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### Microfluidic reactor for lipase-catalyzed regioselective synthesis of neohesperidin ester derivatives and their antimicrobial activity research

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#### 1. Introduction

Recently, microreactor technology has received a great deal of attention [1–3]. A key feature of such devices is their increased surface area-to-volume ratio which is a direct result of their decrease in physical size. Specific surfaces of microstructured devices can be as high as 50 000 m<sup>2</sup> m<sup>-3</sup> whereas conventional laboratory apparatus does not usually exceed 1000 m<sup>2</sup> m<sup>-3</sup>. A consequence of this increase in specific surface is the enhancement of mass and heat transport in the system. The advantages of microreactors have been substantiated by a growing number of examples over the past decade [4–11]. In recent years, there is an increasing interest for the enzymatic synthesis in microreactors [12–22].

Neohesperidin are natural polyhydroxylated diphenylpyrane derivatives that are widely distributed among various plants. In recent years, many studies have been published describing their properties. In particular, flavonoids exhibit strong antioxidant [23,24], anti-inflammatory [25], anti-tumoral [26,27] and antiviral

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#### ABSTRACT

Lipase-catalyzed regioselective synthesis of neohesperidin ester derivatives was performed by Lipase TL IM from *Thermomyces lanuginosus* in a continuous-flow microreactor and then their antimicrobial activity was studied. It appears that neohesperidin, neohesperidin dihydrochalcone with primary OH on the sugar part is the most reactive substrate. Various reaction parameters were investigated including substrate molar ratio, reaction time and temperature. Maximum conversion (92%) was obtained under the optimal condition of substrate molar ratio of 8:1 (vinyl esters: neohesperidin) at 52 °C for about 35 min. Then, the antibacterial activity of modified neohesperidin ester derivatives was examined and showed great improvement against gram negative and gram positive bacteria.

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activities [28,29]. The use of flavonoids in numerous cosmetic and pharmaceutic formulations seems very attractive. Unfortunately, the development of such products is seriously limited due to their poor solubility in apolar media like oils. Therefore, the acylation or the glycosylation of these molecules can be used as a tool to improve their properties. These reactions can be performed either chemically or enzymatically. However, due to the polyhydroxylated nature of these molecules, the enzymatic approach is more selective [30]. Both lipase and protease have been used to the enzymatic acylation of flavonoids. However, some of these methods always need longer reation time and the conversion yields were not ideal for some specific substrates [31–33].

So we report here, for the first time, microfluidic reactor for lipase-catalyzed regioselective synthesis of neohesperidin ester derivatives (Scheme 1) and then their antimicrobial activity were studied too. The aim of this paper is to investigate, under a continuous-flow microreactor, the effect of the flavonoid structure (hesperidin, neohesperidin, neohesperidin dihydrochalcone) and the carbon-chain length of the fatty acids (C2, C12 and C16) on the flavonoid acylation performance. Then we further studied the antimicrobial activity of neohesperidin ester derivatives with different chain lengths compared with the neohesperidin.







Scheme 1. Lipase-catalyzed regioselective acylation of hesperidin, neohesperidin and neohesperidin dihydrochalcone in microreactors.

#### 2. Results and discussion

#### 2.1. Experimental setup

The enzymatic synthesis of flavonoid esters was performed in microreactor. The equipment configuration that was used for the enzymatic synthesis of flavonoid esters reactions starting from neohesperidin and vinyl carboxylate is described in Fig. 1. Harvard Apparatus PHD 2000 syringe pumps were used to deliver reagents from syringes to the reactor. On the syringe pump, a 10 mL syringe with the neohesperidin solution and a 10 mL syringe with vinyl carboxylate in 2-methyl-2-butanol were mounted. Lipozyme TL IM were filled in silica gel tubing (inner diameter ID = 2.0 mm, length = 1 m). The reaction temperature was controlled by water



Fig. 1. Microreactor setup for the continuous-flow synthesis of neohesperidin ester catalyzed by Lipozyme TL IM.

bath, which was to immerse the microchannel in water bath and adjust the temperature of the reaction by controlling the temperature of the water bath. Streams 1 and 2 were mixed together at a flow rate of 8.9  $\mu$ L min<sup>-1</sup> in a Y-mixer at 52 °C and the resulting stream (17.8  $\mu$ L min<sup>-1</sup>) was connected to a sample vial which was used to collect the final mixture.

