocomposite

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Abstract: In the study reported here, magnesium/aluminum (Mg/Al)-layered double hydroxide (LDH) was intercalated with an anticancer drug, protocatechuic acid, using ion-exchange and direct coprecipitation methods, with the resultant products labeled according to the method used to produce them: “PANE” (ie, protocatechuic acid-Mg/Al nanocomposite synthesized using the ion-exchange method) and “PAND” (ie, protocatechuic acid-Mg/Al nanocomposite synthesized using the direct method), respectively. Powder X-ray diffraction and Fourier transform infrared spectroscopy confirmed the intercalation of protocatechuic acid into the inter-galleries of Mg/Al-LDH. The protocatechuic acid between the interlayers of PANE and PAND was found to be a monolayer, with an angle from the z-axis of 8° for PANE and 15° for PAND. Thermogravimetric and differential thermogravimetric analysis results revealed that the thermal stability of protocatechuic acid was markedly enhanced upon intercalation. The loading of protocatechuic acid in PANE and PAND was estimated to be about 24.5% and 27.5% (w/w), respectively. The in vitro release study of protocatechuic acid from PANE and PAND in phosphate-buffered saline at pH 7.4, 5.3, and 4.8 revealed that the nanocomposites had a sustained release property. After 72 hours incubation of PANE and PAND with MCF-7 human breast cancer and HeLa human cervical cancer cell lines, it was found that the nanocomposites had suppressed the growth of these cancer cells, with a half maximal inhibitory concentration of 35.6 μg/mL for PANE and 36.0 μg/mL for PAND for MCF-7 cells, and 19.8 μg/mL for PANE and 30.3 μg/mL for PAND for HeLa cells. No half maximal inhibitory concentration for either nanocomposite was found for 3T3 cells.

Keywords: Mg/Al-LDH, direct coprecipitation method, ion-exchange method, MCF-7, HeLa, 3T3

Introduction

One of the most prolific scientific advancements of the past few decades has been the birth of nanotechnology, which has provided new opportunities for producing materials with markedly varied and unusual properties that have huge potential in many fields such as optics, medical science, catalysis and agriculture.1

Searching for a safe and effective method of delivering bioactive molecules and drugs, especially anticancer drugs, to specific cells is an interesting and intriguing area of research in modern pharmaceutics.2 Conventional drug therapy in cancer does not always provide the desired therapeutic effects because the drugs have limited aqueous solubility, resistance to treatment of cancer diseases, and degrade enzymatically. Moreover, high doses of these drugs are needed that can lead to severe side effects and are not cost-effective.3
Layered double hydroxides (LDHs) are used extensively in medicine as a unique delivery carrier for genes and drugs due to their outstanding properties that make them suitable for delivering to cells. These properties include their good biocompatibility (eg, use as an antacid and anti-peptic agent); less toxic effects compared with other inorganic nanoparticles; high specific surface areas and chemical stability, which have resulted from functionalization and modification of their internal and external surfaces; ability to offer loaded drugs full protection; cell targeting abilities; and ability to be used as controlled-release drug-delivery systems.

LDHs, also known as “hydrotalcite-like compounds,” are a class of anionic clays. Their structure is based on positively charged brucite-like layers containing hydroxides of metal cations $M^{2+}$ and $M^{3+}$. The general formula of an LDH is:

$$\left[M_{x}^{2+}M_{y}^{3+}(OH)_{z}\right]^{m}\left(A^{m-}\right)_{n} \cdot nH_{2}O,$$

where “$M^{2+}$” and “$M^{3+}$” are divalent and trivalent metal cations, respectively, “$A^{m-}$” refers to an interlayer anion with an $m$ charge, $n$ is the number of water molecules in the interlayer space, and $x$ is the layer charge density.

