Synthesis and \textit{in vitro} antimicrobial activity evaluation of coumarin-3-carboxylic acids obtained via cascade reaction using chitosan as a recyclable catalyst†

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In this work, commercial chitosan was used as a recyclable and biodegradable catalyst for efficient one-pot synthesis of coumarin-3-carboxylic acids in water or ethanol:water (3:7, v/v) at 75 °C via Knoevenagel-intramolecular cyclization cascade reaction. Coumarin-3-carboxylic acids (3a–i) were obtained from different substituted salicylaldehydes and Meldrum’s acid under mild conditions in short times (10–45 min) and good isolated yields (77–88%) without requiring laborious purifications. The proposed method combined the advantages of homogeneous catalysis with recovery and recyclability of four catalysts without compromising its catalytic activity and structural integrity. In the evaluation of \textit{in vitro} antimicrobial activity against 4 bacterial and 8 fungal strains, the tested coumarin-3-carboxylic acids 3c–e, 3h, and 3i showed an inhibitory effect on the growth of the microorganisms (1024–256 μg mL\textsuperscript{-1}). Product 3d showed the lowest MIC against fungal and bacterial strains (256 μg mL\textsuperscript{-1}), suggesting a bactericidal and fungicidal nature.

1 Introduction

Coumarins are lactones of natural or synthetic sources, characterized by the presence of a benzo-γ-pyran ring rich in highly conjugated π electrons.\textsuperscript{1} Various medicinal effects are reported for its derivatives, such as use as drug carriers,\textsuperscript{2} antitumor,\textsuperscript{3} anti-HIV,\textsuperscript{4} anti-inflammatory,\textsuperscript{5} antioxidants,\textsuperscript{6} and anticoagulants.\textsuperscript{7} Among them, coumarin-3-carboxylic acid and its derivatives constitute a subclass of compounds differentiated by the presence of the carboxyl group linked to carbon C3 of the pyran component of coumarin ring. These compounds stand out in studies from different areas since the carboxyl group allows the introduction of units that contribute to the increased chemical activity, for example, (i) the formation of coumarin-piperazine hybrids gives rise to compounds with potential anti-Alzheimer activity,\textsuperscript{8} (ii) the production of flexible and changeable films of coumarin-3-carboxylic acids/GdF\textsubscript{3}:Sm\textsuperscript{3+} allows the luminescent detection of pesticides in water even at micro concentrations,\textsuperscript{9} and (iii) the complexation of copper in coumarin-3-carboxylic acids originates efficient luminescent sensors for rapid detection of water in common organic solvents.\textsuperscript{10} Consequently, these compounds are considered promising for innovative work in industry and research.

The usual methods for the synthesis of coumarin-3-carboxylic acids include Knoevenagel-intramolecular cyclization cascade reaction as one of the main routes and uses salicylaldehyde and Meldrum’s acid as reagents.\textsuperscript{11,12} In recent years, it has been possible to highlight the application of potassium carbonate or sodium azide as a reaction promoter in water at room temperature,\textsuperscript{13} the use of amino acids in ethanol or water under reflux,\textsuperscript{14} or still, the using of a bifunctional heterogeneous catalyst based on primary amide in methanol.\textsuperscript{15} Several other conditions for the synthesis of coumarin-3-carboxylic acids can also be found in the literature.\textsuperscript{16–18} However, to the best of our knowledge, the application of natural polymers has not still been reported.

Chitosan is one of the most abundant polymers in nature and shows remarkable properties, such as low cost, biodegradability, biocompatibility, non-toxicity, and solubility only in dilute acid solutions.\textsuperscript{19,20} It is still distinguished for having wanted characteristics in sustainable methodologies, acting mainly in the heterogeneous catalysis of various organic reactions,\textsuperscript{21} including Knoevenagel condensation.\textsuperscript{22,23} However, when used in synthesized coumarin-3-carboxylic acids, chitosan promotes homogeneous catalysis due to the low pKa (H\textsubscript{2}O) = 4.97 of Meldrum’s
acid, which decreases the pH of the reaction medium, facilitating the solubilization of this biopolymer in water.

