

Anti-microbial Nanohybrids Based on Naturally Derived Citric Acid Intercalated Layered Double Hydroxides

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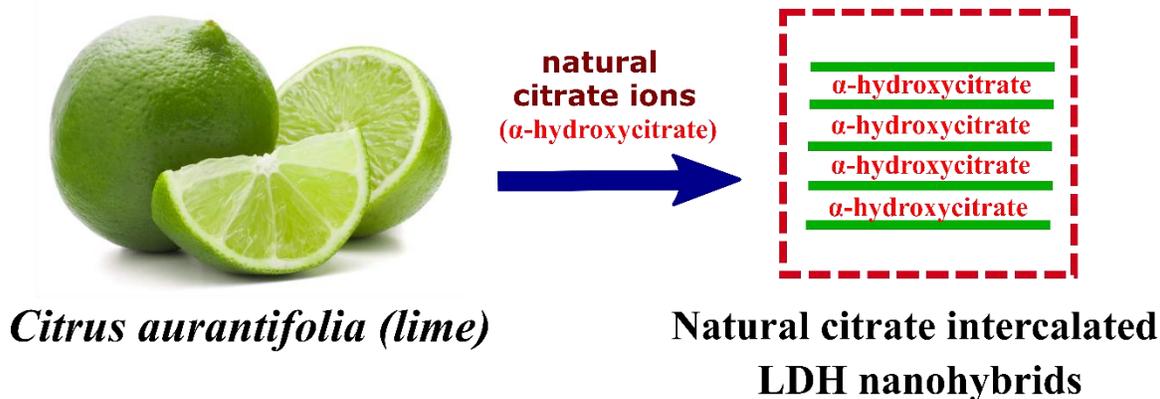
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Abstract

Currently, there is an increased demand for advanced food packages, which can significantly increase the shelf life of food items. In the current context, it is envisaged that nanotechnology has the potential to address stability, toxicity, shelf-life, and low-cost issues of antimicrobials associated with the packaging industry. Antimicrobial nanocomposite systems are believed to be more efficient than their microscale counterparts due to the high surface area to volume ratio and quantum mechanical involvement in deciding their properties. As a result of high surface area, they are able to attach more copies of microbial molecules and cells, thus reducing the quantity of material required while significantly improving their activity. This study focuses on the development of slow-release antimicrobial material based on natural citrate (α -hydroxycitrate) intercalated layered double hydroxide (LDH) nanohybrid. Natural citrate ions available in *Citrus aurantifolia* (lime) were extracted by a simple chemical method and intercalated into Mg-Al-Layered Double Hydroxide following a one-step co-precipitation method. Successful intercalation of the citrate ion was confirmed by powder X-ray diffraction (PXRD) and Fourier transform infrared (FTIR) spectroscopic analysis. Release kinetics of resulted nanohybrid was studied and compared using different release kinetic models. Antimicrobial properties of this novel nanohybrid were confirmed against two common food pathogens, *Colletotrichum gloeosporioides* and *Saccharomyces cerevisiae*, and the results were compared against sodium benzoate, which is the commonly used commercial antimicrobial agent in the food industry. Successful intercalation of natural citrate ions into LDH and its activity against the tested microbes show the potential of using it as a slow-release nanohybrid material in many food-related applications.



Keywords: Layered Double Hydroxide, α -Hydroxycitrate, Natural, Safe, Lime Extract, Slow Release, Antimicrobial

1. Introduction

With the current increase in the global population, it is important to ensure that every human being receives a healthy and active life. According to the United Nations statistics, however, approximately one in nine people on earth suffer from chronic hunger on a daily basis (WFP, 2016). Hence, there's an emerging need to increase global food availability to maintain health standards. One of the major aspects of such improvements is the maintenance of food quality by minimizing the spoilage that arises due to the poor quality of packaging and unsafe food coating techniques used (Robertson, 2016). In this context, the development of advanced food packaging and coating materials with efficient food preservation mechanisms to reduce postharvest loss while ensuring the quality and safety of the food items is crucial.

Concepts of nanotechnology, which play a key role in many foremost advancements in science today, have also been utilized in the food industry, resulting in advanced food packaging materials based on functional nanomaterials (Smolander and Chaudhry, 2010). Specifically, nanotechnology has mainly focused on the development of advanced materials that can control pathogenic microbial growth and delay oxidation (Dong et al., 2016, Lee, 2016, Dainelli et al., 2008). Several studies have reported using silver nanoparticles to develop polymer composites with antimicrobial activity as a novel approach for preserving food and its shelf-life extension (Rhim et al., 2013, Duncan, 2011). Moreover, nanocoatings have also been used extensively in the food industry to avoid moisture and air (Biji et al., 2015, Brody et al., 2008). Also, it has been shown in the literature that the addition of certain nanomaterials can result in light weight, mechanically and thermally stable packaging materials (Vanderroost et al., 2014).