Owing to the efficient drug-delivery feature of LDH nanocarriers, many pharmaceutically active compounds have been successfully intercalated into the interlayer gallery of LDH. Among them are the cardiovascular drugs flavastatin and pravastatin; anti-inflammatory drugs such as diclofenac and fenbufen; antihypertensive drugs like perindopril erbumine; the antihistamine drug cetirizine hydrochloric acid; and anticancer drugs such as cordycepin, which was intercalated into the gallery of magnesium/aluminum (Mg/Al)-LDH. It was observed that the resulting nanohybrid had greater stability and a greater suppression effect on U937 cancer cell growth than free cordycepin and methotrexate (MTX). A MTX-Mg/Al nanohybrid had a much stronger inhibition effect on the proliferation of human MNNG-HOS osteosarcoma cancer cells compared with unbound MTX.

Various methods have been used to intercalate drugs as guest molecules into LDHs, but the most common methods are direct coprecipitation and ion exchange. Compared with the ion-exchange method, the direct coprecipitation method produces a large quantity of the nanocomposite and there is more risk with this method of carbon dioxide uptake and the incorporation of unwanted hydroxide anions in the reaction mixture.

Protopcatechuic acid (3,4-dihydroxybenzoic acid) is a natural phenolic acid isolated from a number of popular medicinal plants such as Sudan mallow (Hibiscus sabdariffa L.), St John’s wort (Hypericum perforatum L.), and Japanese ginkgo (Ginkgo biloba L.). Previous studies have shown that protocatechuic acid has an amazing antioxidant property. Free radicals, including 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), hydroxyl radical, superoxide anion radicals ($O_2^-$), ferric ions ($Fe^{3+}$), cupric ions ($Cu^{2+}$), and ferrous ions ($Fe^{2+}$) attack lipids, carbohydrates, proteins and DNA, which leads to various disorders and diseases. Protocatechuic acid terminates these attacks through its scavenging and chelating activities. Further, protocatechuic acid demonstrates other remarkable pharmacological activities such as anticancer, antitumor, antimutagenic, antibacterial, anti-inflammatory, antigenotoxic, cardioprotective, and chemopreventive. It has been shown to cause markedly apoptotic effects in the treatment of several types of cancer cells, including human leukemia (pa-2000-leukemia), cervix, breast, lung, liver, and prostate. It induces cell death via increasing DNA fragmentation, decreasing mitochondrial membrane potential, lowering Na-K-ATPase activity, and elevating caspase-3 and caspase-8 activities in cancerous cells. Moreover, protocatechuic acid inhibits cell adhesion and the production of vascular endothelial growth factor, interleukin (IL) 6, IL-8, and intercellular adhesion molecule 1 in cancer cells and does not have negative effects on normal human cells.

However, there has been limited research undertaken on the intercalation of protocatechuic acid into LDHs. Thus, in this study, protocatechuic acid was selected as a model for an anticancer drug and was intercalated into an Mg/Al-LDH matrix using both ion-exchange and direct coprecipitation techniques. We focused our study on the spatial orientation of the protocatechuic acid anion between the layers and its thermal stability, physico-chemical features, and release properties at different pH values. In addition, we also investigated the effect of a protocatechuic acid-Mg/Al-LDH nanocomposite on the viability of MCF-7 human breast cancer, HeLa human cervical cancer, and normal cells to assess the delivery efficiency of Mg/Al-LDH vectors for use as drug-delivery nanovehicles.

**Materials and methods**

**Materials**

$\text{Mg(NO}_3\text{)}_2 \cdot 6\text{H}_2\text{O}$ at 99% and protocatechuic acid ($\text{C}_9\text{H}_8\text{O}_5$, molecular weight 154.12 g/mol) at 97% purity were purchased from Acros Organics (Geel, Belgium). $\text{Al(NO}_3\text{)}_3 \cdot 9\text{H}_2\text{O}$ and NaOH were purchased from Friendemann Schmidt (Parkwood, WA, USA). Phosphate-buffered saline
Pristine Mg/Al-LDH (0.36 mg) was used to perform the same protocatechuic acid release experiments as already described. A physical mixture of protocatechuic acid (0.13 mg) and pristine Mg/Al-LDH, 0.49 mg of protocatechuic acid and pristine Mg/Al-LDH, 0.13 mg) and pristine Mg/Al-LDH (0.36 mg) was used to perform the same protocatechuic acid release experiments as already described.