Although the role of chitosan in homogeneous catalysis has been little explored, it is possible to combine the efficiency of this type of catalysis with recovery and recyclability, which are explicit advantages of heterogeneous catalysis. Consequently, the reaction can be carried out without compromising the structure of the biopolymer. Furthermore, the application of chitosan in homogeneous catalysis also provides satisfactory results in short reaction time and high yield values even after recovery cycles, as highlighted by Asghari-Haji and collaborators (2016) and Lal and collaborators (2012). Thus, in continuation of efforts to develop new methodologies for the efficient synthesis of coumarin-3-carboxylic acids and investigate new biological activities, this work proposes the use of commercial chitosan as a reusable catalyst between various salicylaldehydes and Meldrum’s acid in water as a green solvent and the antimicrobial activity evaluation of the products obtained.

2 Results and discussion

2.1 Characterization of catalyst

SEM and CHN elemental analysis were performed as complementary characterization. The morphology of chitosan was observed by SEM and exhibited an irregular surface formed by a smooth, non-porous membranous phase (Fig. 1). This behavior reflects the strong hydrogen bonds among chitosan functional groups. Consequently, catalytic processes must begin with NH2 groups on the surface of the biopolymeric material. The percentages of N (7.59%), C (41.48%), and H (7.00%) were determined using elemental analysis, and the total nitrogen concentration was calculated as being equivalent to 5.42 mol g⁻¹. Utilizing the method of Sabnis and Block (1997), which employs the absorbance intensities from the infrared spectrum of chitosan in the 1655 and 3450 cm⁻¹ regions, it was possible to calculate the degree of N-deacetylation (DD = 83%) of the commercial biopolymer, which is in accordance with that reported by the supplier. Based on this result, the concentration of amino groups was calculated and corresponded to approximately 4.5 mol g⁻¹. It is worth mentioning that amino groups are essential for the progress of the coumarin-3-carboxylic acid synthesis reaction, since they are responsible for catalyzing the Knoevenagel reaction.

2.2 Catalytic activity test

To evaluate the catalytic performance of chitosan in the synthesis of coumarin-3-carboxylic acid (3a), a series of reactions were performed using salicylaldehyde (1) and meldrum acid (2) (Table 1). Initially, the reactions were carried out in the absence and presence of chitosan using water as solvent under heating (entries 1 and 2, Table 1). Interestingly, in the presence of chitosan, product 3a was obtained in only 20 minutes with a yield of 81% (entry 1, Table 1), six times faster than in the reaction without the catalyst under the same conditions (entry 2, Table 1). This result can be explained by the action of free amino groups in chitosan, which serve as essential basic groups for activating the active methylene compound.

When the reactions were performed without solvent in the absence and presence of chitosan (entries 3 and 4, Table 1), it was observed that the reactions occurred more slowly due to the
A mechanistic proposal for the production of coumarin-3-carboxylic acids using chitosan as a catalyst based on mechanisms reported in the literature is demonstrated in Fig. 2. Briefly, the reaction proceeds through a Knoevenagel condensation between 1 and 2, followed by intramolecular cyclization. The amino groups of chitosan are believed to activate 2 by deprotonating the alpha carbon of this active methylene compound, making it more nucleophilic (I). Subsequently, the formation of the Knoevenagel adduct occurs through nucleophilic attack on 1 (II) followed by dehydration (III). Through the nucleophilic attack of the hydroxyl of 1 on the carbonyl of 2, an intramolecular cyclization (IV) occurs, eliminating acetone and generating product 3a with the return of the catalyst as the final step (V). It is noteworthy that protonated chitosan solubilizes in the aqueous reaction medium, promoting efficient homogeneous catalysis. After restitution, it precipitates in the medium, allowing recovery and reuse in new reactions.

