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Nano-Microemulsions of $CaCO_3$-Encapsulated Curcumin Ester Derivatives With High Antioxidant and Antimicrobial Activities and pH Sensitivity

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In this study, we synthesized nano-microemulsions of calcium carbonate ($CaCO_3$)-encapsulated curcumin (Cur)-Ferulic acid (FA) ester derivatives of diverse mass ratios by using the solution casting approach. The structures, antioxidant and antimicrobial activities, physical properties, and potential of hydrogen (pH) sensitivity of these products were examined. Compared with microparticles of $CaCO_3$, those of $CaCO_3$@Cur-FA exhibited excellent antimicrobial and antioxidant properties. Response to pH was indicated through the release of Cur-FA from $CaCO_3$@Cur-FA in solutions having different pH values. The results demonstrated that Cur-FA was released more quickly from $CaCO_3$@Cur-FA at pH 5.5 than at pH 7.4. $CaCO_3$@Cur-FA demonstrated good antioxidant capacities through its ability to scavenge 2,2′-amino-di(2-ethyl-benzothiazoline sulphonic acid-6)ammonium salt ($ABTS^+$) and 1,1-diphenyl-2-picrylhydrazyl (DPPH). These activities were three-fold more than those observed in $CaCO_3$ microparticle control groups; additionally, the antimicrobial activity against *Aspergillus niger* and *Escherichia coli* increased by 40.5 and 54.6%, respectively. Overall, the microparticles of $CaCO_3$@Cur-FA outperformed Cur-FA in terms of antimicrobial properties by inhibiting the growth of certain zoonotic pathogens.

Keywords: antioxidant activity, pH response, antimicrobial property, $CaCO_3$@Cur-FA, biological activity

HIGHLIGHTS

- $CaCO_3$@Cur-FA microspheres showed spherical morphology with uniform size.
- $CaCO_3$@Cur-FA exhibited excellently antioxidant activity.
- $CaCO_3$@Cur-FA exhibited excellently antibacterial activity.
- $CaCO_3$@Cur-FA exhibited excellently pH responsivity activity.
- $CaCO_3$@Cur-FA could as a new food additive, which applicated in the food industry,

INTRODUCTION

Curcumin (diferuloylmethane; Cur) is a leading curcuminoid extracted from the rhizome of *Curcuma longa* plant (1) and has been used as a traditional herbal medicine across Asian countries. This is because it has antioxidant, anticancer (2), antiviral (3), anti-inflammatory, and antimicrobial properties that help in controlling chronic disorders (4). In addition, curcumin, as a common natural pigment, is extensively used in various food industries such as those of canned food, sauces, and brine products. Cur has high solubility in solvents with lower polarity (for example, ethanol and propylene glycol), and it is deemed to be a lipophilic substance (4). Ferulic acid (FA) is suggested to markedly modulate the incidences of inflammation, oxidation, and metabolic syndrome (5). FA is easily soluble in solvents with high polarity (such as ethanol, water, and methanol) and is deemed to be a hydrophilic substance (6). Lipophilic and hydrophilic phytochemicals are extensively distributed in daily foods (such as plants, fruits, herbs, grains, and vegetables) and exhibit antiviral, antimicrobial, and antioxidant activities when used in combination (7). Based on this interesting finding, we designed a molecule in which Cur and FA were connected via an esterification reaction; this molecule was designated as Cur-FA and could be used as a novel food additive.

At present, nanotechnology has offered measures to cope with diverse technical challenges in various fields such as the food industry (8, 9). Calcium carbonate ($CaCO_3$) delivery systems (10) play an important role in various fields including biomedicine, healthcare, and industrial manufacturing (11). They can be used easily and extensively because of their high yield, cost-effectiveness, non-toxicity, biodegradability, biocompatibility, and appropriate decomposition efficiency, apart from their stability even in a basic environment (12). Therefore, $CaCO_3$ together with its modified substances could be used as a delivery vehicle and immobilizing carrier for food research, thus reducing the effective dose required (13). Besides, these materials have small droplet sizes, which enhances their antimicrobial bioactivity by allowing their penetration into the cell membrane, resulting in lipid bilayer destabilization.