Among the many potential nanomaterials in food applications, layered double hydroxides (LDHs) can be named as one of the most promising candidates owing to their excellent biocompatibility, presence of tunable inter-layer regions capable of intercalating a broad spectrum of guest molecules and high aspect ratio resulting due to the presence of nanometer-thick layers (Bugatti et al., 2011). LDHs have widely been explored as carriers for active drugs, particularly for active molecules which are highly unstable under standard environmental conditions (Rives et al., 2013, Park et al., 2015, Rives et al., 2014). Moreover, they are promising in the development of slow-release stable drug compositions and currently, some of these drugs are commercially available (Perera et al., 2015, Samindra et al., 2014). However, not much

attention has yet been devoted to the development of LDH-based functional nanomaterials for applications in food industry. Hence, herein we have explored the potential of using natural α -hydroxycitrate anion intercalated LDH nanohybrids as an antimicrobial food packaging material. In fact, citrate has long been used in the preservation of food and, therefore, can be named as a promising candidate for food packaging.

Antimicrobial activity of citrate-LDHs, pure citrate and sodium benzoate (commercially available) were tested against the growth of *Colletotrichum gloeosporioides* and *Saccharomyces ascomycetes* fungi which are common microbes that attack many crops, including fruits and vegetables leading to brown blotch disease and anthracnose (Kang et al., 2003). As an example, *Colletotrichum spp* can spoil bananas, mangoes and papaya, leading to anthracnose, crown rot disease and stem-end-rot (Al Zaemey et al., 1993). In the biology of *Colletotrichum sp* form white-colored colonies in the Potato Dextrose Agar (PDA) medium, which turned into light pink with time. *Saccharomyces cerevisiae* which is one of the genetic engineering developments of Yeasts, has the ability to spoil fruits and vegetables by forming lesions and patches on the surface of the fruit. *Saccharomyces cerevisiae* also grows as light-coloured colonies in the PDA medium, which turn into light yellow with time. The activity of the organic acids in growth inhibition of food spoiling microorganisms as *Saccharomyces spp* has been studied in attempts to improve the shelf life of food (Shokri, 2011).

These organic acids can inhibit the respiration of the microorganisms, which ultimately leads to their death. For example, in a medium of acetic acid, fungal growth is inhibited. But, the fungus is able to grow in the same normal medium when acetic acid is eliminated, showing that the growth inhibition may have resulted from an inhibition of respiration from acetic acid than structural damage to the cells of the fungus (Kang et al., 2003). The antimicrobial activity of citrate has been shown against *E-coli*, *Salmonella spp*, *Bacillus spp*, *Staphylococcus aureus*, etc. (Pathan et al., 2012, Onyeagba et al., 2004), and it is readily used in the medicinal applications. Moreover, citrate is a natural plant extract and therefore leads to greener food packaging materials without any potential toxicity to human beings. However, to our knowledge, food packaging materials based on naturally occurring substances like citrate have not been developed yet. Hence, with the current emerging trends of exploring the potential use of naturally available active compounds in applications in the food industry, we have explored the potential of using α -hydroxycitrate anion intercalated LDH nanohybrids as antimicrobial food packaging and coating materials. This nanohybrid can be introduced as a pellet or a thin coating into food packaging.

2. Materials and Methods

2.1. Reagents

All chemicals and reagents used for the experiments were of analytical grade, purchased from Sigma Aldrich-USA and distilled water was used for experimental work. Natural citrate ions were extracted from *Citrus aurantifolia* (lime) as explained (Talibi et al., 2011). Lime was purchased from the local market.

Extraction of natural citrate from Citrus aurantifolia (lime)

Ca(OH)₂ (5.0 g) was added to the lime juice solution (100.00 cm³) and heated for 1 min below the boiling point of citric acid (175 °C) while stirring. Then, the solution was cooled down to room temperature and filtered. The resulting solid was dissolved in conc. H₂SO₄ (100.00 cm³) and heated the solution at 70 °C for 10 min while stirring. Then, the solution was filtered to remove solid CaSO₄. The solution was cooled down to room temperature, filtered, and washed with distilled water for several times to get solid citric acid. The purity of the citric acid was confirmed using thin layer chromatography.

2.2. Synthesis of LDHs

Synthesis of Mg-Al-NO₃-LDH (nitrate-LDH) - standard material

Nitrate-LDH was prepared as explained (Li et al., 2004) and used as the standard material for comparison. A solution containing 0.25 mol of Mg((NO₃)₂·6H₂O) and 0.125 mol of Al((NO₃)₃·9H₂O) in 250.00 cm³ of distilled water was added drop-wise to a solution containing 0.25 mol of NaNO₃ in 250.00 cm³ of distilled water under nitrogen atmosphere and vigorous stirring condition. A solution of 2.0 mol dm⁻³ NaOH was used to maintain the pH at 10. Then, the solution was stirred overnight, and the precipitate obtained was separated, washed thoroughly with distilled water and dried at 60 °C.