Cell culture
MCF-7, HeLa, and 3T3 cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum, L-glutamine 15 mM, penicillin 100 µg/mL, and streptomycin 100 µg/mL, and incubated at 37°C in humidified 5% CO₂.
Cytotoxicity assay using 3-(4, 5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide (MTT) was performed by plating the cells into a 96-well plate at a density of $1.0 \times 10^5$ cells/well in 100 µL of cell culture medium and allowing to attach overnight. PANE and PAND and protocatechuic acid stock solution were prepared in dimethyl sulfoxide (DMSO), and subsequently diluted in culture medium to dilute to the various concentrations of 1.562–50.000 µg/mL and give a final volume of 200 µL in each well. Cell viability was assessed using an MTT solution after exposure to the nanocomposites and protocatechuic acid. After 72 hours exposure time, a 20 µL aliquot of the MTT solution at a concentration of 5 mg/mL was added to each well and incubated at 37°C for 4 hours. To solubilize the formazan after 4 hours of incubation, 100 µL of DMSO was added to each well and the plates were kept in a dark place within a shaker at room temperature for 30 minutes. Absorbance of the test solution in the 96-well plates was measured at 570 nm using a microplate reader. Presented cell cytotoxicity data are expressed as the percentage of cell viability compared with untreated cells under the same experimental conditions.

Results and discussion

Powder X-ray diffraction

Figure 1 illustrates the powder XRD patterns of the pristine LDH, PANE and PAND, and free protocatechuic acid. The two intense lines at a low 2θ value of 11.09° and 22.21° in the powder XRD pattern of the pristine Mg/Al-LDH sample (Figure 1A), which was used for preparation of PANE, correspond to diffractions by planes 003 and 006, respectively, with a basal spacing value of 7.9 Å and 3.9 Å, respectively.16 The interlayer distance ($d_{003}$) value of the pristine LDH sample (Figure 1A) is 7.9 Å, clearly lower than 8.2 Å. This is due to the low nitrate anions that lie in the center of the interlayer galleries.77

For PANE, a new diffraction pattern at $d_{003} = 8.3$ Å appears (Figure 1B), whereas for PAND a new diffraction pattern at $d_{003} = 9.5$ Å appears (Figure 1C). The change in the 003 basal reflection pattern of PANE and PAND compared with the pristine Mg/Al-LDH sample indicates the successful intercalation of protocatechuic acid into the nanocomposites.

The slight discrepancy in the $d_{003}$ different value between PANE and PAND is probably due to the content of water in the interlayer galleries and different charge density of the layers.78

Spatial orientation of the protocatechuic acid intercalated into PANE and PAND

Figure 2A shows the molecular size dimensions of protocatechuic acid estimated by ChemOffice software (Cambridge, MA, USA). From the XRD patterns, we determined that the $d$ spacing ($d_{003}$) of PANE was 8.3 Å (obtained by averaging the higher two-order peaks) and 9.0 Å (obtained by averaging the higher three-order peaks) for PAND. The thickness of the brucite-like layer of Mg/Al-LDH is 4.8 Å.39 Therefore, the gallery height of the LDH after the intercalation of protocatechuic acid could be calculated by the $d$ spacing minus the thickness of the LDH layer, which were 3.5 Å (8.3–4.8 Å) and 4.2 Å (9.0–4.8 Å) for PANE and PAND, respectively. The three axes of protocatechuic acid were 9.0 Å, 6.8 Å, and 2.9 Å, for the x-, y-, and z-axis, respectively.
The gallery height of the nanocomposites were 3.5 and 4.2 Å for PANE and PAND, respectively, both of which are much smaller than the value of the long and short axes (9.0 and 6.8 Å, respectively) and slightly larger than the thickness of protocatechuic acid (2.9 Å). This suggests that the protocatechuic acid anions in PANE and PAND were accommodated as a monolayer in each nanocomposite with an angle from the z-axis of 8° in PANE and 15° in PAND, as shown in Figure 2B.

