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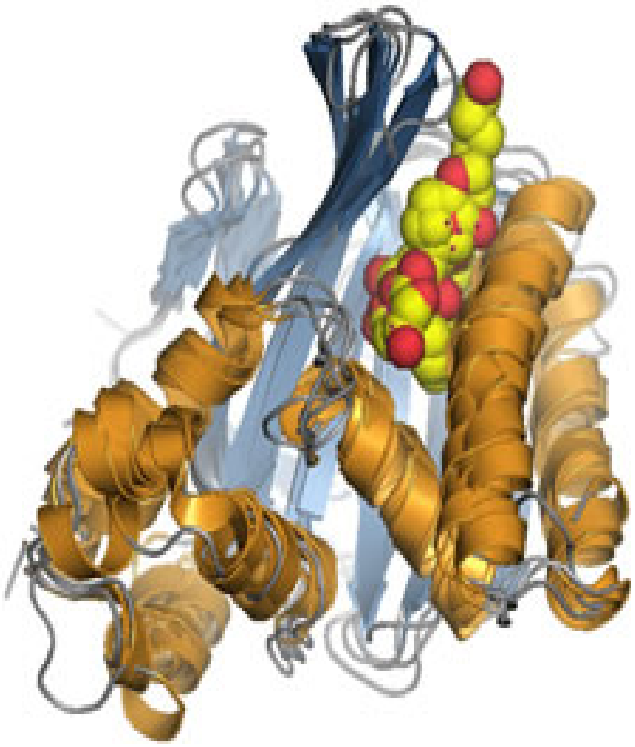

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Chiral Separation by HPLC and Biological Evaluation of Some Heterocyclic Derivatives of Hesperetin

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Abstract. *The separation of enantiomers of great interest to the pharmaceutical, chemical and food industries. This work has focused on studying the properties of chiral HPLC separation of some mono-and bis-heterocyclic derivatives Hesperetin possessing a stereogenic center in position 2. The chiral separation of these molecules was performed by HPLC using two chiral stationary phases derived polysaccharides (Chiralcel OJ, Chiralpak IA) and various mobile phases (alkane / alcohol). The Chiralpak IA column showed a better enantioseparation using methanol alone as polar mobile phase. In addition, these compounds have undergone antibacterial tests. The majority of compounds have an inhibitory overlooked all the microorganisms tested power. The antioxidant activity overlooked the DPPH have been shown that these derivatives showed a very important activity of free radicals.*

***Key Words*:** Hesperetin, Heterocyclic derivatives, Enantioseparation, DPPH, β-carotene, antimicrobial

1. Introduction

Much of the current research interest focuses on the study of chiral molecules such as flavanons. Due to their polyphenolic structure, these compounds have health-related properties, which are based on their antioxidant activity as well as anticancer, antiviral and anti-inflammatory activities [1, 2]. Among the different types of flavanones most studied, we are interested in this work on Hesperetin [5,7-dihydroxy-2-(3'-hydroxy-4'-methoxyphenyl) chroman-4-one] is a flavanone obtained by the hydrolysis acid of hesperidin. Hesperetin and its glycoside is also mainly present in citrus fruits. The aglycone is less dominant in nature than the glycoside. The most widely distributed glycoside of hesperetin is hesperidin, which is conjugate with rhamnosyl-β-1,6-glucose. Hesperidin (hesperetin-7-rutinoside) is present in higher extents in lemons, limes, sweet oranges, tangerine and tangor species of citrus fruits [3]. The flavanone Hesperetin is a chiral molecule exists in two enantiomeric forms due to the presence of an asymmetric carbon at position 2 [4], and it shows significant reactivity centers allowing him heterocyclic derivative formation [5]. It has in its structure two important

reactive centers in positions 3 and 4, which allows him training two mono iminohesperetin (**1**, **2**), a bis-iminohesperetin (**3**) and an enamin (**4**), these derivatives possess in their structures a chiral center, this has led us to reflect on their chiral separation of enantiomers by HPLC we used in this separation the stationary phase CSP based on polysaccharides.