Synthesis of citrate intercalated LDH (citrate-LDH)

Both synthetic and natural citrate intercalated LDH (referred to as synthetic citrate-LDH and natural citrate-LDH, respectively) were synthesized. A similar procedure as explained for nitrate LDH was used for the synthesis of synthetic citrate-LDH except for using 0.2 mol dm⁻³ citrate anion solution instead of NaNO₃.

For the synthesis of natural citrate intercalated LDH (natural citrate LDH), the natural citrate anion extracted according (0.2 mol dm⁻³) to the procedure given above was used as the anion to be intercalated.

2.3 Characterization

PXRD analysis for powders of LDHs was done using a Bruker D8 focus X-ray powder diffraction meter using Cu K α radiation (wavelength–1.54 Å) over a 2 theta range from 2° to 70° with the step size of 0.02°. FTIR spectroscopic analysis was done using Bruker Vertex80 in the range of 600 cm⁻¹ to 4000 cm⁻¹. For powdered samples, diffuse reflection mode was used. Here, the sample was mixed with potassium bromide in 1:100 ratio, and then the mixture was ground to a fine powder, and a thin pellet was prepared. For liquid samples, Attenuated Total Reflectance (ATR) mode was used. The FTIR spectrum was obtained in the frequency range from 400–4000 cm⁻¹. UV- 2602 Labomed, Inc. single beam scanning spectrophotometer was used to study the slow release characteristics of citric acid intercalated LDHs as a function of time at different pH values. The total amount of citrate intercalated into the LDH was quantified by the same technique.

The absence of any nitrate impurities arising from parent nitrate solutions within the layered structure was verified using the Kjeldahl method. SEM imaging was carried out to understand the internal morphology of the nanohybrid. Hitachi SU 6600 SEM was used in the secondary electron mode. All

samples were coated with a thin layer of Au prior to analysis. Elemental analysis was carried out using the secondary electron mode using the X-ray detector.

2.4 Release behavior studies

Release behavior studies of the natural and synthetic citrate LDHs and pure citrate were conducted using the standard method explained in the literature (Tronto et al., 2004). The amount of release of citrate was measured in an aqueous medium at different pH values of 3, 4 and 5. Here, the pH of the medium was controlled using a phosphate buffer. The amount of citrate ions released was measured using a UV visible spectrophotometer and quantified using a calibration plot.

2.5 Determination of antimicrobial activity

A series of solutions from 1–100 ppm standard citric acid was prepared using a stock solution of 1000 ppm. The same sets of solutions were prepared for the natural and synthetic citrate intercalated LDH samples. Here, the stock solution contained 1000 ppm of citrate-LDH.

Preparation of agar medium

Potato dextrose agar (PDA) medium (9.75 g in 250 ml) was prepared, autoclaved and allowed to cool slightly above room temperature (Elgayyar et al., 2001, Atehnkeng et al., 2008). Then, the media was poured immediately on the plates, which were oven-dried before use and were allowed to settle in a sterile environment.

Inoculum Preparation

Inoculants were prepared from well-isolated colonies of *Colletotrichum gloeosporioides* and *Saccharomyces cerevisiae*. The colonies were regrown by transferring into a new agar medium and then were incubated at room temperature for 24 h.

Inoculation and Incubation of the medium

Sodium benzoate, the commercially used antimicrobial agent for food preservation, was used as the positive control, while distilled water was used as the negative control. Different concentrations of citrate-LDH and pure citrate (5 ppm, 10 ppm, 15 ppm, 20 ppm, 30ppm, 40 ppm and 50 ppm), and the two controls were spot into each well prepared on the agar surface using a 40 µl micropipette. Sterilized distilled water was used as the negative control, whose MIC value is 0 ppm.

Then, inoculation was carried out with *Colletotrichum gloeosporioides* and *Saccharomyces cerevisiae*. Inoculated agar plates were allowed to stand for 24 h and incubated at room temperature. The diameter of the inhibition zone for pure citrate, citrate-LDHs and standards were measured after 24 h and the diameter of the inhibition zone vs concentration was plotted.

Determination of Minimum Inhibitory Concentration (MIC)

Using a graphical method, the minimum inhibitory concentration (MIC) of citrate-LDHs was determined (Figure 1).