After optimizing the reaction conditions, the same protocol was applied with a variety of substituted salicylaldehydes (Table 4). It was observed that monosubstituted salicylaldehydes by either electron-donating or electron-withdrawing groups reacted efficiently to generate the desired coumarin-3-carboxylic acids (3b–g) with times of less than 30 minutes and yields greater than 81% (entries 2-7, Table 4). This methodology provided results superior to those achieved by various methods related in the literature as sustainable and eco-friendly using homogeneous catalysts and water as solvent. However, when using monosubstituted salicylaldehyde derivatives containing bulky groups (entry 8, Table 4) or disubstituted (entry 9, Table 4), the reactions proceeded slowly. In this case, their reaction showed low yields, likely due to the low solubility of these substrates in water. This behavior was also observed by Kamat and collaborators (2020), who synthesized coumarin-3-carboxylic acids under conditions close to those used in this work, however, using β-cyclodextrin as a homogeneous catalyst.

In order to improve the solubility of salicylaldehydes from entries 8 and 9 of Table 4 in the reaction medium, new tests were carried out using the binary mixture ethanol:water as solvent (Table 5). Applying the ratio 3:7 (v/v) of the binary ethanol:water mixture in both reactions (entry 1, Table 5), the total conversion of reactants into products was observed in 45 minutes, achieving yields higher than previously obtained (entries 8 and 9, Table 4). The presence of ethanol in the solvent allowed better homogenization of the systems, contributing to the enhanced reaction performance. Even increasing the amount of ethanol in the binary mixture (ethanol:water 1:1, v/v) in both reactions (entry 2, Table 5), the reactants were still observed after 60 minutes, and the yields were lower than those achieved with the binary mixture ethanol:water (3:7, v/v). This finding reinforces the preference for water as the favored medium for these reactions.

Table 2 Catalyst quantity test in the syntheses of coumarin-3-carboxylic acid (3a)a

<table>
<thead>
<tr>
<th>Entry</th>
<th>Catalyst mass (mg)</th>
<th>Timeb (min)</th>
<th>Isolated yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>20</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
<td>83</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>10</td>
<td>83</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>10</td>
<td>81</td>
</tr>
</tbody>
</table>

a Reaction conditions: 1 (0.5 mmol), 2 (0.5 mmol), H2O (2.5 mL). b The reactions were monitored by TLC until the disappearance of 1.

Table 3 Solvent test for obtaining coumarin-3-carboxylic acid (3a)a

<table>
<thead>
<tr>
<th>Entry</th>
<th>Solvent</th>
<th>Timeb (min)</th>
<th>Isolated yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Water</td>
<td>10</td>
<td>83</td>
</tr>
<tr>
<td>2</td>
<td>Ethanol:water (3:7 v/v)</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>Ethanol</td>
<td>24</td>
<td>83</td>
</tr>
<tr>
<td>4</td>
<td>THF</td>
<td>24</td>
<td>83</td>
</tr>
<tr>
<td>5</td>
<td>Toluene</td>
<td>24</td>
<td>Traces</td>
</tr>
</tbody>
</table>

a Reaction conditions: 1 (0.5 mmol), 2 (0.5 mmol), solvent (2.5 mL), chitosan (10 mg). b The reactions were monitored by TLC until the disappearance of 1. c Reaction performed under reflux. d Time in hours.
2.3 Antimicrobial activity evaluation