Infrared spectroscopy

The Fourier transform infrared (FTIR) spectra of protocatechuic acid, Mg/Al-LDH, PANE, and PAND are shown in Figure 3. The FTIR spectrum of Mg/Al-LDH (Figure 3B) shows a strong absorption band at 1385 cm⁻¹ that can be attributed to the stretching vibration of NO₃⁻. In the low-frequency region, the absorption peaks indicate that the lattice vibration modes can be attributed to M–O and O–M–O at 610 and 837 cm⁻¹, respectively. In the FTIR spectrum of pristine protocatechuic acid (Figure 3A), there is a stretching vibration band for the OH group at 3202 cm⁻¹, which can be attributed to the hydrogen bonding of the carboxylic group. The C=O stretching vibration of the carboxylic group at 1664 cm⁻¹ can be seen to have vanished from the FTIR of the nanocomposites. At the same time, intense peaks at 1507 and 1364 cm⁻¹ for PANE and 1506 and 1367 cm⁻¹ for PAND are due to asymmetric and symmetric stretching, respectively, of the COO⁻ group can be clearly observed. The band at 550 cm⁻¹ and at 566 cm⁻¹ for PANE and PAND, respectively, can be attributed to M–O and O–M–O, respectively.

Elemental analysis

The carbon, hydrogen, nitrogen and sulphur (CHNS) analysis and inductively coupled plasma elemental data were used to determine the drug and inorganic layer compositions of PANE and PAND. As shown in Table 1, PANE and PAND contained both drug and inorganic layers, indicating that both of these were constituents of the nanocomposites. This shows that protocatechuic acid was intercalated into the Mg/Al-LDH inorganic interlayers. Table 1 shows that the Mg²⁺ to Al³⁺ molar ratio in PANE and PAND was 3.3 and 2.9, respectively, compared with the initial molar ratio of 4.0. As a result of the elemental chemical analysis and thermogravimetric studies, the empirical formula for PANE was determined to be [Mg₀.₇₇Al₀.₂₃(OH)₂][PA⁻₀.₁₇(NO₃)₀.₀₅₃]yH₂O, while it was [Mg₀.₇₄Al₀.₂₆(OH)₂][PA⁻₀.₂₉(NO₃)₀.₀₅₁]yH₂O for PAND.

Thermal analysis

Results of thermal analysis of pure protocatechuic acid, PANE, and PAND are shown in Figure 4A–C, respectively. For protocatechuic acid (Figure 4A), four thermal events were clearly observed. The first event, which occurred in the region of 55°C–129°C, was attributed to the removal of absorbed water molecules, corresponding to a sharp peak in the differential thermogravimetric curve at 101°C, with a weight loss of 2.9%. This was followed by a second stage at 175°C–299°C due to the decomposition combustion of protocatechuic acid, which corresponded to a strong peak at 250°C and a weight loss of 79.4%. The third and fourth weight losses occurred in the region of 299°C–408°C and 408°C–593°C, respectively, with 6.4% and 12.2% weight loss, respectively.

Figure 4B and C show the thermal decomposition of PANE and PAND, respectively. As shown in the figure, it...
Table 1 Elemental chemical composition for protocatechuic acid and its nanocomposites

<table>
<thead>
<tr>
<th>Sample</th>
<th>C (%) (w/w)</th>
<th>N (%) (w/w)</th>
<th>Mg (%) (w/w)</th>
<th>Al (%) (w/w)</th>
<th>X (%) (w/w)</th>
<th>Anion (%) (w/w)</th>
<th>Mg/Al</th>
<th>Empirical formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>PANE</td>
<td>13.37</td>
<td>0.676</td>
<td>19.1</td>
<td>6.1</td>
<td>0.23</td>
<td>24.5</td>
<td>3.3</td>
<td>[Mg,Al(OH)_2]PA_2(NO_3)_4 · yH_2O</td>
</tr>
<tr>
<td>PAND</td>
<td>14.98</td>
<td>0.607</td>
<td>17.3</td>
<td>6.7</td>
<td>0.26</td>
<td>27.5</td>
<td>2.9</td>
<td>[Mg,Al(OH)_2]PA_2(NO_3)_4 · yH_2O</td>
</tr>
</tbody>
</table>

Notes: *Estimated from CHNS analysis; †estimated from inductively coupled plasma analysis.