In the present study, we hypothesized that the nano-microemulsion of $CaCO_3$-encapsulated curcumin ester derivatives would have a higher antimicrobial activity against pathogens. This may be because the nano-microemulsion has a small droplet size, which can facilitate its penetration into the microbial cell membrane for destroying its activity and inducing death. This study characterized the nano-microemulsion by using various approaches. Additionally, the nano-microemulsion was examined for its antimicrobial ability against seven zoonotic pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Rhizopus*, and *Aspergillus niger*).

MATERIALS AND METHODS

Sodium carbonate (Na_2CO_3) and calcium chloride ($CaCl_2$) were provided by Xiqiao Science Co., Ltd. (Shantou, China). Cur-FA: ^{1}HNMR ($CDCl_3$, 400 MHz) δ:3.83 (s, 6H), 3.85 (s, 3H), 3.87 (s, 3H), 4.59 (s, 2H), 5.59(s, 2H), 6.31 (d, 1H), 6.79 (d, 2H), 6.91 (d, 3H), 6.93 (d, 1H), 6.99 (d, 2H), 7.06 (d, 1H), 7.10 (d, 1H), 7.11 (d, 2H), 7.20 (s, 1H), 7.23 (s, 1H), 7.27 (d, 1H), 7.48 (d, 1H), 7.60 (d, 3H), 9.5 (s, 2H). HRMS (ESI) calculated for [M + H]+$C_{42}H_{38}O_{12}$: 734.75 found 734.69.

Microbial Cultures

Four foodborne pathogenic strains were provided by the Microbiology Laboratory of the National Research Centre (Egypt), which included *E. coli, S. aureus, Rhizopus, A. niger*. Microbial cultures were maintained in a suitable agar medium inclined at an angle of at 4°C (slant culture), which served as the stock cultures. The pathogens were grown in Mueller–Hinton agar (MHA) or Mueller–Hinton broth (MHB).

$CaCO_3$ and $CaCO_3$@Cur-FA Microparticle Preparation

In this study, $CaCO_3$@Cur-FA microparticles were prepared by a method described previously. Briefly, water: acetone (3:1) solution was mixed with Na_2CO_3 (0.5 M) and $CaCl_2$ (0.5 M) with Cur-FA1 (0.1 M), Cur-FA2 (0.2 M), or Cur-FA3 (0.3 M) to prepare the stock solutions. The $CaCl_2$ solution was mixed with Cur-FA (Cur-FA1, Cur-FA2, or Cur-FA3) in a 50-mL beaker, followed by 10 min of stirring with the 85-1 constant temperature magnetic stirrer (Shanghai Zhiwei Electric Appliance Co., Ltd) to prepare $CaCO_3$@Cur-FA microparticles. After the rapid addition of Na_2CO_3 solution to the aforementioned mixture, the obtained solution was subjected to 5 min of stirring at 500 rpm at 40°C, followed by centrifugation to collect the products. $CaCO_3$ microparticles were synthesized using a similar method without adding Cur-FA solution.

Characterization of $CaCO_3$@Cur-FA Microparticles

The Zetasizer Nano-Zeta potentiometer (Nano ZS90, Malvern, UK) was used to measure zeta-potential and particle size of the $CaCO_3$@Cur-FA microparticles. The scanning electron microscope (SEM, Model S-4800 II FESEM, Hitachi, High-Technologies Co., Ltd., Japan) was used to observe the particle morphology.

pH Sensitivity of $CaCO_3$@Cur-FA Microparticles

To assess the pH sensitivity of $CaCO_3$@Cur-FA microparticles in a food microenvironment, the $CaCO_3$@Cur-FA samples (0.2 mg, 2 mL) were dissolved in phosphate buffered saline (PBS) of various pH (5.5, 6.8, and 7.4) and added into dialysis tubes (MWCO 3500). Thereafter, these tubes were soaked in 10 mL of PBS with corresponding pH (within the centrifuge tube) by constant shaking below 37°C. We took out 1 mL dialysate at pre-determined time intervals to measure the absorption, thereby assessing the amount of released Cur-FA. Then, 1 mL fresh PBS was added to the centrifuge tube.