The in vitro antifungal and antibacterial activity of the synthesized coumarin-3-carboxylic acids (3a–i) was investigated using two strain species, four bacterial and eight fungal (Table 6). The MIC values, expressed in micrograms per milliliter compared to fluconazole and gentamicin, showed products 3a, b, f, and g were ineffective or did not present activity against all strains evaluated. Products 3c–e, h, and i tested from a concentration of 1024 μg mL^{−1} showed an inhibitory effect on the growth of the microorganisms. However, only products 3e and 3i were able to inhibit strains of A. niger and P. citrinum species with the highest MIC used (MIC = 1024 μg mL^{−1}). Product 3d presented the lowest MIC compared to the others, and, compared to gentamicin, it presented the same MIC for P. aeruginosa and E. coli strains. In relation to fluconazole, the 3d product showed similar activity for the strains C. albicans, C. tropicalis (ATCC-13803 and LM-77), and A. flavus (LM-26 and LM-55). On the other hand, the species A. niger and P. citrinum showed resistance to the product above. The minimum bacterial concentration (MBC) and minimum fungicide concentration (MFC) were determined at a concentration of 256 μg mL^{−1}, suggesting that product 3d is bactericidal and fungicidal in nature. According to the criteria of Holetz et al. (2022),31 Sartoratto et al. (2004),32 and Houghton et al. (2007),33 products 3c–e, h, and i present strong (up to 600 μg mL^{−1}) or moderate (600–1500 μg mL^{−1}) biological activity.

2.4 Catalyst recyclability studies

Although in this work chitosan acts as a homogeneous catalyst, its precipitation was achieved by washing with NaOH solution (0.4 mol L^{−1}). Therefore, the catalyst was subjected to reuse tests to verify the catalytic activity and structural integrity of the biopolymer after each synthesis cycle of coumarin-3-carboxylic acid (3a) between 1 and 2 under optimization (entry 8, Table 1). During the cycles, there were no significant losses in the isolated yield of compound 3a, as shown in Fig. 3. Furthermore, the similarity between the FT-IR spectra of the initial (a) and recovered (b) catalyst after the four cycles supports the maintenance of the polymer structure after recovery and reuse (Fig. 4). No additional bands were observed in the spectrum of the catalyst after recovery, mainly from product 3a (c), attesting to the efficiency of the washings to remove any residual substance impregnated in the catalyst completely. Comparing the ^1H NMR spectra of chitosan solubilized in the binary mixture D_{2}O: CD_{3}COOD (1:1, v/v) before (a) and after reuse (b), the appearance of new signals after recycling was also not observed, indicating once again the retention of the polymeric structure and the efficiency of the treatment after recovery Fig. 5.
Table 4  Application of substituted salicylaldehydes to obtain various coumarin-3-carboxylic acids

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>Product</th>
<th>Time\textsuperscript{b} (min)</th>
<th>Isolated yield (%)</th>
<th>M.p. [°C] this work/[lit]\textsuperscript{ref.}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H</td>
<td><img src="image" alt="3a" /></td>
<td>10</td>
<td>83</td>
<td>188–190 [189–191]\textsuperscript{13}</td>
</tr>
<tr>
<td>2</td>
<td>5-OCH\textsubscript{3}</td>
<td><img src="image" alt="3b" /></td>
<td>10</td>
<td>88</td>
<td>198–201 [198–200]\textsuperscript{13}</td>
</tr>
<tr>
<td>3</td>
<td>3-OCH\textsubscript{3}</td>
<td><img src="image" alt="3c" /></td>
<td>15</td>
<td>82</td>
<td>214–215 [216–217]\textsuperscript{16}</td>
</tr>
<tr>
<td>4</td>
<td>5-Br</td>
<td><img src="image" alt="3d" /></td>
<td>30</td>
<td>81</td>
<td>189–192 [193–195]\textsuperscript{13}</td>
</tr>
<tr>
<td>5</td>
<td>5-NO\textsubscript{2}</td>
<td><img src="image" alt="3e" /></td>
<td>20</td>
<td>82</td>
<td>227–229 [232–234]\textsuperscript{13}</td>
</tr>
<tr>
<td>6</td>
<td>5-Cl</td>
<td><img src="image" alt="3f" /></td>
<td>15</td>
<td>81</td>
<td>123–122 [122–123]\textsuperscript{13}</td>
</tr>
<tr>
<td>7</td>
<td>4-OH</td>
<td><img src="image" alt="3g" /></td>
<td>15</td>
<td>88</td>
<td>264–265 [260–262]\textsuperscript{13}</td>
</tr>
<tr>
<td>8</td>
<td>4-N(CH\textsubscript{2}CH\textsubscript{3})\textsubscript{2}</td>
<td><img src="image" alt="3h" /></td>
<td>120\textsuperscript{c}</td>
<td>74</td>
<td>224–226 [224–225]\textsuperscript{14}</td>
</tr>
<tr>
<td>9</td>
<td>4,6-OCH\textsubscript{3}</td>
<td><img src="image" alt="3i" /></td>
<td>120\textsuperscript{c}</td>
<td>33</td>
<td>232–234 [234–236]\textsuperscript{16}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reaction conditions: salicylaldehyde (0.5 mmol), 2 (0.5 mmol), solvent (2.5 mL), chitosan (10 mg). \textsuperscript{b} The reactions were monitored by TLC until the disappearance of salicylaldehyde. \textsuperscript{c} Interrupted reactions.
3 Conclusions