#### 2.2. Substrate specificity of flavonoids

The effect of flavonoid structure (Fig. 2) on the performance of this reaction was investigated using quercetin, hesperidin, neohesperidin and neohesperidin dihydrochalcone with vinyl laurate as an acyl donor.

Fig. 3. summarises the conversion yields of flavonoids obtained for all substrates studied. These results indicate that neohesperidin was the most reactive substrate. In fact, conversion yields were of about of 92, 80 and 5%, respectively, for neohesperidin, neohesperidin dihydrochalcone and hesperidin. For an aglycon flavonoid, such as quercetin, no product was observed. The high conversion yield obtained with neohesperidin can be explained by the presence of the primary OH sugar on this molecule which is more reactive than the secondary OH group of hesperidin.

#### 2.3. Acyl donor structure effect

The effect of the carbon-chain length of the acyl donors on the performance of the flavonoids acylation was demonstrated using fatty acid vinyl esters with carbon number varying from 2, 12 to 16 as acyl donor and neohesperidin as flavonoid. The results obtained (Fig. 4) show that the conversion yields are affected by the chain length of acyl donors. The lowest conversion yield was obtained with vinyl acetate (60%). For higher chain length (C12 and C16), no significant difference was observed. The conversion yield was to 92% and 88%, respectively, for vinyl laurate and vinyl palmitate.

#### 2.4. Molar ratio (acyl donor/neohesperidin) effect

The effect of molar ratio (acyl donor/neohesperidin) on the performance of the flavonoids acylation was investigated from 1:1 to 12:1. Fig. 5 shows the strong effect of this parameter on the enzymatic synthesis of 6"-O-lauroyl-neohesperidin. As for the long chain acyl donor such as vinyl laurate and vinyl palmitate, the best result can be obtained with the ratio of acyl donor/ neohesperidin = 8:1. As for the short chain acyl donor such as vinyl



Fig. 3. Flavonoid structure effect on the acylation performance with vinyl laurate carried out in microreactors using from Thermomyces lanuginosus lipase.



Fig. 4. The effect of carbon-chain length on the acylation performance of neohesperidin carried out in microreactors using from Thermomyces lanuginosus lipase.

acylate, the best result can be obtained with the ratio of acyl donor/ neohesperidin = 21:1.



Neohesperidin

Neohesperidin dihydrochalcone

Fig. 2. Structures of different flavonoid derivatives.



Fig. 5. The influence of vinyl laurate/neohesperidin on the conversion of 6"-O-lauroylneohesperidin catalysed by Lipozyme TL IM from *Thermomyces lanuginosus* in a flow microreactor.

#### 2.5. Reaction time/flow rate effect

The influence of reaction time/flow rate on the conversion of 6"-O-lauroyl- neohesperidin was also studied. Fig. 6 shows that the best conversion and regioselectivity of 6"-O-lauroyl-neohesperidin was observed for a residence time of 35 min and at a flow rate of 17.8  $\mu$ L min<sup>-1</sup>.

Finally, to explore the scope and limitations of this new highly efficient neohesperidin ester synthesis, hesperidin **1a**, neohesperidin **1b** and neohesperidin dihydrochalcone **1c**, 3 vinyl esters **2a-c** were subjected to the general reaction conditions, both using a single-mode shaker reactor and a continuous flow/microreactor processing. For the shaker experiments, reaction times always need to be about 24 h or even more to obtain ideal conversion (Method A). Using lipase-catalyzed regioselective acylation of neohesperidin under continuous-flow conditions, 6 flavonoid monoesters were synthesized in parallel in a single experiment at the same flow rate (Method B, C), the results were better with the flow/microreactor processing than with the single-mode shaker (Table 1, entry 1–6).



Fig. 6. The influence of reaction time on the conversion of 6"-O-lauroyl-neohesperidin catalysed by Lipozyme TL IM from *Thermomyces lanuginosus* in a flow microreactor.

Importantly, applying continuous flow/microreactor processing, a conversion of 90% or even more to 6"-monoester can be obtained, with negligible formation of diesters. This allows us to control the production of 6"-monoester and simplify the purification of products.