Antioxidant Activity Assay

The antioxidant activity of $CaCO_3$@Cur-FA was assessed by measuring its ability to scavenge 2,2′-azino-bis (3-ethylbenzothiazoline-6 sulfonic acid; $ABTS^+$) and 1,

1-diphenyl-2-picrylhydrazyl (DPPH) free radicals. In brief, 0.2 g $CaCO_3$@Cur-FA was dissolved in 2 mL distilled water before the test. Further, 2 mL of the $CaCO_3$@Cur-FA supernatant was added into ∼2 mL of DPPH methanol solution (0.1 mM) to react for 30 min in dark. Subsequently, the ultraviolet spectrophotometer (Shimadzu, UV-2007, Japan) was used to measure the absorbance (optical density; OD) at 517 nm. The test tube was shaken for ensuring solution uniformity prior to measurements. For the composite film, its ability to scavenge $ABTS^+$ free radicals were determined using the modified approach.

The $ABTS^+$ or DPPH scavenging ability [R (%)] was determined through (Eq. 1)

$$R(\%) = (A2 - A1)/A0 \quad (1)$$

A0 is the OD value of DPPH ($ABTS^+$/PBS) in distilled water;

A1 is the OD value of $CaCO_3$@Cur-FA solution in distilled water mixed with the DPPH methanol solution (ABTS+/PBS mixed solution), and

A2 indicates the OD value of DPPH methanol solution ($ABTS^+$/PBS mixed solution).

Antimicrobial Assays

In this study, we adopted agar–well diffusion approach to perform antimicrobial studies. In brief, 1 mL of active strain culture (10^5 cells /mL) was added to 20 mL MHB (Becton Dickinson, USA) and poured into petri dishes containing MHA. When the agar solidified, wells (diameter, 5 mm) were cut using a sterile borer. Further, 50 μL of the nano-microemulsion or bulk extract was added to each well. The plates were incubated for 2 h under ambient temperature, so that the solutions in the wells diffused into the agar. Moreover, the plates were incubated for 24 h at 37°C. Afterwards, we determined the microbial growth inhibition rate and inhibition zone diameters. Every sample was measured thrice (penicillin G, bulk extract, and nano-microemulsion), and the experiment was performed in triplicates.

Discs containing penicillin G (10 U) and ethanol were used as the positive and negative controls, respectively.