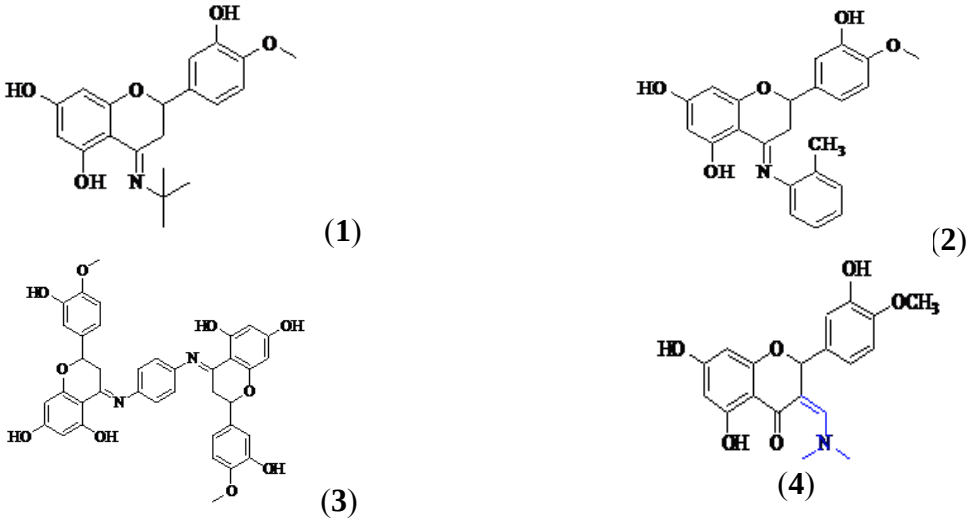


Figure 1: Chemical structure of iminohesperetin (**1**), iminohesperetin (**2**), bis-iminohesperetin (**3**) and enamin (**4**).

In recent years, there has been great interest in the discovery of new antimicrobial agents, due to an alarming increase in the rate of infections with microorganisms resistant to antibiotics. Natural and synthetic flavonoids and flavanones have attracted considerable attention because of their interesting biological activities such as antimicrobial, antifungal, antiviral, antioxidant, anti-inflammatory, etc...[6-8]. To search for new antimicrobial agents, we studied in vitro the antimicrobial power of derivatives of hesperetin with the disk diffusion method on agar solid medium.

Flavanones are a large number of pharmacological properties such as anti-cholesterol [9], antioxidant [10-15], anti-inflammatory, diuretic [16], diabetes [17] activities. Given the biological importance of hesperetin, our main goal for this part is the biological evaluation of derivatives Hesperetin synthesized.

2. Material and methods

2.1. Solvents and samples. The solvents used were grade HPLC, n-heptane from Haën Reidel (Seelze, Germany), isopropanol Merck KGaA (darrnstadt Germany), n-hexane, methanol and ethanol from Sigma-Aldrich (Seelze, Germany) solutions of each compound (1 mg / ml) were dissolved in methanol.

2.2. Apparatus and procedures. The analytical chiral HPLC experiments were performed on a unit composed of different modules that make up the HPLC Shimadzu®20-A of this facility are a Shimadzu LC 20-AD pump ® (Kyoto, Japan), a degasser Shimadzu DGU ®-20 A5 (Kyoto, Japan), a manual injection system valve Rheodyne 1907 loop 20µL. An injection syringe 20 µl Hamilton. Shimadzu ® UV detector SPD-20A (Kyoto, Japan), cell volume 12 µl, a system of command and control (Shimadzu® CBM-20Alite) which allows you to manage different modules an operating system (Shimadzu ® LC solution) to record and

process the chromatograms, the mobile phases are first filtered (sintered glass 0.45 microns) by the degasser. All columns are used at an ambient temperature. Two columns used in this study are commercially known as Chiralcel OJ, Chiralpak IA were obtained from Chiral Technologies Europe (Illkirch Cedex, France). Chiralpak IA is immobilized on the silica, which is based on the tris (3,5 dimethylphenylcarbamate) amylose, Chiralcel OJ is based on cellulose (4-methylbenzoate) on the silica deposited. A "screening" of the selectors must be made available in order to find an appropriate method of the enantiomers of our products synthesized. For the optimization of the method, we used different composition of the mobile phase with their appropriate rate and different solutions. All columns are used at room temperature. The apparent retention factor k ', the apparent enantioselectivity α, the efficiency of the N column, and the resolution Rs are determined by the same relations mentioned above, and therefore can be calculated by the operating software (Shimadzu LC ® solution).