Abbreviations: C, carbon; N, nitrogen; Mg, magnesium; Al, aluminum; X, aluminum mole fraction; Y, the mole of water molecules; PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method; CHNS, carbon, hydrogen, nitrogen, sulphur.

progressed through two major stages of weight loss; these occurred at temperature maxima of 93°C and 115°C, and 462°C and 500°C, with weight losses of 20.1% and 15.2%, and 34.8% and 40.7% for PANE and PAND, respectively. The first stage of weight loss in the range 39°C–270°C was due to the removal of water physisorbed on the external surface of the LDH as well as structured water. The second weight loss in the range 277°C–642°C and 262°C–997°C, with a total weight loss of 34.8% and 40.7%, for PANE and PAND, respectively, can be attributed to de-hydroxylation of the metal hydroxide layers and decomposition of nitrate ions.

Decomposition of protocatechuic acid in PANE and PAND occurred at 462°C and 500°C, respectively, both of which are higher temperatures than that for the decomposition of the free protocatechuic acid (250°C). This suggests that the thermal stability of protocatechuic acid in the nanocomposites was enhanced due to the intercalation process as a result of the electrostatic attraction between the negatively charged carboxylate group of the protocatechuic acid and the positively charged interlayer space of the LDH.
echuic acid and the positively charged brucite-like layers of Mg/Al-LDH.

Surface properties
The surface morphologies of Mg/Al-LDH, PANE, and PAND are shown in Figure 5. The micrographs in Figure 5 were obtained using a field-emission scanning electron microscope (Figure 5A and D at 10,000×; Figure 5B, E, and G, at 25,000×; and Figure 5C, F, and H at 50,000 × magnification). As shown in this figure, all samples had typical, nonuniform, irregular agglomerates of compact and nonporous plate-like structures.

As shown in Figure 6A, all isotherms are Type IV (according to the International Union of Pure and Applied Chemistry classification), indicating a mesopore-type material. It can be seen that the adsorbate uptake of Mg/Al-LDH was slow at the relative pressure range of 0.0–0.4, but rapid adsorption was observed after this, with an optimum uptake of about 18 cm$^3$/g, indicating a high capacity for nitrogen gas uptake. However, for PANE and PAND, the uptake was slow until a relative pressure of 0.8 was reached. Further increase in the relative pressure beyond 0.8 resulted in rapid adsorption of the absorbent and an optimum uptake of 9.0 cm$^3$/g and 3.0 cm$^3$/g for PANE and PAND, respectively. The desorption branch of the hysteresis loop for Mg/Al-LDH was different from that of PANE and PAND. PANE displayed an H1-type branch (open-ended cylindrical pore), while PAND was found to have an H2-type branch (with open slit-shaped capillaries). As a result of nitrogen adsorption, the surface area of the materials determined using the Brunauer, Emmet, and Teller (BET) method decreased from 9 m$^2$/g for Mg/Al-LDH to 3 and 2 m$^2$/g for PANE and PAND, respectively. This was due to the change in the porous texture as a result of the formation of the nanocomposite compounds.

Figure 6B shows plots of the Barret–Joyner–Halenda (BJH) desorption pore size distribution for Mg/Al-LDH, PANE, and PAND. As shown in the figure, a single-peaked pore size distribution was observed for Mg/Al-LDH, centered at around 60 Å. In contrast, a single peak each for PANE and PAND appears at 88 and 43 Å, respectively. Due to the intercalation process, the BJH pore volume decreased from 0.03 for Mg/Al-LDH to 0.01 cm$^3$/g for PANE and PAND.

