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Synthesis of some Thymol derivatives for enhanced antibacterial activity

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Abstract

Thymol derivatives were synthesized by condensation reactions implicating its phenolic hydroxyl group. Spectral analysis by 13C, 1H NMR, FT-IR and Mass Spectroscopy confirmed their chemical structures as the following compounds: 2-isopropyl-5-methylphenylformate (1), 2-isopropyl-5-methylphenyl benzoate (2), 5-methyl-2-propylphenyl acetate (3), 2-(2-(2-isopropyl-5-methylphenoxy)ethoxy) ethanol (4) and 2-isopropoxy-1-isopropyl-4-methylbenzene (5). Their antibacterial activity compared to Thymol was assessed by a standard broth microdilution method against five referenced bacterial strains: *Escherichia coli* a CIP 54127, *Salmonella typhimurium* an ATCC 133115, *Staphylococcus aureus* a CIP 4.83, *Pseudomonas aeruginosa* ATCC 15442 and *Klebsiella pneumonia* a CIP 104216. Obtained results confirmed enhancement of antibacterial activity of product (5) and moderately of product (3), while products 1, 2 and 4 exhibited low activity comparing to Thymol. These results suggest that the phenolic hydroxyl group of Thymol is involved in the interactions with bacterial structures leading to inhibition of their growth and even killing them at higher doses.

Keywords: *Thymol, Hemi Synthesis, Phenolic Hydroxyl Group, Antibacterial Activity, Active Site.*

1.Introduction

Bacterial resistance to antibiotics and the emergence of multi-drug resistant bacteria is one of the major health problems worldwide. Current research is trying to discover new products with higher and more targeted antibacterial activity that can overcome this problem [1-2]. The use of essential oils or their main terpenic constituents has proved to be an important alternative in the fight against pathogenic bacteria, nosocomial infections and to increase hygiene conditions in hospitals and food industries [3]. Terpenic compounds are also considered as safe products and are used in many pharmaceuticals, cosmetics, hygiene products such as antiseptics, biocides and also as bio-based solvents [4-5]. The antibacterial activity of the terpenic phenolic compounds is well known and the strong antibacterial activity of some essential oils (for example from Thymus species) can be attributed principally and most probably to the presence of phenolic compounds such as Thymol and Carvacrol at high amounts in these essential oils [6]. Adding to that, numerous studies have shown that some of these phenolic compounds act effectively in synergy with antibiotics and can restore their action against pathogenic bacterial strains [7-9]. Furthermore, previous works prove that monoterpenes are good precursors for the synthesis of active molecules [10]. Effectively, numerous monoterpenes were usefully used as precursors of catalysts and are of great of interest due to their successfully use in enantioselective syntheses [11-13]. Considering this, we focused in this work on Thymol as an interesting starting material that could be used to obtain active antibacterial molecules. Thymol which is naturally biosynthesized by many aromatic plants, particularly from the Labiatae Family is also attracting more and more interest because of its many biological activities [14-18]. Thymol in its crystalline solid state is very slightly soluble in water which represents a problem for the formulation of liquid products (for example antiseptics and biocides as Bio-products). Another problem is that Thymol at certain amounts may also present important levels of toxicity for living organisms and cells [19-20]. In one hand, a chemical modification of this phenolic compound must lead to a change in its physical properties importantly the synthesis of liquid products and if possible soluble in water which may facilitate their handling. On another hand, biological activities, in particular antimicrobial ones must be preserved or as well as possible enhanced which means reduction of its effective antimicrobial dose and minimization of its side effects. Thus, we suggest in this work to improve the antibacterial activity of Thymol by organic synthesis. In this vision, we first look here to carry out a preliminary study focused on some modifications at the phenolic hydroxyl group of Thymol (PHG) by condensation reactions. This will allow us later to understand the role and the mechanism of action of this (PHG) in the antibacterial activity of Thymol. Under specific experimental conditions and as part of one pot reaction, condensation reactions seem to be among the methods of choice to target the (PHG) of Thymol with good yields [21-22]. The antibacterial activity of the synthesized compounds compared to Thymol will be thereafter assessed by a standardized method using referenced bacterial strains. In this work, we report so a synthesis of some Thymol derivatives and evaluation of their antibacterial activities comparing to Thymol as starting material.

2. Materials and methods

All synthesis reactions were realized under argon atmosphere (Ar). All Chemical products are of pure quality for analysis purchased from Merck and were used as received. TLC spots were detected under UV light (254 nm). Fourier-transform Infrared spectra were obtained using a Perkin-Elmer Frontier FT-IR spectrometer. ^{1}H and ^{13}C spectra were recorded on a Bruker DRX 400 spectrometer operating at 400 MHz. Chemical shifts were expressed in ppm (δ) relative to residual protons of $CDCl_3$ ($CDCl_3$, δ = 7.26 ppm for ^{1}H NMR and δ = 77.16 ppm for ^{13}C). Coupling constant *J* values are expressed in Hz. Chromatographic separations were performed by flash chromatography using silica gel (Kieselgel 300-400 mesh). LC/DAD/MS analysis were performed on an Agilent 1290

infinity equipped with a Photo Diode Array Detector and a 6120 Series Quadrupole LC MS System operating at 70 eV.