2.3. Determination of antioxidant activity. To investigate the antiradical activity of the different synthesized products, we opted for the method that uses the (diphenyl picryl-hydrayl) DPPH as a relatively stable free radical, to perform this test, we introduced 0.01 g of each product was dissolved in 1 ml of DMF extract the plate silica gel 60 F254 (Merck) with an aluminum frame with a capillary tube. Then visualized with the aid of a solution of DPPH at the concentration of 2 mg / ml in methanol. In the presence of antioxidants, DPPH is reduced from the color purple to yellow. On TLC plates, areas of antiradical activities highlighted in yellow - light purple background after an optimal time of 30 minutes [18].

2.4. Determination of antimicrobial activity. Culture medium used Mueller Hinton. The tests are performed on four bacterial strains *Staphylococcus aureus, Escherischia coli, Pseudomonas aeruginosa* and *Bacillus cereus*, mainly from the Pasteur Institute in Algiers (Table 2).
Masses of each product were dissolved in appropriate volumes of dichloromethane, to obtain a series of 5 µl of a solution corresponding to the quantity of 75 µg of product was used to impregnate sterile paper discs of 6 mm diameter. The discs were then dried at room temperature to remove any trace of solvent before the antibacterial test. Masses of each product were dissolved in appropriate volumes of DMF, for a series of 5µl of a solution corresponding to the quantity of 25µg, 50µg, 75µg and 100 micrograms of product was used to impregnate sterile paper discs diameter 6 mm. The discs were then dried at room temperature to remove any trace of solvent before the antifungal test.
To determine the antibacterial activity, the bacterial suspension prepared was cast on Mueller Hinton (MH) for the four bacterial strains. After the flood the entire surface of the medium with the bacterial suspension, the supernatant was discarded by aspiration with a transfer pipette. Each box has received five discs placed on an identification number stamped on the bottom face of the box. The dishes were then incubated in an oven at 37 ° C for about 24 hours. After incubation, we proceeded to measure the diameter of the inhibition zones, in the case of sensitivity around the discs of 6 mm diameter.

3. Results and discussion

3.1. Chiral separation screening. Polysaccharides such as cellulose and amylose have a potential application in chiral separation. It is evident that this is the supramolecular structure of these polymer responsible for their chirality [19-21]. These polymers consist of repeating glucose units which have five asymmetric carbons: this is essential to give the PSC the enantioselective properties. Moreover, each unit has three hydroxyl groups potentially

derivable [22]. Four compounds heterocyclic derivative of Hesperetin were analyzed on Chiralcel OJ, Chiralpak IA. The enantioselectivity of the PSC studied by analyzing the compounds using normal phase and polar organic phase fashion (100% alcohol), this column is characterized by silica particles coated with modified cellulose. Separation results are shown in table 1.

Separating the diastereomers of compounds **3** and **1** on the Chiralcel OJ was not successful on chiral stationary phase in this condition of polar organic phase (100% isoprpanol), wherein these compounds are more polar, can therefore be reduced polarity of the mobile phase was improve the quality of separation. The results separation on the Chiralcel OJ column that was carried out in normal fashion stage alkane/alcohol. There was a separation for compound **3** with Rs = 0.88, but which is insufficient with the mobile phase hexane / isopropanol 80: 20% for compound **2**with Rs = 0.65.On increased polarity to mobile phase of hexane / isopropanol 70: 30% leads to an resolution Rs = 0.77 for compound **1**.