![Figure 5](https://www.dovepress.com/)

**Figure 5** Field-emission scanning electron micrographs of: Mg/Al-layered double hydroxide at (A) 10,000×, (B) 25,000×, and (C) 50,000 × magnification; PANE at (D) 10,000×, (E) 25,000×, and (F) 50,000 × magnification; and PAND nanocomposite (G) 25,000× and (H) 50,000 × magnification.

**Abbreviations:** PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method.
whereas the BJH average pore diameter increased from 65 Å for Mg/Al-LDH to 147 and 152 Å for PANE and PAND, respectively (Table 2).

### Release behavior of protocatechuic acid from PANE and PAND

The release profiles of protocatechuic acid from the nanocomposites and the physical mixture of protocatechuic acid and pristine Mg/Al-LDH are shown in Figure 7. The physical mixture of protocatechuic acid and pristine Mg/Al-LDH exposed to pH 4.8 and pH 7.4 environments show the release of protocatechuic acid very quickly, within 10 minutes (Figure 7A). In contrast, the release rate of protocatechuic acid from PANE and PAND was slower than from the physical mixture, indicating that PANE and PAND have the potential to be used for the controlled release of drugs. This result may be attributed to the interaction between the negatively charged protocatechuic acid and the positively charged inorganic LDH layers.

In addition, the release rate of protocatechuic acid from PANE and PAND was dependent on the pH of the environment. The release rate at pH 7.4 was markedly lower than that at pH 5.3 and 4.8 for both nanocomposites.

![Figure 6 Adsorption–desorption isotherms (A) and BJH pore size distribution (B) for Mg/Al-LDH, PANE, and PAND.](https://www.dovepress.com/)

**Abbreviations:** BJH, Brunauer, Emmet, and Teller (method); LDH, layered double hydroxide; PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method; STP, standard temperature and pressure.

In Figure 7B, the percent release of protocatechuic acid from PANE can be seen to have reached about 85% within about 1000 minutes when exposed to a pH of 4.8 and to have reached 82% by 3894 minutes at a pH of 5.3. About 59% of release had occurred within about 7500 minutes when exposed to a pH 7.4 environment. Figure 7C shows that the release profile of the drug from PAND reached about 98% and 83% when exposed to pH 4.8 and 7.4 within 900 and 4000 minutes, respectively. About 87% of the drug was released within about 1349 minutes when exposed to a pH 5.3 environment. The difference in release rates at pH 4.8, 5.3, and 7.4 may be due to a difference in release mechanism between protocatechuic acid and the nanocomposites. At acidic pH (4.8 or 5.3), LDHs are unstable and can easily dissolve, therefore drug release can occur if the LDH interlayers are removed. At pH 7.4, LDHs are more stable compared with at pH 4.8 or 5.3 and, as a result, the release would occur primarily through the ion exchange between the protocatechuic acid and the negative anions available in the PBS.

### Release kinetics of protocatechuic acid from PANE and PAND

There are various kinetic models that can be used to describe the total release of drugs from the interlayers of nanocomposites. Common models used include pseudo-first order, pseudo-second order, and parabolic diffusion. The pseudo-first order kinetic equation is:

\[
\ln (q_e - q_t) = \ln q_e - k_1 t,
\]

where “\(q_e\)” and “\(q_t\)” are the equilibrium release amount and the release amount at time \(t\), respectively, and “\(k_1\)” is the corresponding release rate constant. A plot between \(\ln (q_e - q_t)\)
<table>
<thead>
<tr>
<th>Samples</th>
<th>pH</th>
<th>Saturation release (%)</th>
<th>Pseudo-first order</th>
<th>R²</th>
<th>Pseudo-second order</th>
<th>Parabolic diffusion</th>
<th>Pseudo-first order</th>
<th>Rate constant, k (mg/min)</th>
<th>Rate constant, k (min⁻¹)</th>
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<th>Rate constant, k (min⁻¹)</th>
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<td>7.4</td>
<td>59</td>
<td>0.9804</td>
<td></td>
<td>0.9989</td>
<td>0.8860</td>
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<td>5.3</td>
<td>82</td>
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<td>0.8517</td>
<td>4.9 × 10⁻⁴</td>
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<td>4.8</td>
<td>85</td>
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<td>0.8334</td>
<td>3.1 × 10⁻³</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>7.4</td>
<td>83</td>
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<td>0.8266</td>
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<td>PANE</td>
<td>5.3</td>
<td>87</td>
<td>0.9543</td>
<td>0.9652</td>
<td>0.9796</td>
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<td>2.1 × 10⁻³</td>
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<tr>
<td>PANE</td>
<td>4.8</td>
<td>98</td>
<td>0.9805</td>
<td>0.8272</td>
<td>0.9563</td>
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</table>