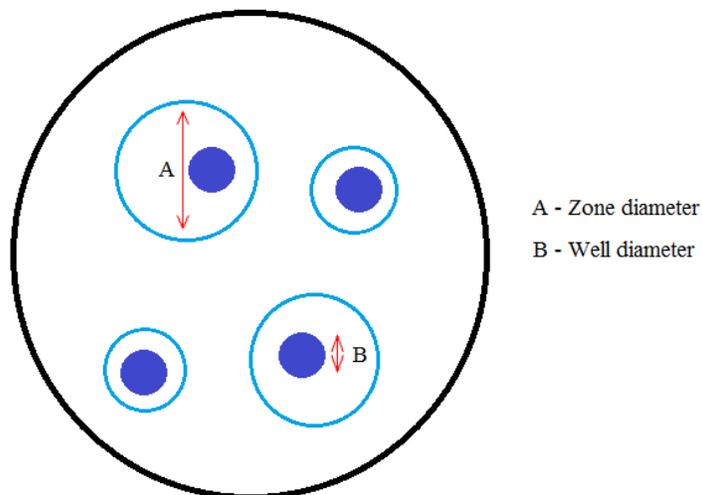


Figure 1. Inhibition zones developed in an agar medium.

Using graphs of X^2 against the concentration of citrate were drawn.

$$X^2 = \left(\frac{\text{Zone diameter} - \text{Well diameter}}{2} \right)^2$$

MIC value is determined by extrapolating the graph to meet the X-axis (Figure 2).

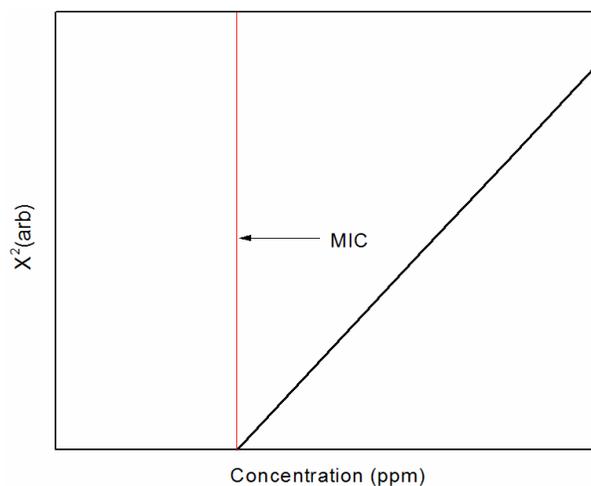


Figure 2. Plot for determination of MIC value.

For the LDH, at the MIC value, the amount of citrate intercalated was determined using UV –Vis spectrophotometric analysis.

3. Results and Discussion

3.1 Characterization of extracted citrate

Thin layer chromatography

Thin layer chromatography conducted in a methanol: ethanol (1:1) system confirmed the presence of one spot suggesting a successful extraction of citrate ions from lime.

PXRD characterization

Purity and the crystallinity of the extracted citrate from lime were confirmed using PXRD patterns, where PXRD pattern suggests that there are no detectable crystalline impurities in the citrate extract. However, in this study, 100% purity is not required since LDHs composed of positively charged layers, are capable of selectively intercalating anions in the medium.

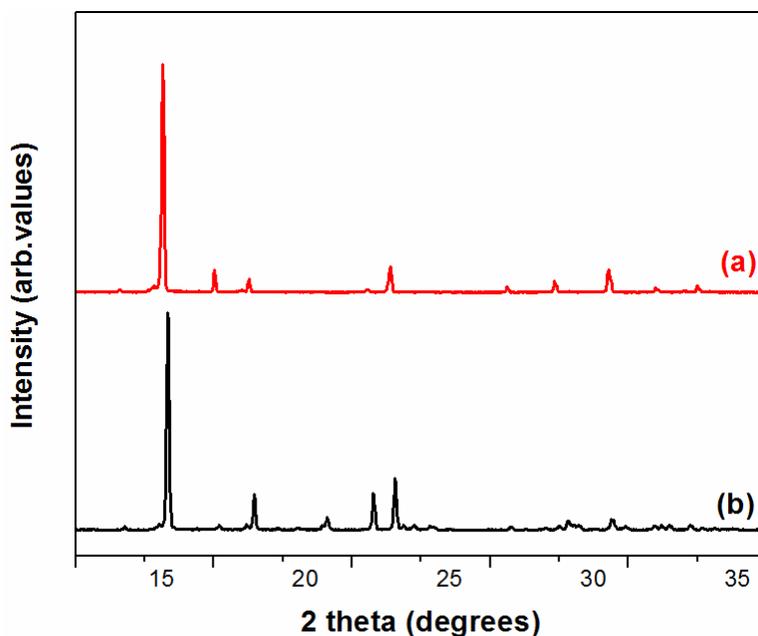


Figure 3. Comparison of the PXRD patterns of (a) extracted natural citrate from lime (b) synthetic citrate.