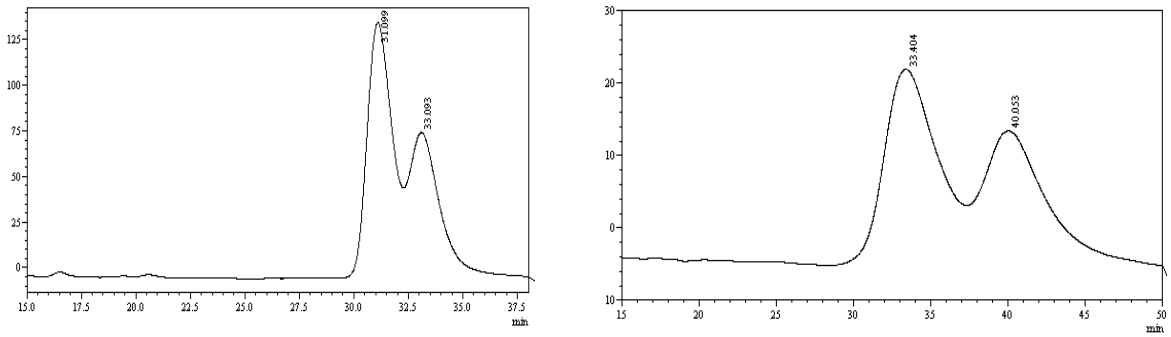
The separation of analytes was performed on the second-generation PSC amylose Chiralpak IA conditions in polar organic phase (100% methanol). The chromatographic results obtained by HPLC under these conditions are shown in Table 1.

Table 1: Result chromatography on Chiralcel OJ column and Chiralpak IA.

	Compound	Eluent (alcohol%)	rate of flow	t_{r1}	t_{r2}	*Rs*	*α*
Chiralcel® OJ Chiralpack® IA	**1**	30[a]	0.5	31.09	33.09	0.77	1.08
	1	100[a]	0.2	24.96	-	-	-
	2	20[a]	0.5	29.50	35.78	0.65	1.26
	3	20[a]	0.5	33.40	40.05	0.88	1.20
	3	40[b]	0.5	26.89	28.39	0.45	1.08
	3	100[a]	0.2	25.02	-	-	-
	1	100[c]	0.5	13.18	17.30	3.92	1.60
	2	100[c]	0.5	13.37	17.53	3.80	1.48
	3	100[c]	0.5	13.29	17.53	3.94	1.60
	4	100[c]	0.5	13.71	18.08	3.99	1.32

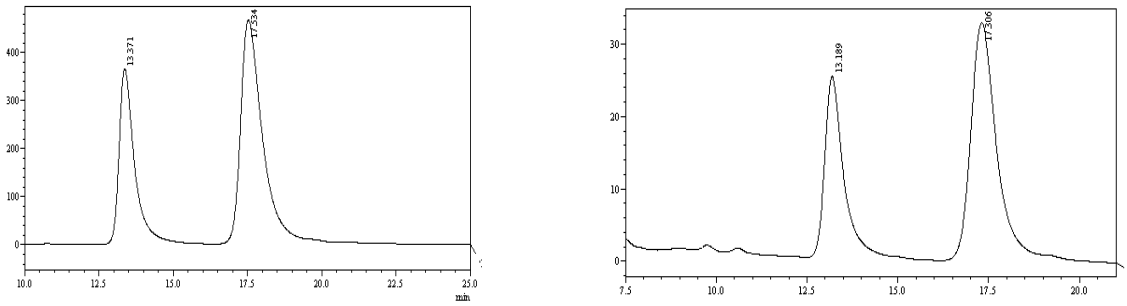
Note: [a] hexane / isopropanol, [b] heptane / isopropanol, [c] methanol.
Mobile phase: hexane /isopropanol, heptane /isopropanol, methanol (100%), flow rate: 0.5 ml / min,
Mobile phase: isopropanol (100%), flow rate 0.2 ml / min.