#### 2.6. In vitro antibacterial activity

The antibacterial test results are presented in Table 2. The compounds **3d**, **3e** and **3f** exhibited their excellent antibacterial activity, especially against *Escherichia coli*. While the products **3a**, **3b** and **3c** were active only against Gram negative *Escherichia coli*. The result revealed that the compounds **3a-f** showed an increased antibacterial activity and the antibacterial activity of the derivatives can be attributed to the their higher lipophilicity, which could increase their ability to interact with the cell membrane or even to penetrate throw the cell membrane.

#### 3. Conclusion

In conclusion, we have developed a new method of microfluidic reactor for lipase-catalyzed regioselective synthesis of neohesperidin ester derivatives and further studied the antibacterial activity of acylated modified neohesperidin ester derivatives. Compared with neohesperidin, the modified neohesperidin ester derivatives showed an increased antibacterial activity especially against Escherichia coli. The salient features of this microfluidic reactor synthesis of neohesperidin ester derivatives include mild reaction conditions (52 °C), fast reaction times (35 min), high yields as well as high regioselectivities that make our methodology a valuable contribution to the field of neohesperidin monoester synthesis. The method of enzymatic synthesis in a microreactor environment described here provides us a new way of thinking in organic synthesis, and it may have more applications to synthetic organic chemistry catalyzed by enzyme in the future.

 
 Table 1

 Shaker and continuous flow synthesis of flavonoid monoesters catalysed by Lipozyme TL IM.

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Entry	Product <sup>a</sup>	Method <sup>b</sup>	t	Yield [%] <sup>c</sup>	Regioselectivity [%] <sup>d</sup>		
1	3a	А	24 h	55	85		
		В	35 min	60	100		
2	3b	А	24 h	83	95		
		С	35 min	92	100		
3	3c	А	24 h	80	92		
		С	35 min	88	99		
4	3d	Α	24 h	50	82		
		В	35 min	58	100		
5	3e	Α	24 h	72	90		
		С	35 min	86	99		
6	3f	Α	24 h	78	92		
		С	35 min	84	99		

<sup>a</sup> Reactions and the structure of the products **3a-3f** see Scheme 1.

<sup>b</sup> Method A: Shaker reactor, 2-methyl-2-butanol/DMSO = 4:1 (4 mL), 0.17 g Lipozyme TL IM (43 mg mL<sup>-1</sup>), 24 h. Method B: continuous flow microreactor, 8.91  $\mu$ L min<sup>-1</sup> feed **1** (0.05 M solution of neohesperidin in 2-methyl -2-butanol/DMSO = 4:1) and 8.91  $\mu$ L min<sup>-1</sup> feed **2** (1.03 M solution of vinyl acetate in 2-methyl -2-butanol) at 52 °C (residence time 35 min), Lipozyme TL IM 0.87 g. Method C: It is similar to method B except the feed 2 is 0.39 M solution of vinyl palmitate/vinyl laurate in 2-methyl -2-butanol.

<sup>c</sup> Isolated yield.

<sup>d</sup> Corresponding to the amount of 6"-O-neohesperidin ester to the total amount of neohesperidin esters.

Table 2
Antibacterial activity of the compounds 1b, 1c, 3a-3f using agar diffusion method. <sup>b</sup>

strain	Concentration (mg ml <sup>-1</sup> )	Zone of §	Zone of growth inhibition (mm) <sup>a</sup>								
		1b	3a	3b	3c	1c	3d	3e	3f	AMP	
E. coli S. aureus	50	24.2	26.0	28.1	29.5	26.3 11.2	29.0 12.5	30.1 13.7	32.0 15.0	31.0 11.8	
E. coli S. aureus	25	21.0	23.2	24.6 -	26.0 -	24.0 9.0	24.5 10.5	26.0 11.0	29.0 12.6	30.8 12.0	
E. coli S. aureus	12.5	17.4 -	19.0 -	21.0 -	22.9 -	19.2 8.0	20.0 8.9	21.3 10.0	23.0 11.5	31.2 12.3	

<sup>a</sup> The diameters of the distinctly clear zones were measured using a metric ruler. All experiments were run in triplicate, "-" no effect.