3.2 Characterization of LDHs

Morphological Studies using SEM

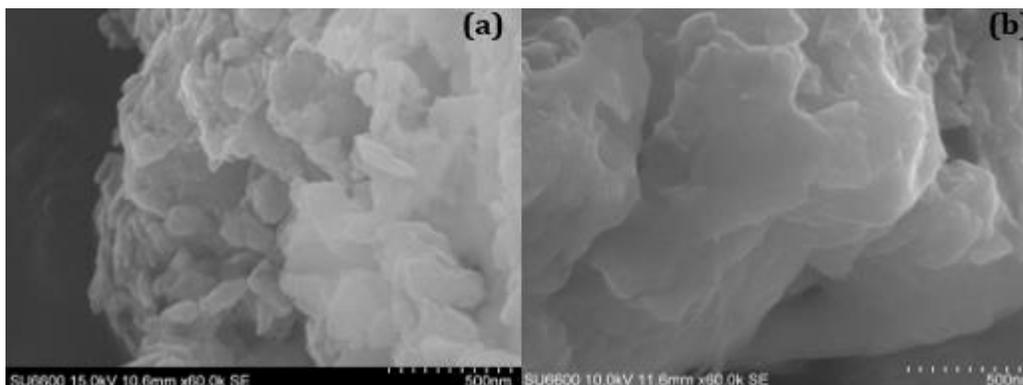


Figure 4. SEM images of (a) nitrate-LDH and (b) natural citrate-LDH.

As depicted in Figure 4, the morphology of the (a) nitrate-LDH and (b) natural citrate-LDH was examined by scanning electron microscopy. Both pure LDH and citric acid intercalated LDH showed the conventional layered structure consisting of thick platelets of nanometer size. After the intercalation of citric acid to nitrate-LDH, a reduction of crystallinity was observed, which was further confirmed by PXRD studies.

PXRD Characterization

PXRD techniques were used to confirm the structure and the crystallinity of synthesized nitrate-LDHs and the successful intercalation of citrate ions into the LDHs. PXRD patterns of synthesized nitrate-LDH clearly matched with previously reported patterns (Perera et al., 2015). All patterns were indexed based on a hexagonal unit cell. The cell parameters of the nitrate-LDH unit cell were found to be 2.52 nm (c) and 0.27 nm (a and b), respectively. The interlayer spacing of nitrate-LDHs was calculated considering peaks corresponding to the 003 and successive orders and yielded an interlayer spacing of 0.84 nm, which agrees with the previously reported value. Assuming the layer thickness of 0.48 nm, a gallery height of 0.36 nm is suggested. This gallery height leads to a vertical arrangement of nitrate anions within the layers considering the size of nitrate ions as 0.4 nm (Perera et al., 2015).

In citrate-LDHs the basal peaks show a lower wavenumber shift while there is no noticeable change in the non-basal reflections. This suggests that there is an increase in the interlayer spacing upon intercalation of both synthetic and natural citrate ions. This interlayer expansion confirms the successful intercalation of citrate ions.

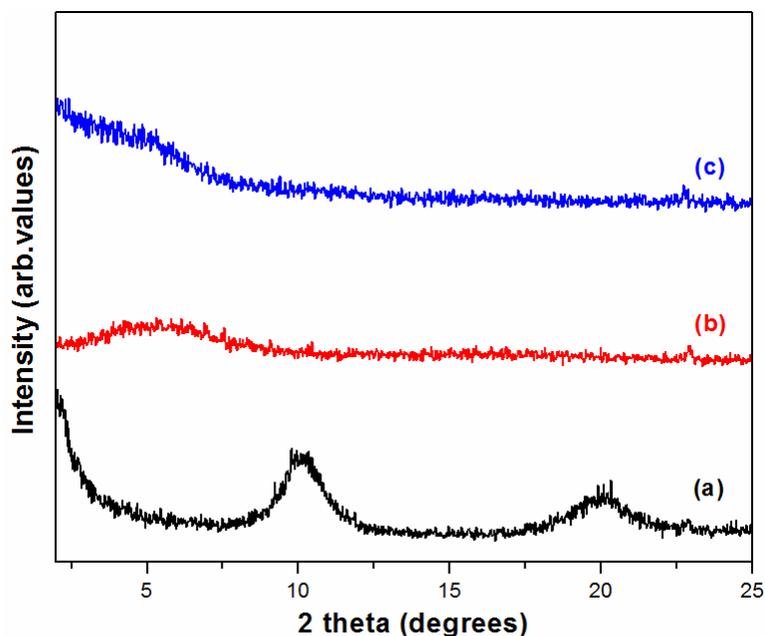


Figure 5. PXRD patterns for (a) nitrate-LDH, (b) synthetic citrate-LDH and (c) natural citrate-LDH.

The observed interlayer spacing for synthetic citrate LDH is 1.5 nm, and that for natural citrate LDH is 1.8 nm. Assuming the size of citrate 0.72 nm a vertical arrangement of citrate anions within the interlayer region can be suggested.