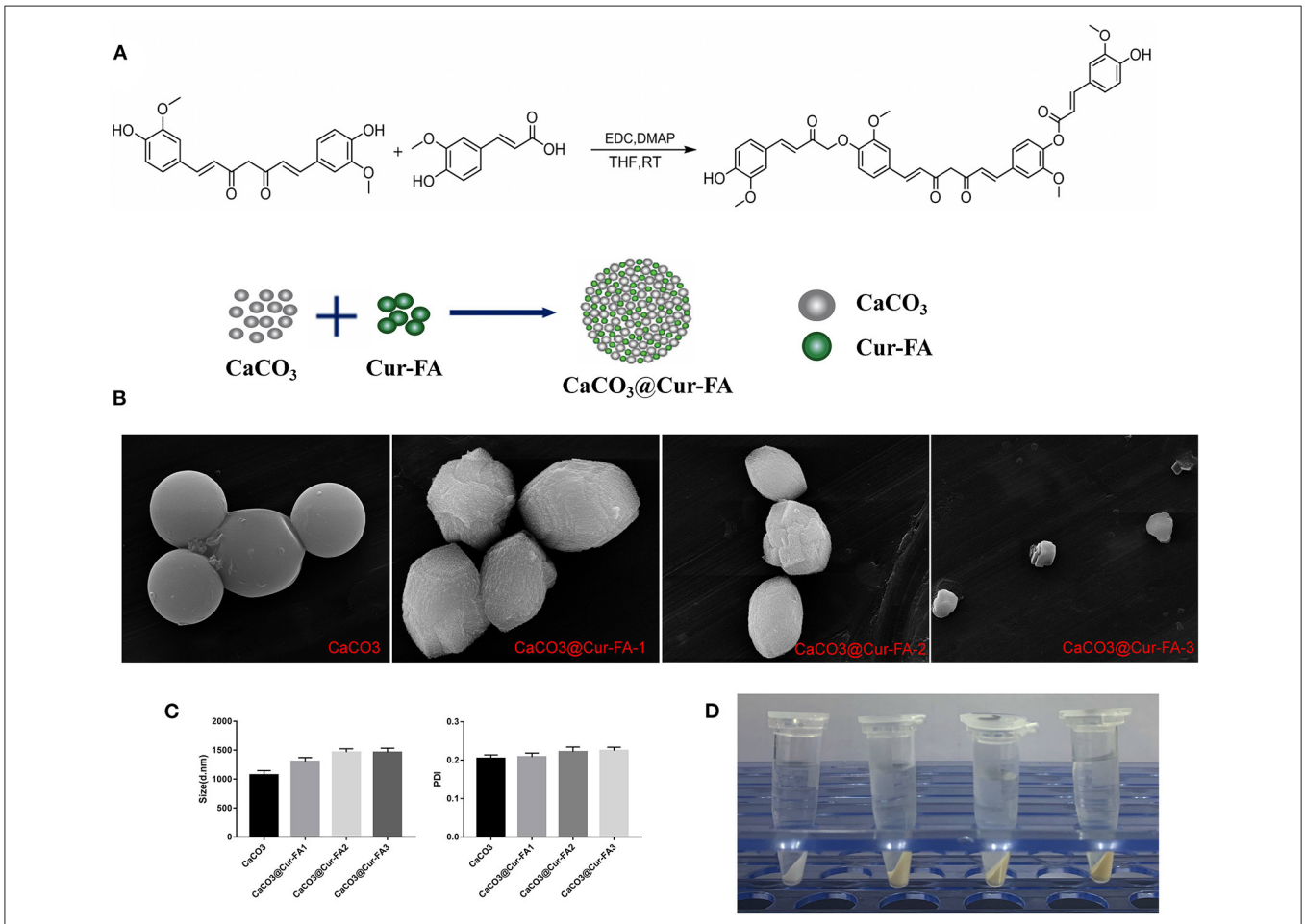

FIGURE 1 | Characterization of $CaCO_3$ and $CaCO_3$@Cur-FA. **(A)** Synthesis of Cur-FA and $CaCO_3$@Cur-FA. **(B)** SEM images of $CaCO_3$ and $CaCO_3$@Cur-FA. Scale bar = 5 μm. **(C)** Particle size and PDI for $CaCO_3$ and $CaCO_3$@Cur-FA. **(D)** The images of $CaCO_3$ and $CaCO_3$@Cur-FA.

Statistical Analysis

The differences among the samples were analyzed using ANOVA. Statistical analysis was performed using SPSS software. The significance of difference ($P < 0.05$) was compared by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Characterization of $CaCO_3$ and $CaCO_3$@Cur-FA Microparticles

This study successfully prepared $CaCO_3$ microparticles using the mineralisation approach (**Figure 1A**). As shown in **Figure 1B**, the SEM data of $CaCO_3$ and $CaCO_3$@Cur-FA microparticles clearly revealed the spherical morphology as well as the almost even size distribution. The sample particle size is given in **Figure 1C**. **Table 1** displays the sample zeta potential. The average hydrodynamic sizes of the microparticles of $CaCO_3$, $CaCO_3$@Cur-FA1, $CaCO_3$@Cur-FA2, and $CaCO_3$@Cur-FA3 were 1070.1 ± 76 nm, 1303.5 ± 66 nm, 1464.8 ± 58 nm, and 1460.7 ±72 nm, respectively. Thus, the optimal concentration of Cur-FA required for preparing uniform-sized $CaCO_3$@Cur-FA microparticles was 0.2 M. The mean size of $CaCO_3$@Cur-FA mildly increased relative to that of the unmodified $CaCO_3$ microparticles, indicating the successful encapsulation of Cur-FA into the microemulsion. In addition, no droplet aggregation was observed, which indicated that $CaCO_3$@Cur-FA maintained its identity in the process of drying. The image of $CaCO_3$@Cur-FA microparticles is shown in **Figure 1D**.

TABLE 1 | Characterization of $CaCO_3$, $CaCO_3$@Cur-FA-1, $CaCO_3$@Cur-FA-2, $CaCO_3$@Cur-FA-3.