<sup>b</sup> Agar diffusion method: the microbial culture containing indicator bacteria was transferred in a sterile Petri dish. The Oxford cup was vertically placed on culture medium, and were filled with 50 μl compounds, dimethyl sulfoxide (DMSO) solvent was used as negative control, ampicillin sodium (4 mg/mL) as the positive control group. After the plates were incubated at 37 °C for 24 h.

#### 4. Experimental

#### 4.1. General methods

Unless otherwise stated, all chemicals and solvents were obtained from commercial sources and used without further purification. Lipozyme TL IM from Thermomyces lanuginosus was purchased from Novo Nordisk. Vinyl palmitate and vinyl laurate were purchased from Aldrich. Hesperidin (Sigma), neohesperidin (Sigma), neohesperidin dihydrochalcone (Fluka) were used as flavonoid substrates. Harvard apparatus PHD 2000 syringe pumps were purchased from Harvard apparatus. Analytical TLC was performed on silica gel 60 plates (Merck) using ethyl acetate/methanol/ $H_2O$  (25:3.6:1, by vol) as eluent. Spots were detected by  $I_2$  and ultraviolet irradiation at 254 nm. The reaction was monitored by HPLC analysis using a 4.6  $\times$  200 mm, 5  $\mu$  Kromasil ODS column with a gradient of methanol/water. For the analysis of neohesperidin esters of lauric acid, methanol/water 90:10 (v/v) was used as mobile phase (flow rate 1.0 mL  $min^{-1}$ ), and for esters of palmitic acid methanol/water 95:5 (v/v) was used (flow rate, 1.0 mL min<sup>-1</sup>). Flavonoid and flavonoid ester were detected at 285 nm. The conversion vield was defined as the ratio between the molar concentration of flavonoid ester and the initial molar concentration of the flavonoid used. After purification of the synthesized products by column chromatography, the chemical structures of flavonoid esters were determined by <sup>1</sup>H NMR and ESI-MS. <sup>1</sup>H NMR spectra were recorded on 500 MHz NMR spectrometer with DMSO-d as solvent. Data for <sup>1</sup>H NMR are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, dd = doublet of doublet, t = triplet, m = multiplet) and coupling constants in Hertz (Hz).

#### 4.2. Synthesis

# 4.2.1. General procedure for neohesperidin esters synthesis under shaker conditions

**Method A:** neohesperidin (0.1 mmol) was added to 4 mL of solvent (2-methyl-2-butanol/DMSO = 4:1). The biocatalyst lipozyme TL IM (45 mg mL<sup>-1</sup>, 0.18 g) was then added and the suspension maintained at 52 °C for 24 h under shaker conditions. Vinyl laurate (0.8 mmol, 0.26 g) was then added. The reactions were performed in the presence of 3 Å molecular sieves. Aliquots were withdrawn at different times, analyzed by TLC and HPLC. When the conversion of neohesperidin to neohesperidin monopalmitate reached the maximum value (determined by TLC and HPLC), the mixture was cooled and filtered. The tert-amyl alcohol was evaporated under reduced pressure, and the oily residue was submitted to column chromatography on silica gel (200–300 mesh). The products were eluted with a gradient of ethyl acetate/methanol/ $H_2O$  (25:2.5:0.6, by vol). The purification was monitored by TLC. The

fractions containing the main products were pooled, the solvent evaporated, and the residue analyzed by <sup>1</sup>H NMR.

# 4.2.2. General procedure for 6"-O-ethanoyl-neohesperidin (**3a**) and 6"-O-ethanoyl-neohesperidin dihydrochalcone (**3d**) synthesis in continuous flow microreactors

**Method B:** 0.49 mmol of the neohesperidin was dissolved in 10 mL 2-methyl -2-butanol/DMSO = 4:1 (feed 1, ~0.049 M) and 10.29 mmol vinyl acetate were dissolved in 10 mL 2-methyl -2-butanol (feed 2; ~1.03 M). Lipozyme TL IM (0.87 g) were filled in silica gel tubing (inner diameter ID = 2.0 mm, length = 1m). Streams **1** and **2** were mixed together at a flow rate of. 8.91  $\mu$ L min<sup>-1</sup> in a Y-mixer at 52 °C and the resulting stream (17.82  $\mu$ L min<sup>-1</sup>) was connected to a sample vial which was used to collect the final mixture. The final mixture was then evaporated, and the oily residue was submitted to column chromatography on silica gel (200–300 mesh). The products were eluted with a gradient of ethyl acetate/methanol/H<sub>2</sub>O (25:2.5:0.6, by vol). The purification was monitored by TLC. The fractions containing the main products were pooled, the solvent evaporated, and the residue analyzed by <sup>1</sup>H NMR.