The results of chromatographic system based on immobilized CSP (Chiralpak IA) show a very good resolution ranging from 3.80 and 3.99 for all compounds with a shorter retention time (13.29 and 17.43 min for compound **3**) by using a mobile phase polar organic (100% methanol). Almost all compounds were solved with a good resolution with the mobile phase and the elution order is similar, and the same retention time that is closer this can be linked to their common structural feature. So this separation was performed successfully in polar organic phase when using methanol as mobile phase polar however not give any resolution with other alcohol such as isopropanol or ethanol. The resolution depends on the nature of alcoholic modifier [23]. The selection of the mobile phase it depends on the stationary phase properties of the racemic compound [24], it is very clear in this type of PSC chirapakIA when we used methanol which dissolve our samples, their character polaire has no harmful influence on this type of PSC immobilized. The enantioselectivity of the enantiomers was achieved on the derivative of amylose better than the cellulose derivative.



product 2 **product 3**

Figure 1: Chromatogram of the enantiomers of different products synthesized on the Chiralcel OJ column Mobile phase: hexane / isopropanol:80/20, flow rate: 0.5ml/min, injection volume: 20 µl, detection: 230 nm



product 2 **product 1**

Figure 2: Chromatogram of the enantiomers of different products synthesized on the Chiralpak IA column Mobile phase: methanol, flow rate: 0.5ml/min, injection volume: 20 µl, detection: 230 nm

3.2. DPPH radical scavenger effect. The antioxidant activity of different products against the DPPH radical was evaluated spectrophotometrically by following the reduction of the radical which is accompanied by the transition from purple to yellow

The activity of free radicals was investigated in four synthesized products. The results showed that all our products have given an positive reaction to DPPH. Depending on the intensity of the spots. We used as reference vitamin C (or ascorbic acid).

Our study showed that the derivatives of hesperetin showed significant antioxidant activity. All products presented yellow spots with a different fluorescent.

The antioxidant test performed on TLC plates gave many antiradical spots. All synthesized products were subjected to this test and all have responded positively. The highest activity was obtained with the DPPH test as:

Compounds	Color after 30 min	severity
Vc	dark yellow	+++
1	dark yellow	+++
2	dark yellow	+ ++
3	dark yellow	+++
4	yellow	+

3.3. Antimicrobial activity. The results of the antifungal and antibacterial activity of derivatives hesperetin obtained are given in the tables 2.

Table 2: Average diameters of the zones of inhibition (in mm) of products vis-à-vis the strains tested

Product	strain	ATCC N°	Diameter (mm)
1			**10.8**
2	*Escherichia coli*	25 922	**10.8**
3			**10.2**
4			**11.2**
1			**12.8**
2	*Staphylococcus aureus*	25 923	**10.6**
3			**10**
4			**10.6**
1			**13**
2	*Pseudomonas aeruginosa*	27 853	**12.2**
3			**8**
4			**9.2**
1			**11.6**
2	*Bacillus cereus*	11 778	**8.8**
3			**11**
4			**10.8**

To search for new antimicrobial agents, we studied in vitro the antimicrobial power of derivatives of hesperetin with the disk diffusion method on agar solid medium. The antibacterial activity of derivatives of hesperetin were investigated against four pathogenic bacteria, namely *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Bacillus cereus*. The test results are shown in Tables 2. Generally, there are a fairly well-defined activity on the growth of most strains tested (the inhibition diameters range from 8 to 13). From this table, we see that each synthetic compound acts differently on microorganisms. That is to say that certain compound can have a very significant effect on the organism (the sensitivity of *Pseudomonas* to compounds 2, 4 respectively). So else may have a lesser share, or even zero on another. Several studies have been conducted to elucidate the mechanisms of action of flavonoids on bacteria, yeasts and molds. The antibacterial activity of flavonoids can be explained by the mechanism of toxicity against microorganisms that is either non-specific interactions such as the establishment of hydrogen bonds with the cell wall proteins or enzymes, chelation metal ions, inhibition of bacterial metabolism, sequestration necessary for bacterial growth substances.

4. Conclusion.

The Chiralpak IA show a higher enantioselectivity than Chiralcel OJ with better resolution of almost all the excesses. The conditions that gave the best separation associated with the use of methanol as the mobile phase, which is why the resolution depends the nature of alcoholic modifier. In this work, we evaluated the antioxidant and antibacterial activities of hesperetin derivatives. The discovery of new bioactive products can be beneficial to public health in poor countries.

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