**Notes:** ¹Rate release constant for pseudo-second order; ²rate release constant for pseudo-first order; ³rate release constant for parabolic diffusion.

**Abbreviations:** PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method.

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**Figure 7** (A) Release profiles of physical mixture of protocatechuic acid with Mg/Al-layered double hydroxide. (B) Release profiles of protocatechuic acid from PANE at pH 7.4 (a), pH 5.3 (b), and pH 4.8 (c). (C) Release profiles of protocatechuic acid from PAND at pH 7.4 (d), pH 5.3 (e), and pH 4.8 (f). Insets show the release details.

**Abbreviations:** PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method.
against time, \( t \), gives a straight line and the rate constant value can be calculated.

In the pseudo-second order kinetic model, the release behavior of drugs from a nanocomposite can be described as:\(^{46}\)

\[
t/q_t = 1/k_q q_e^2 + t/q_e
\]

By plotting \( t/q_t \) against \( t \), a straight line is obtained and the rate constant, \( k_2 \), as well as \( q_e \) can be calculated using the relation:

\[
k_2 = 1/q_e^2 \cdot \text{intercept}
\]

The parabolic diffusion kinetic model can be written as:\(^{47}\)

\[
1 - (M_t/M_0)/t = k_3 t^{-0.5} + b,
\]

where “\( M_0 \)” and “\( M_t \)” are the drug content remaining in the LDH at release time 0 and \( t \), respectively, “\( k_3 \)” is the corresponding release rate constant, and \( b \) is a constant.

Using the above three kinetic models, it was found that the pseudo-second order model was more satisfactory for describing the release kinetic processes of protocatechuic acid from PANE at pH 7.4 and 4.8 and from PAND at pH 7.4, with a coefficient of determination (\( R^2 \)) of 0.9989, 0.9601,

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**Figure 8** Fitted data for the release of protocatechuic acid from PANE samples at (A) pH 7.4, (B) 5.3, and (C) 4.8 and from PAND samples at (D) pH 7.4, (E) 5.3, and (F) 4.8. Abbreviations: PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method; min, minute.
and 0.9910, respectively (Figure 8 and Table 3). The release of protocatechuic acid from PAND at pH 4.8 obeyed the pseudo-first order kinetic model, with $R^2 = 0.9805$ (Table 3). The release kinetic process of protocatechuic acid from PANE and PAND in a pH 5.8 environment obeyed the pseudo-first order and parabolic diffusion model, respectively, with an $R^2$ of 0.9147 and 0.9796, respectively.

For PANE (Table 3), the rate release constant ($k$) value was $2.2 \times 10^{-5}$ and $4.1 \times 10^{-5}$ mg/min at pH 7.4 and 4.8, respectively, and for PAND the corresponding value was $2.3 \times 10^{-5}$ mg/min at pH 7.4 (pseudo-second order) and $2.1 \times 10^{-3}$ min$^{-1}$ at pH 4.8 (pseudo-first order). The rate constant for pseudo-first order at pH 5.3 was $4.9 \times 10^{-4}$ min$^{-1}$, while for the parabolic diffusion model it was $3.1 \times 10^{-2}$ min$^{-1}$.