The d spacing of 0.1 nm for 110 peak corresponding to the intermetallic distance in the base lattice observed for nitrate LDH has not changed upon intercalation of citrate anion. This observation suggests that the base of the lattice has not been changed due to the intercalation reaction; therefore, its layered nature is maintained.

FTIR characterization

In order to confirm the successful intercalation of citrate ions into the LDH host matrix, FTIR characterization was utilized and peak positions, broadening, and peak shifts from pure citric acid and standard nitrate LDH were taken into consideration.

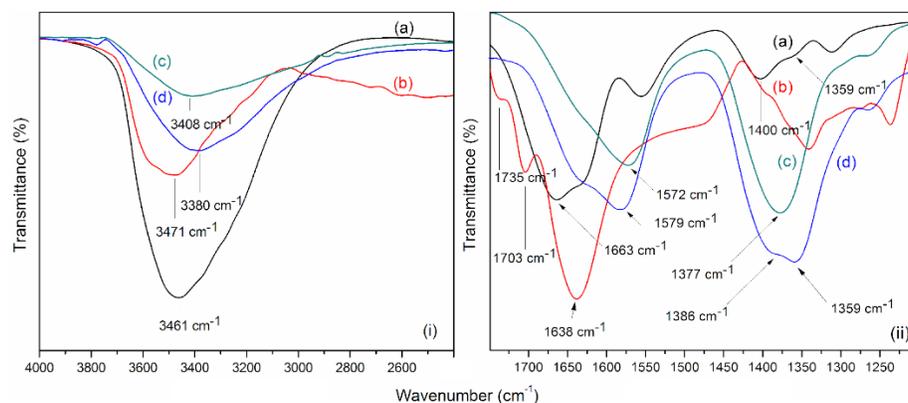


Figure 6. FTIR spectra for the (i) O-H and (ii) carboxylate stretching regions of (a) nitrate-LDH, (b) pure citric acid, (c) natural citrate-LDH and (d) synthetic citrate-LDH.

Hydroxyl stretching frequency of free citrate ion, which appears around 3471 cm^{-1} as an intense broad band, has been shifted to a lower wavenumber after intercalation to 3408 cm^{-1} and 3380 cm^{-1} for natural citrate-LDH and synthetic citrate-LDHs, respectively. This peak for nitrate-LDH, appears approximately at 3461 cm^{-1} . This shift accounts for the strengthening of the H-bonding network within the layered compound due to the presence of highly polar citrate molecules instead of nitrate molecules (Perera et al., 2015). The peak broadening in the hydroxyl stretching region is due to the presence of H-bonding with different degrees of strength.

The appearance of carboxylate stretching frequencies in the citrate-LDHs is one of the most significant observations supporting the argument for the successful incorporation of the organic acid into the layered material. The sharp peaks observed at 1579 cm^{-1} and 1357 cm^{-1} for synthetic citrate-LDH and at 1572 cm^{-1} and 1377 cm^{-1} for the natural citrate-LDH account for the asymmetric and symmetric stretching modes of carboxylate groups, respectively (Collins et al., 2014, Wang et al., 2015). A clear absence of the peak position of carbonyl stretching at 1735 cm^{-1} (Lori et al., 2016) and at 1703 cm^{-1} of pure citric acid (Shi et al., 2007, Răuciu et al., 2006) further confirmed the citrate ion encapsulation to LDH and the presence of a strong H-bonding network within the new hybrid material. The disappearance of the bending vibrations of interlayered and incorporated water molecules of LDH and citric acid, respectively at $1660\text{-}1640\text{ cm}^{-1}$ wavenumbers (Singha et al., 2011, Nilwala et al., 2014) suggests the successful intercalation of citrate ions to LDHs. Interestingly, the disappearance of nitrate absorption bands around 1400 cm^{-1} and 1359 cm^{-1} of nitrate LDH further confirms the intercalation of natural citrate into LDH interlayer spacing (Baikousi et al., 2013, Wang et al., 2015, Nilwala et al., 2014).

During the synthesis of citrate intercalated LDHs in the presence of nitrate ions originating from the metallic nitrates, there is a competition for intercalation between the citrate anions and nitrate anions. Although the nitrate concentrations are high in the medium, it was further confirmed by Kjeldahl analysis that there is no detectable N in the citrate intercalated LDHs. This can be attributed to the intercalation affinity of the two anions, where the intercalation affinity of anions shows the following sequence, tri valent > di valent > monovalent. When nitrate ions are occupied in the interlayer spacing, a larger number of monovalent nitrate ions are required to neutralize the charge in the layers, which leads to the formation of a more sterically crowded LDH. On the other hand, with multi-valent ions, a lower number of molecules are needed for the charge neutralization; hence, the interlayer region is less crowded. Also, trivalent ions such as citrate ions are capable of anchoring two layers leading to a more stabilized layered structure.