4.2.2.1. 6"-O-ethanoyl-neohesperidin (**3a**). **R**<sub>f</sub>: 0.35. <sup>1</sup>**H-NMR** (**DMSO-d**<sub>6</sub>, **500 MHz**, **ppm**):  $\delta$  12.03 (s, 1H, -OH<sub>5</sub>), 9.12 (s, 1H, -OH<sub>3'</sub>), 6.95 (s, 1H, H<sub>5'</sub>), 6.92 (d, 1H, *J* = 2.9 Hz, H<sub>6</sub>'), 6.87 (m, 1H, H<sub>2'</sub>), 6.10 (d, 1H, *J* = 2.2 Hz, H<sub>8</sub>), 6.06 (d, 1H, *J* = 2.2 Hz, H<sub>6</sub>), 5.49 (m, 1H, H<sub>2</sub>), 5.44 (m, 1H, H<sub>1"</sub>), 5.41 (m, 1H, 2"'-OH of neohesperidin), 5.16 (m, 1H, H<sub>1"''</sub>), 5.10 (s, 1H, 4"'-OH of neohesperidin), 4.74 (d, 1H, *J* = 4.8 Hz, 4"-OH of neohesperidin), 4.69 (d, 1H, *J* = 4.4 Hz, 3"-OH of neohesperidin), 4.50 (d, 1H, *J* = 5.8 Hz, 3"'-OH of neohesperidin), 4.26 (d, 1H, *J* = 11.9 Hz, H<sub>6"</sub> acylated), 4.03 (m, 1H, H<sub>6"</sub> acylated), 3.77 (s, 3H of -OCH<sub>3</sub> of phenyl), 3.73–3.14 (m, 8H, H of rhamnoglucosyl), 3.22 (m, 1H, H<sub>3</sub>), 2.73 (d, 1H, *J* = 3.2 Hz, H<sub>3</sub>), 1.94 (s, 3H, -COCH<sub>3</sub>), 1.17 (m, 3H of CH<sub>3</sub> of rhamnosyl). **IR (KBr, cm<sup>-1</sup>)**: 3408 (OH), 1736 (C=O), 1640 (C=C); **ESI-MS (m/z)**: 688 (M<sub>1</sub>+2H<sub>2</sub>O-H)<sup>-</sup>, M<sub>1</sub> corresponding exactly to the molecular weight of 6"-O-ethanoylneohesperidin.

4.2.2.2. 6"-O-ethanoyl-neohesperidin dihydrochalcone (**3d**). R<sub>f</sub>: 0.25. <sup>1</sup>**H-NMR (DMSO-d<sub>6</sub>, 500 MHz, ppm**):  $\delta$  12.30 (s, 1H, -OH<sub>5</sub>), 8.80 (s, 1H, -OH<sub>3'</sub>), 6.78 (d, 1H, J = 10.5 Hz, H<sub>5'</sub>), 6.64 (d, 1H, J = 2.5 Hz, H<sub>6'</sub>), 6.60 (m, 1H, H<sub>2'</sub>), 5.98 (s, 2H, H<sub>6</sub> and H<sub>8</sub>), 5.43 (m, 1H, H<sub>1"</sub>), 5.40 (m, 1H, 2<sup>'''</sup>-OH of neohesperidin dihydrochalcone), 5.10 (m, 1H, H<sub>1"</sub>), 5.06 (d, 1H, J = 5.1 Hz, 4<sup>'''</sup>-OH of neohesperidin dihydrochalcone), 4.70 (d, 1H, J = 4.8 Hz, 4<sup>'''</sup>-OH of neohesperidin dihydrochalcone), 4.68 (d, 1H, J = 5.8 Hz, 3<sup>'''</sup>-OH of neohesperidin dihydrochalcone), 4.24 (d, 1H, J = 12.9 Hz, H<sub>6"</sub> acylated), 4.05 (m, 1H, H<sub>6"</sub> acylated), 3.71 (s, 3H of -OCH<sub>3</sub> of phenyl), 3.68–3.15 (m, 8H, H of rhamnoglucosyl), 3.22 (m, 2H, H<sub>3</sub>), 2.71 (t, 2H, J = 7.7 Hz, H<sub>2</sub>), 2.00 (m, 3H, -COCH<sub>3</sub>), 1.20 (m, 3H of CH<sub>3</sub> of rhamnosyl); **IR (KBr, cm<sup>-1</sup>)**: 3408 (OH), 1736 (C=O), 1640 (C=C). **ESI-MS (m/z)**: 690 (M<sub>1</sub>+2H<sub>2</sub>O-H)<sup>-</sup>, M<sub>1</sub> corresponding exactly to the molecular weight of 6″-O-ethanoyl- neohesperidin dihydrochalcone.