**Cytotoxicity assay of protocatechuic acid, PANE, and PAND samples against 3T3, HeLa, and MCF-7 cell lines**

The beneficial efficiency of PANE and PAND compared with free protocatechuic acid was investigated by cytotoxicity assay using two cancer cells, the HeLa cervical cancer cell line and the MCF-7 breast cancer cell line. In addition, the 3T3 cell line of normal fibroblast cells was used to evaluate drug-induced toxicity. Figure 9A shows that protocatechuic acid, PANE, and PAND did not show any toxic effects against normal fibroblasts. Figure 9B and C show the dose-dependent cell viability of HeLa and MCF-7 cells, respectively. Overall, the protocatechuic acid, PANE, and PAND gradually suppressed the tumor cell growth in a dose-dependent manner.

Figure 9B and Table 4 clearly show that the tumor suppression (cytotoxicity) efficiency can be described in the following order: PANE > PAND > protocatechuic acid in HeLa cells, with half maximal inhibitory concentration values of 19.8, 30.3, and 38.4 µg/mL, respectively. Figure 9C and Table 4 indicate that protocatechuic acid did not show any significant cytotoxicity against MCF-7 cells, and that PANE had a higher tumor suppression efficiency than PAND, with half maximal inhibitory concentration values of 35.6 and 36.0 µg/mL, respectively.

Figure 9B shows that when the concentration was increased from 1.562 to 50.000 µg/mL, there was a rapid decrease in cell viability. The maximum suppression effects of protocatechuic acid, PANE, and PAND were observed at the concentration of 50 µg/mL, with 44.8%, 14.8%, and 14.3% cell viability, respectively. For MCF-7 cells, the corresponding value for protocatechuic acid, PANE, and PAND at 50 µg/mL was 50.3%, 20.6%, and 41.8%, respectively.
Table 4 The half maximal inhibitory concentration (IC\textsubscript{50}) values for protocatechuic acid, PANE, and PAND samples against 3T3, HeLa, and MCF-7 cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
<th>3T3</th>
<th>HeLa</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Free drug</td>
<td>PANE</td>
<td>PAND</td>
</tr>
<tr>
<td>IC\textsubscript{50}</td>
<td>No cytotoxicity</td>
<td>38.8</td>
<td>19.8</td>
</tr>
</tbody>
</table>

Notes: 3T3, fibroblast cell; MCF-7, human breast cancer cell; HeLa, human cervical cancer cell. Abbreviations: PAND, protocatechuic acid-Mg/Al nanocomposite synthesized by direct method; PANE, protocatechuic acid-Mg/Al nanocomposite synthesized by ion-exchange method.

Conclusion
In the study reported here, protocatechuic acid, an active anticancer drug, was incorporated into the Mg/Al-LDH matrix by ion-exchange and direct coprecipitation methods to form intercalated nanocomposite products. XRD and FTIR studies indicate the successful intercalation of protocatechuic acid into the inorganic inter-galleries of the LDH. Results of thermogravimetric and differential thermogravimetric analyses show that the thermal stability of the intercalated protocatechuic acid exhibited a notable increase compared with that of pristine protocatechuic acid. This finding indicates that, for PANE and PAND, the protocatechuic acid moiety was oriented as a monolayer with an angle from the z-axis of 8° in PANE and 15° in PAND. The intercalated amount of protocatechuic acid in PANE and PAND was 24.5% and 27.5% (w/w), respectively.

The in vitro protocatechuic acid release from PANE and PAND was markedly lower than that from the corresponding physical mixture of the drug with pristine LDH at pH 4.8, 5.3, and 7.4. In addition, the release rate of protocatechuic acid from the nanocomposites at pH 7.4 was markedly lower than that at pH 4.8 and 5.3, and this is due to a possible difference in the release mechanism. At pH 7.4, the mechanism possibly occurred through ion exchange, while at pH 4.8 and 5.3 it possibly occurred through both the dissolution of LDH layers and ion exchange.

Results of the in vitro cytotoxicity study indicate that the tumor suppression efficiency in HeLa and MCF-7 cells can be described in the following order: PANE > PAND > protocatechuic acid. The cytotoxicity of PANE was greater than that of PAND and this is in parallel with the higher protocatechuic acid in PANE compared with in PAND.

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Disclosure
The authors declare no conflicts of interest in this work.

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