3.4. Release behavior study of LDHs

The release behavior of natural citrate-LDH was studied at pH mediums 3, 4 and 5 (Fig. 7 (a)). These pH values correspond to those at which most of the microbes are active. A rapid release for pure citrate is observed as described in the literature (Perera et al., 2015), and all ions were released within the first 1.5 h for all pH mediums. According to the above results, natural citrate-LDH shows a gradual release of the citrate anions as a function of time. Citrate has released in a slow manner within the first 8 h. Then, it shows a constant releasing behavior.

The amount of release of citrate at pH 3 is higher than that of pH 4 and pH 5, which is in well agreement with Perera et al. When the acid within the layers is protonated in acidic medium it gradually comes out. This facilitates the release of intercalated ions.

The pH of ripened fruits varies between 3.5–4.5 pH range. At these pH values, citrate-LDHs demonstrate the best slow-release behavior, thus demonstrating the potential for use as antimicrobial compounds in food storage applications.

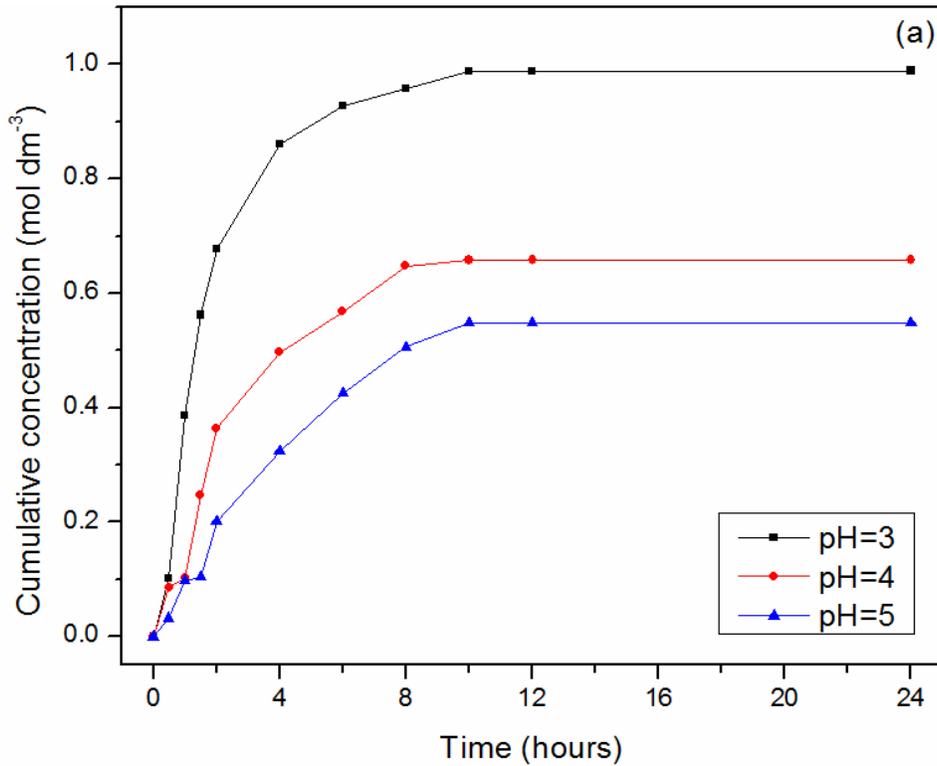


Figure 7. Release behavior of natural citrate-LDH.

Since the release contains a burst release followed by a sustainable release, models were fitted to the initial release stages. According to the R^2 values obtained, the Higuchi model gave the highest R^2 of 0.98 at pH=5 for citrate-LDH release, whereas R^2 of 0.95 was obtained for the Korsmeyer-Peppas model. All other models, such as Zeroth order, First Order and Hixon model, exhibited R^2 below 0.9 even at pH=5. Therefore, it can be further explained that the release mechanism is similar to the release of water-soluble citrate molecules from a homogeneous matrix followed by a diffusion mechanism (Costa and Sousa Lobo, 2001, Kottegoda et al., 2017).