#### 4.2.3. General procedure for 6"-O-lauroyl-neohesperidin (3b), 6"-O-palmitoyl- neohesperidin (3c), 6"-O-lauroyl-neohesperidin dihydrochalcone (3e) and 6"-O-palmitoyl-neohesperidin dihydrochalcone (3f) synthesis in continuous flow microreactors

**Method C:** 0.49 mmol of the neohesperidin was dissolved in 10 mL 2-methyl -2-butanol/DMSO = 4:1 (feed **1**, ~0.049 M) and 3.92 mmol vinyl laurate or vinyl palmitate were dissolved in 10 mL 2-methyl-2-butanol (feed **2**; ~0.39 M). Lipozyme TL IM (0.87 g) were filled in silica gel tubing (inner diameter ID = 2.0 mm, length = 1 m). The next reaction procedure is similar to Method B.

4.2.3.1. 6"-O-lauroyl-neohesperidin (**3b**). **R**<sub>f</sub>: 0.45. <sup>1</sup>**H-NMR (DMSOd**<sub>6</sub>, **500 MHz, ppm**):  $\delta$  12.03 (s, 1H, H<sub>5</sub>), 9.11 (s, 1H, -OH<sub>3'</sub>), 6.93 (s, 1H, -OH<sub>5'</sub>), 6.92 (d, 1H, J = 2.9 Hz, H<sub>6'</sub>), 6.87 (m, 1H, H<sub>2'</sub>), 6.10 (d, 1H, J = 2.2 Hz, H<sub>8</sub>), 6.06 (d, 1H, J = 2.2 Hz, H<sub>6</sub>), 5.49 (m, 1H, H<sub>2</sub>), 5.44 (m, 1H, H<sub>1"</sub>), 5.41 (m, 1H, 2<sup>'''</sup>-OH of neohesperidin),5.16 (m, 1H, H<sub>1"''</sub>), 5.10 (s, 1H, 4<sup>'''</sup>-OH of neohesperidin), 4.74 (d, 1H, J = 4.8 Hz, 4<sup>''</sup>-OH of neohesperidin), 4.69 (d, 1H, J = 4.4 Hz, 3<sup>''</sup>-OH of neohesperidin), 4.50 (d, 1H, J = 5.8 Hz, 3<sup>'''</sup>-OH of neohesperidin), 4.26 (d, 1H, J = 11.9 Hz, H<sub>6"</sub> acylated), 4.03 (m, 1H, H<sub>6"</sub> acylated), 3.77 (s, 3H of -OCH<sub>3</sub> of phenyl), 3.73–3.14 (m, 8H, H of rhamnoglucosyl), 3.22 (m, 1H, H<sub>3</sub>), 2.73 (d, 1H, J = 3.2 Hz, H<sub>3</sub>), 2.22 (m, 2H, -CH<sub>2</sub>-CO-), 1.17 (m, 3H of CH<sub>3</sub> of rhamnosyl), 1.26 (m, 18H of (CH<sub>2</sub>)<sub>9</sub> of lauroyl), 0.85 (t, 3H, J = 7.0 Hz, CH<sub>3</sub>). **IR (KBr, cm<sup>-1</sup>)**: 3415 (OH), 1738 (C=O), 1647 (C=C). **ESI-MS (m/z)**: 828 (M<sub>1</sub>+2H<sub>2</sub>O-H)<sup>-</sup>, M<sub>1</sub> corresponding exactly to the molecular weight of 6"-O- lauroyl-neohesperidin.