Nanoparticles	Size	PDI	Zeta (mV)
$CaCO_3$	1070.1 ± 76	0.205 ± 0.009	4.75 ± 0.34
$CaCO_3$@Cur-FA-1	1303.5 ± 66	0.208 ± 0.010	−12.34 ± 1.23
$CaCO_3$@Cur-FA-2	1464.8 ± 58	0.222 ± 0.012	−13.45 ± 3.08
$CaCO_3$@Cur-FA-3	1460.7 ± 72	0.225 ± 0.010	−12.14 ± 1.19

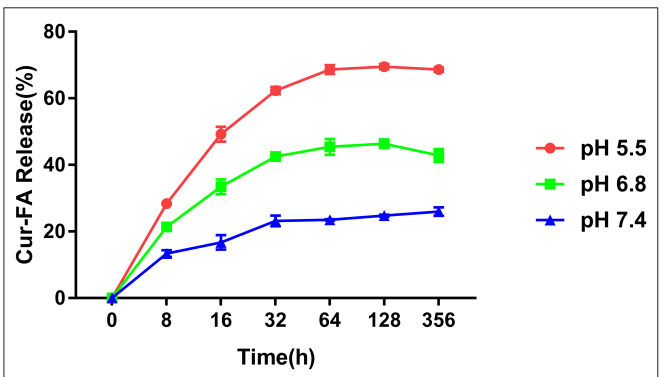

FIGURE 2 | pH sensitivity of CaCO3@Cur-FA. Cur-FA was released from $CaCO_3$@Cur-FA at pH 7.4, 6.8, and 5.5.

pH Sensitivity of $CaCO_3$@Cur-FA2 Microparticles

The pH sensitivity of $CaCO_3$@Cur-FA2 microparticles was evaluated in PBS of pH 7.4, 6.8, and 5.5. As shown in **Figure 2**, $CaCO_3$@Cur-FA2 released only 24.5% of loaded Cur-FA2 at pH 7.4 after 128 h. On the contrary, 70.1 and 45.6% of Cur-FA2 was released from $CaCO_3$@Cur-FA2 at pH 5.5 and 6.8, respectively, which was associated with $CaCO_3$ decomposition under the acidic condition. The results demonstrated that $CaCO_3$@Cur-FA could be used as a food additive with a visible pH sensitivity.

Antioxidant Activity of $CaCO_3$@Cur-FA2 Microparticles

Antioxidant activity plays a vital role in the food industry. It is the ability of inhibiting or delaying additional molecular oxidation (14). Previous results have demonstrated that Cur

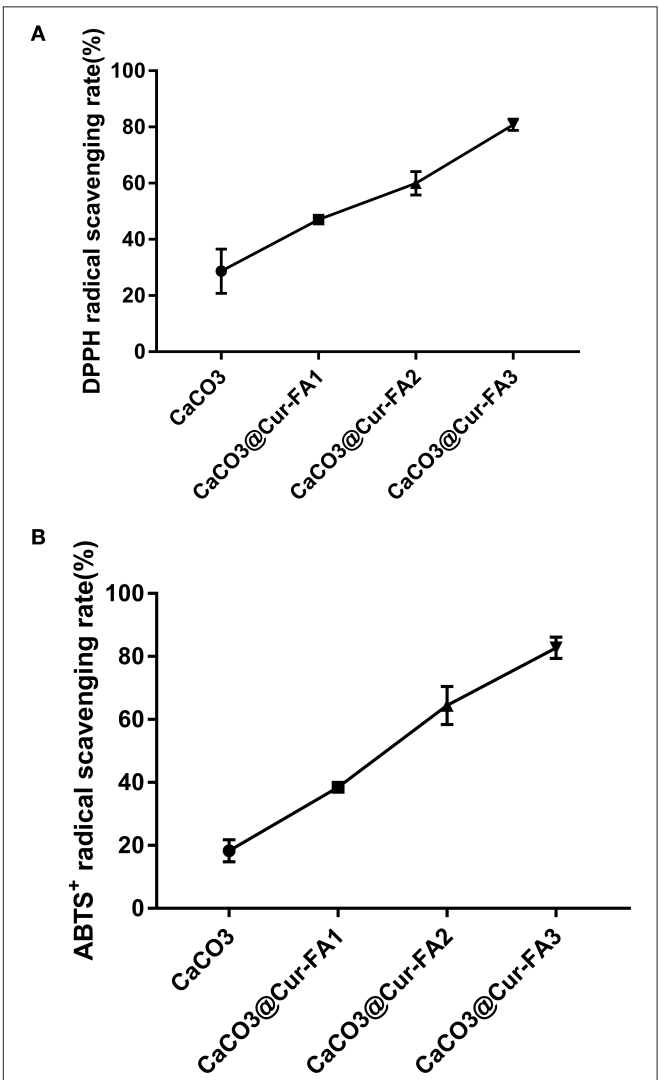

FIGURE 3 | Abilities of $CaCO_3$ and $CaCO_3$@Cur-FA to scavenge DPPH **(A)** and $ABTS^+$ **(B)** radicals.