This study concludes that it is possible to obtain a variety of coumarin-3-carboxylic acids in short times (10–45 min) and good isolated yields (77–88%) through a one-pot reaction between various salicylaldehydes and Meldrum’s acids in water or ethanol/water (3:7, v/v) at 75 °C and using commercial chitosan as a recyclable catalyst. Although, in this case, chitosan promotes homogeneous catalysis, the protocol developed allows its recovery and reuse four times in a simple and efficient way (approx. 82%), guaranteeing the combination of advantages of homogeneous and heterogeneous catalysis. Furthermore, the moderate reaction conditions, the use of water as a green solvent, and the use of chitosan as a recyclable, biodegradable, and commercially available catalyst ensure advantages for the developed method, configuring it as an attractive proposal and ecologically friendly for further organic reactions. In the evaluation of antimicrobial
activity, the coumarin-3-carboxylic acids 3c–e, h, and i tested showed an inhibitory effect on the growth of microorganisms. Product 3d had the lowest MIC against fungal or bacterial strains, suggesting a bactericidal and fungicidal nature.

4 Experimental section

4.1 General information

Low molecular weight chitosan biopolymer with a deacetylation degree in the range of 75–85% was purchased from Sigma-Aldrich. The NaOH and Na$_2$SO$_4$ used were obtained from Alphatec. The aldehydes and Meldrum’s acid used for coumarin-3-carboxylic acids synthesis were purchased from Sigma-Aldrich. The solvents ethanol (200 proof), toluene, and tetrahydrofuran of analytical grade were obtained from Tedia. The ethanol/water mixtures were prepared in volumetric proportions (3:7, v/v). All chemicals were used without additional purification processes.

FT-IR spectra were recorded using KBr pellet technique in the range 400–4000 cm$^{-1}$ on a Shimadzu spectrophotometer model IRPRESTIGE-21. The scanning electron microscopy (SEM) was performed in FESEM JOEL JSM-7401F at 5.0 kV after the gold metallation step. The elemental analysis of carbon, hydrogen, and nitrogen (CHN) was performed on a PerkinElmer model 2400 series ii elemental microanalyzer. The nuclear magnetic resonance (NMR) spectra were accomplished in DMSO-$d_6$ or binary mixture D$_2$O:CD$_3$COOD (1:1, v/v) on a VARIAN mercury 500 MHz/125 MHz and 400 MHz/100 MHz. Chemical shifts ($\delta$) were reported in ppm, downfield from internal TMS standard. Precoated silica gel (E. Merck Kiesegel 60F254, layer thickness 0.25 mm) was applied for the thin layer chromatography (TLC). Melting points (M.p.) were determined in open capillaries in Fisatom model 431 and are uncorrected. Details and discussions of Fourier transform infrared spectroscopy (FT-IR), X-ray powder diffraction (XRD), thermogravimetric analysis (TGA), and differential thermal analysis (DTA) of the commercial chitosan can be found in our previous work (Abrantes and collaborators, 2022).$^{34}$