4.2.3.2. 6"-O-palmitoyl-neohesperidin (3c). R<sub>f</sub>: 0.48. <sup>1</sup>H-NMR **(DMSO-***d*<sub>6</sub>, **500 MHz**, **ppm**): δ 12.04 (s, 1H, -OH<sub>5</sub>), 9.10 (s, 1H, -OH<sub>3'</sub>), 6.92 (s, 1H, H<sub>5'</sub>), 6.91 (d, 1H, J = 2.9 Hz, H<sub>6'</sub>), 6.87 (m, 1H, H<sub>2'</sub>), 6.10 (d,  $1H, J = 2.2 Hz, H_8), 6.06 (d, 1H, J = 2.2 Hz, H_6), 5.49 (m, 1H, H_2), 5.48$ (m, 1H, H<sub>1"</sub>), 5.46 (m, 1H, 2<sup>"'</sup>-OH of neohesperidin), 5.15 (m, 1H,  $H_{1'''}$ ), 5.10 (s, 1H, 4'''-OH of neohesperidin), 4.74 (d, 1H, J = 4.8 Hz, 4"-OH of neohesperidin), 4.68 (d, 1H, J = 4.4 Hz, 3"-OH of neohesperidin), 4.30 (d, 1H, J = 5.8 Hz, 3'''-OH of neohesperidin), 4.28 3H of -OCH<sub>3</sub> of phenyl), 3.73–3.15 (m, 8H, H of rhamnoglucosyl), 3.22 (m, 1H, H<sub>3</sub>), 2.73 (d, 1H, J = 3.2 Hz, H<sub>3</sub>), 2.22 (m, 2H, -CH<sub>2</sub>-CO-), 1.17 (m, 3H of CH<sub>3</sub> of rhamnosyl), 1.23 (m, 26H of (CH<sub>2</sub>)<sub>13</sub> of palmitoyl), 0.85 (t, 3H, J = 7.0 Hz, CH<sub>3</sub>). **IR (KBr, cm<sup>-1</sup>)**: 3415 (OH), 1740 (C=O), 1643 (C=C). ESI-MS (m/z): 884 (M<sub>1</sub>+2H<sub>2</sub>O-H)<sup>-</sup>, M<sub>1</sub> corresponding exactly to the molecular weight of 6"-O- palmitoylneohesperidin.

4.2.3.3. 6"-O-lauroyl-neohesperidin dihydrochalcone (**3e**). **R**<sub>f</sub>: 0.40. **<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 500 MHz, ppm)**:  $\delta$  12.33 (s, 1H, -OH<sub>5</sub>), 8.80 (s, 1H, -OH<sub>3'</sub>), 6.79 (d, 1H, *J* = 8.3 Hz, H<sub>5'</sub>), 6.65 (d, 1H, *J* = 1.9 Hz, H<sub>6'</sub>), 6.58 (m, 1H, H<sub>2'</sub>), 5.99 (s, 2H, H<sub>6</sub> and H<sub>8</sub>), 5.44 (m, 1H, H<sub>1"</sub>), 5.39 (m, 1H, 2"'-OH of neohesperidin dihydrochalcone), 5.09 (m, 1H, H<sub>1"</sub>), 5.06 (d, 1H, *J* = 5.1 Hz, 4"'-OH of neohesperidin dihydrochalcone), 4.70 (d, 1H, *J* = 4.9 Hz, 4"'-OH of neohesperidin dihydrochalcone), 4.68 (d, 1H, *J* = 4.4 Hz, 3"-OH of neohesperidin dihydrochalcone), 4.50 (d, 1H, *J* = 5.8 Hz, 3"''-OH of neohesperidin dihydrochalcone), 4.52 (d, 1H, *J* = 12.9 Hz, H<sub>6"</sub> acylated), 4.10 (m, 1H, H<sub>6"</sub> acylated), 3.72 (s, 3H of -OCH<sub>3</sub> of phenyl), 3.67–3.16 (m, 8H, H of rhamnoglucosyl), 3.22 (m, 2H, H<sub>3</sub>), 2.73 (t, 2H, *J* = 7.7 Hz, H<sub>2</sub>), 2.27 (m, 2H, -CH<sub>2</sub>-CO-), 1.26 (m, 18H of (CH<sub>2</sub>)<sub>9</sub> of lauroyl), 1.20 (m, 3H of CH<sub>3</sub> of rhamnosyl), 0.83 (t, 3H, *J* = 7.0 Hz, CH<sub>3</sub>). **IR (KBr, cm<sup>-1</sup>)**: 3415 (OH), 1738 (C=O), 1648 (C=C). **ESI-MS (m/z)**: 830 (M<sub>1</sub>+2H<sub>2</sub>O-H)<sup>-</sup>, M<sub>1</sub> corresponding exactly to the molecular weight of 6"-O- lauroyl - neohesperidin dihydrochalcone.