TABLE 2 | Bacteriostatic activity of films.

Items	*Escherichia coli*	*Escherichia coli*	*Aspergillus niger*	*Rhizopus*
$CaCO_3$	8.2 ± 0.2^{d}	7.1 ± 0.3^{c}	10.5 ± 0.4^{d}	4.2 ± 0.1^{d}
$CaCO_3$@Cur-FA1	13.2 ± 0.3^{a}	8.3 ± 0.2^{b}	12.5 ± 0.6^{cd}	4.3 ± 0.5^{b}
$CaCO_3$@Cur-FA2	14.3 ± 0.4^{a}	9.1 ± 0.3^{b}	13.1 ± 0.4^{b}	5.1 ± 0.4^{bc}
$CaCO_3$@Cur-FA3	14.6 ± 0.2^{a}	9.3 ± 0.4^{a}	13.6 ± 0.3^{a}	5.6 ± 0.3^{a}

Comparison of differences between groups (a, b, c, and d).

(15) and FA (16) have very high antioxidant activity because of phenolic hydroxyl groups in their molecular structure. In our previous study, the antioxidant activity of Cur ester derivatives was shown to be significantly increased. A higher Cur-FA level resulted in the higher antioxidant activity of the Cur-FA film. The DPPH scavenging activity of $CaCO_3$@Cur-FA1 microparticles was increased three times compared with that of $CaCO_3$ microparticles (**Figure 3A**). The $ABTS^{+}$ scavenging ability of CaCO3@Cur-FA was similar to the DPPH scavenging ability (**Figure 3B**). The free radical scavenging ability of $CaCO_3$@Cur-FA microparticles was increased by 40% compared with that of $CaCO_3$ microparticles. Thus, $CaCO_3$@Cur-FA exhibited good antioxidant ability.

Antimicrobial Activity

$CaCO_3$@Cur-FA exhibited antimicrobial activity, which is significant for its application as a food additive (16). In the present work, we adopted the agar–well diffusion method to detect the ability of $CaCO_3$@Cur-FA to inhibit *Rhizopus*, *A. niger*, *E. coli*, *and S. aureus*, and the inhibition zones were found to be 5.6 ± 0.3, 13.6 ± 0.3, 14.6 ± 0.2, and 9.3 ± 0.4 mm, respectively (**Table 2**). Thus, $CaCO_3$@Cur-FA inhibited the growth of these pathogens. $CaCO_3$@Cur-FA microparticles demonstrated superior antimicrobial activity than $CaCO_3$ microparticles, indicating that adding $CaCO_3$ promoted the antimicrobial activity of Cur-FA.

CONCLUSION

A novel food additive comprising Cur-FA was designed and synthesized in this study. Herein, we successfully engineered a simple, efficient, well-characterized, and stable $CaCO_3$@Cur-FA nano-microemulsion as a food additive. The results demonstrated that $CaCO_3$@Cur-FA is a natural antimicrobial food additive with excellent antioxidant effects. The antimicrobial activity of $CaCO_3$@Cur-FA was irreversibly changed with the change in the environmental pH, and our results demonstrated that CaCO3@Cur-FA could be used as a food additive with a visible pH sensitivity, therefore, it could be used as an antibacterial preservative, or as a protective agent for some drugs that are easy to be damaged by gastric acid. Additionally, $CaCO_3$ enhanced the antimicrobial and antioxidant activities of Cur-FA. In conclusion, $CaCO_3$@Cur-FA is a natural material with high safety and degradability and may be extensively used as a food additive.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

LW and ZZ: research concept, methodology, data extraction, analysis, and draft writing. YY: resource searching, verification, formal analysis, supervision, and manuscript reviewing and editing. XW and DL: resources, methodology, project administration, supervision, and manuscript reviewing and editing. ZG: resource searching and manuscript reviewing and editing. Mengying Xia: methodology and manuscript reviewing and editing. All authors reviewed and approved the final manuscript.

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