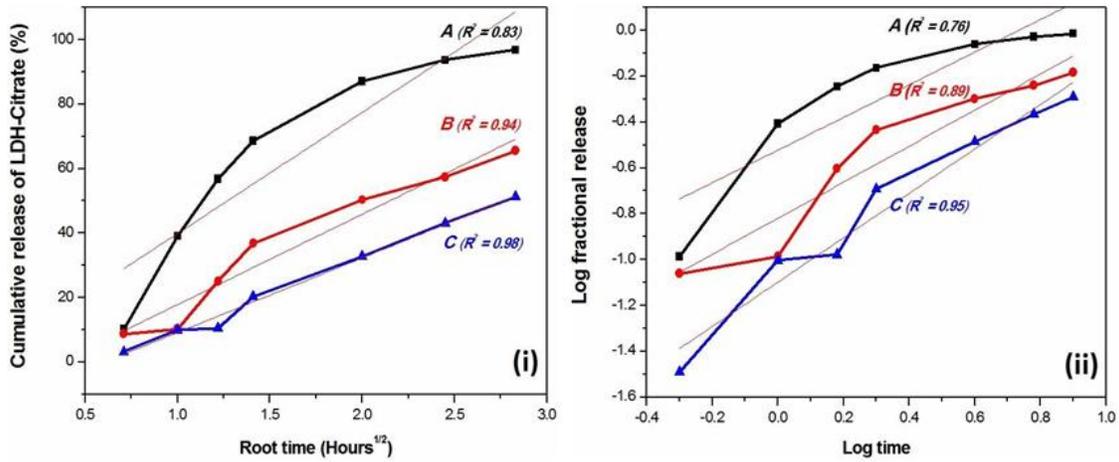


Figure 8. Comparison of the release kinetics of natural citrate-LDHs at (A) pH=3, (B) pH=4, and (C) pH=5 in water using (i) Higuchi model, and (ii) Korsmeyer-Peppas model.

3.5. Determination of Antimicrobial Property of LDHs

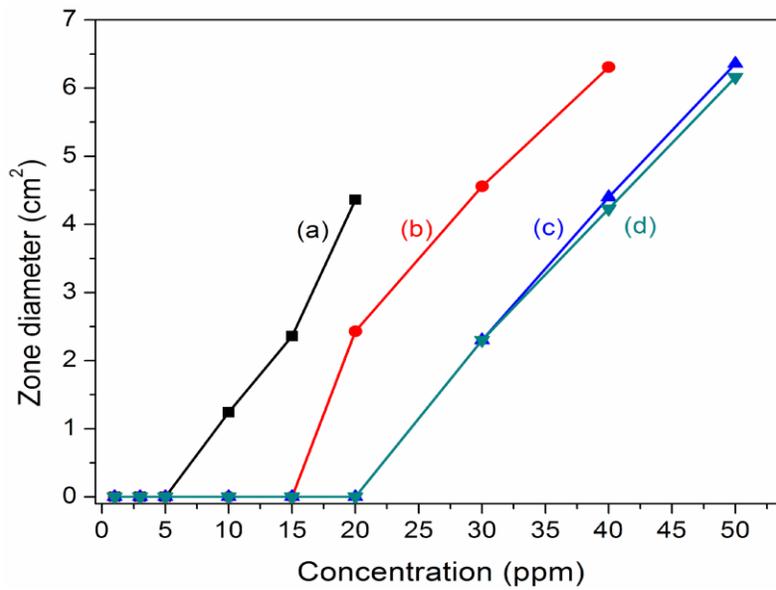


Figure 9. Minimum inhibitory concentrations for (a) Sodium benzoate, (b) pure citric acid, (c) synthetic citrate-LDH, and (d) natural citrate-LDH.

Table 1: Minimum Inhibitory Concentration (MIC) for LDHs.

Substance	MIC Value for <i>Colletotrichum gloeosporioides</i> (ppm)	MIC Value for <i>Saccharomyces cerevisiae</i> (ppm)
Sodium benzoate	10	25
Pure citrate	15	50
Synthetic citrate LDH	20 (equivalent to 6.6 mg of citrate)	70 (equivalent to 20 mg of citrate)
Natural citrate LDH	20 (equivalent to 6.6 mg of citrate)	70 (equivalent to 20 mg of citrate)

According to the above results (Fig. 9), citrate-Mg-Al-LDH demonstrated activity against both *Colletotrichum gloeosporioides* and *Saccharomyces cerevisiae*. It can be concluded that the citrate-LDH is as efficient as the sodium benzoate. However, when pure citrate is used under environmental conditions the activity may deteriorate quickly and as a result, higher concentrations than the citrate-Mg-Al-LDH may be needed.

4. Conclusion

Natural α -hydroxy citrate extracted from *Citrus aurantifolia* (lime) has been successfully intercalated into Mg-Al layered double hydroxides following a one-step co-precipitation method. The successful intercalation of citrate ions within the gallery region of the LDH has been proven by referring to PXRD and FTIR characterization techniques. It was concluded that once citrate ions are intercalated between the nanolayers of an LDH the thermal stability of citrate ions improves significantly. Further, the natural citrate intercalated Mg-Al-LDH demonstrated significant activity against two common food pathogens, *Colletotrichum gloeosporioides* and *Saccharomyces cerevisiae*, with MIC values of 6.6 and 20 mg, respectively. Hence, with the current emerging trends of exploring the potential use of naturally available active compounds, this nanohybrid can be introduced as a pellet or a thin coating into food packaging.

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