4.2.3.4. 6"-O-palmitoyl-neohesperidin dihydrochalcone (**3f**). **R**<sub>f</sub>: 0.45. <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>, 500 MHz, ppm): δ 12.33 (s, 2H, -OH<sub>5</sub>), 9.15 (s, 1H,  $-OH_{5'}$ ), 7.02 (d, 2H, J = 8.4 Hz,  $H_{2'}$  and  $H_{6'}$ ), 6.67 (d, 2H, J = 8.5 Hz, H<sub>3'</sub> and H<sub>5'</sub>), 5.99 (s, 2H, H<sub>6</sub> and H<sub>8</sub>), 5.44 (m, 1H, H<sub>1"</sub>), 5.40 (m, 1H, 2<sup>m</sup>-OH of neohesperidin dihydrochalcone), 5.11 (m, 1H,  $H_{1'''}$ ), 5.08 (d, 1H, I = 5.1 Hz, 4'''-OH of neohesperidin dihydrochalcone), 4.70 (d, 1H, I = 4.8 Hz, 4"-OH of neohesperidin dihydrochalcone), 4.68 (d, 1H, J = 4.4 Hz, 3"-OH of neohesperidin dihydrochalcone), 4.50 (d, 1H, I = 5.8 Hz, 3<sup> $\prime\prime\prime$ </sup>-OH of neohesperidin dihydrochalcone), 4.26 (d, 1H, J = 11.9 Hz,  $H_{6''}$  acylated), 4.09 (m, 1H, H<sub>6"</sub> acylated), 3.71–3.20 (m, 8H, H of rhamnoglucosyl), 3.25 (m, 2H, H<sub>3</sub>), 2.78 (t, 2H, *J* = 7.7 Hz, H<sub>2</sub>), 2.28 (m, 2H, -CH<sub>2</sub>-CO-), 1.26 (m, 26H of (CH<sub>2</sub>)<sub>13</sub> of palmitoyl), 1.20 (m, 3H of CH<sub>3</sub> of rhamnosyl), 0.86 (t, 3H, J = 7.0 Hz, CH<sub>3</sub>). IR (KBr, cm<sup>-1</sup>): 3415 (OH), 1740 (C=O), 1643 (C=C). **ESI-MS (m/z)**: 886  $(M_1+2H_2O-H)^-$ ,  $M_1$  corresponding exactly to the molecular weight of 6"-O-palmitoyl-neohesperidin dihydrochalcone.

#### 4.3. Vitro antibacterial experiment methods

The antibacterial activity of neohesperidin ester derivatives was performed against gram positive bacteria *Staphylococcus aureus* (ATCC 25923) and the gram negative bacteria *Escherichia coli* (ATCC 25922) by using cup plate method. Ampicillin sodium was employed as standard to compare the results. The tests were carried out using a suspension containing the overnight culture of bacteria ( $\sim 10^8$  CFU mL<sup>-1</sup>). The molten agar containing the microbial culture was transferred in a sterile Petri dish. Wells of 8 mm in diameter were vertically placed on the previously seeded agar plates and were filled with 50 µL compounds, DMSO solvent was used as a control to observe the solvent effects. After the plates were incubated at 37 °C for 24 h, the diameters of the distinctly clear zones were measured using a metric ruler. All the experiments were performed in triplicate. The antibacterial test results are presented in Table 2.

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