The microorganisms used belong to the MICOTECa of the Mycology Laboratory, Department of Pharmaceutical Sciences (DCF), Health Sciences Center (CCS) of the Federal University of Paraíba (UFPB). Samples of fungal species were kept in ASD at a temperature of 4 °C (Refrigerator). Fluconazole and gentamycin (Sigma-Aldrich/Merck KGaA) were the antifungals used as controls.

4.2 General procedure for coumarin-3-carboxylic acids synthesis using chitosan

In a round bottom flask, deionized water (2.5 mL), 1 (0.5 mmol), chitosan (10 mg), and 2 (0.5 mmol) were heated to 75 °C under magnetic stirring. The reaction was monitored by TLC using hexane:ethyl acetate mixture (7:3, v/v) as eluent until the disappearance of 1. After completion of the reaction, the flask was uncovered to evaporate the solvent and ethyl acetate or methanol was added to the system while still being heated, allowing the solubilization of the product and the separation of solid chitosan by filtration under reduced pressure. The solubilized product was dried in anhydrous Na$_2$SO$_4$ and recrystallized in the solvent used previously. The chitosan was washed three times with a NaOH solution (0.4 mol L$^{-1}$) to ensure total precipitation and removal of product residues impregnated on its surface, deionized water, and ethanol. I was subsequently filtered and dried under reduced pressure for 4 hours for future use. The product was characterized by $^1$H-NMR and $^{13}$C-NMR.

4.3 Antimicrobial activity of coumarin-3-carboxylic acids

For biological activity assays, 12 microbial species were used, four strains of bacteria and eight strains of fungi: Staphylococcus aureus ATCC-13150, Staphylococcus epidermidis ATCC-12228,
To prepare the inoculum, colonies obtained from cultures of bacterial strains in BHI medium and fungi in ASD medium were suspended in a sterile 0.9% physiological solution and adjusted according to the 0.5 tube on the Mc Farland standard scale to obtain 10⁶ CFU mL⁻¹. Antimicrobial activity assays were conducted according to the protocols of Hadacek and Greger (2000).37,38 The determination of the minimum inhibitory concentration (MIC) of samples on bacterial and fungal strains was carried out using the broth microdilution technique with a cell culture plate (TPP/SWITZERLAND/EUROPA) containing 96 wells with a “U” bottom. Initially, 100 μL of double-concentrated RPMI 1640 broth was distributed into the wells of the microdilution plates. Then, 100 μL of the solubilized molecules were dispensed into the wells in the first row of the plate. Through serial dilution at a ratio of two, concentrations of 1024 up to 2 μg mL⁻¹ were obtained. Finally, 10 μL of the fungal species suspensions were added to the wells, where each column of the plate specifically refers to a species. The prepared plates were aseptically closed and subjected to incubation at 35 ± 2 °C for 24–48 hours for yeast assays and at RT (28–30 °C)/5–7 days for filamentous fungi.

The product was considered active when it inhibited at least 50% of the microorganisms used in biological activity tests.37,39 The MIC was considered and interpreted as active or inactive, according to the following criteria: up to 600 μg mL⁻¹ = strong activity; 600–1500 μg mL⁻¹ = moderate activity; > above 1500 μg mL⁻¹ = weak activity or inactive product.31–33

Data availability

Data availability is not applicable to this article. No primary research results, software or code have been included and no new data were generated or analysed as part of this review. The authors confirm that the data supporting the findings of this study are available within the article and it ESI.†

Conflicts of interest

There are no conflicts to declare.

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