2.1. Chemistry

2.1.1. Synthesis of 2-Isopropyl-5-methylphenylformate (Product 1)

This reaction was conducted under inert atmosphere of argon gaz. A Vilsmeier-Haack reagent was prepared in ice bath by adding 3.1 mL of DMF (40 mmol) to 3.1 mL of POCl3 (33 mmol). This mixture was added slowly under argon to 5 g of Thymol (33 mmol) previously dissolved in Acetonitrile (MeCN). Under refluxing and continuous stirring (rt), this reaction was monitored by TLC and conducted during 4 hours. After this time, the obtained mixture was first washed by crushed ice and then two fold by cold water wish leads to the isolation of an upper yellow oily organic phase containing a new product with traces of Thymol. This organic layer was dried over anhydrous sodium sulfate, vacuum filtered and then separated by flash chromatography which led to the isolation of a liquid, viscous and colorless new product who has the smell of mint with a yield of 79%. Colorless viscous oily liquid, Cyclohexane / EtOAc 9.5:0.5 ; Rf. 0.7; Yield 79%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm), 8.30 (s,1H, OCHO),7.24 (d, J = 7.9 Hz, 1H, Ar-H), 7.07 (d, J = 7.9 Hz, 1H, Ar-H), 6.85 (s, 1H, Ar-H), 3.09-3.02 (m, J = 6.9 Hz, 1H, CH), 2.34 (s, 3H, CH_3), 1.21 (d, J = 6.9 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz, δ, ppm): 159.9 (C=O), 147.25 (CAr), 137.11 (CAr), 136.99 (CAr), 127.75 (CHAr), 126.86 (CHAr), 122.28 (CHAr), 27.07 (CH), 23.1 (2 x CH_3), 20.92 (CH_3).

2.1.2. Esters synthesis

We performed the esterification reaction between acyl chloride (R_1COCl) and Thymol using the general procedure: to a solution of thymol (5 g, 33 mmol) and triethylamine (5.57 ml, 44 mmol) in dichloromethane (DCM), acyl chloride (R_1COCl, 44 mmol : 5,1 mL for PhCOCl and 3,13 mL for CH_3COCl) was added at room temperature (rt) under stirring under inert atmosphere of argon gas. This reaction mixture was refluxed for about 2 hours and reaction progress was monitored by TLC. After completion, the reaction mixture was washed with distilled water and extracted with dichloromethane (3 x 25 ml). The combined organic layers were then washed with distilled water and dried over anhydrous Na_2SO_4. After removal of the solvent by a rotary evaporator, the residue was purified by CC using silica gel with hexane / ethyl acetate (9: 1) as eluent to give the pure ester derivatives (products 2 and 3).

A. 2-Isopropyl-5-methylphenyl benzoate (Product 2): Colorless oily liquid, Cyclohexane / EtOAc 9:1, Rf 0.67, Yield 97 %, 1H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.27 (d, J = 7.5 Hz, 2H,Ar-H), 7.69 (t, J = 7.5 Hz, 1H, Ar-H), 7.57 (t, J = 8.0 Hz, 2H, Ar-H), 7.29 (d, J = 8.0 Hz, ^{1}H, Ar-H), 7.12 (d, J = 7.5 Hz, 1H, Ar-H), 6.99 (s, 1H, Ar-H), 3.07-3.15 (m, 1H, CH), 2.39 (s, 3H, CH_3), 1.26 (d, J = 6.5 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz, δ, ppm): 165.4 (C=O), 148.1 (CAr), 137.2 (CAr), 136.7 (CAr), 133.5 (CAr), 130.1 (2xCHAr), 129.6 (CHAr), 128.6 (2xCHAr), 127.2 (CHAr), 126.5 (CHAr), 122.9 (CHAr), 27.3 (CH), 23.1 (2xCH_3), 20.9 (CH_3).

B. 5-methyl-2-propylphenyl acetate (Product 3): Yellow oily liquid, Cyclohexane / EtOAc 9:1, Rf 0.56, Yield 72 %, 1H NMR (CDCl3, 400 MHz, δ, ppm): 7.30 (d, J = 8 Hz, 1H, Ar),7.11 (d, J = 7.5 Hz, ^{1}H, Ar), 6.83 (s, 1H, Ar),2.90-3.11 (m, 1H, CH), 2.32 (s, 6H, CH_3), 1.21 (d,J = 7, Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz, δ ppm): 170.1 (C=O), 148.3 (CAr), 138.4 (CAr), 135.3 (CAr), 126.3 CHAr), 124.2 (CHAr), 123.5 (CHAr), 28.2 (CH), 26.5 ($COCH_3$), 23.8 (2 x CH_3), 21.5 (CH_3).

2.1.3. Ethers synthesis

We performed the etherification reaction between acetyl chloride (R_1COCl) and Thymol using the following method: to a solution of thymol (2.5 g, 16.64 mmol) and triethylamine (2.24 ml, 16.64 mmol) in diethyl ether, excess of alkyl chloride was added at room temperature under stirring and an inert atmosphere of argon gas. The reaction mixture was stirred for about 10 hours at room temperature (rt). The progress of the reaction was monitored by TLC. The reaction mixture was quenched with distilled water and extracted with diethyl ether (3 x 15 ml). Finally, the combined organic layers were washed with distilled water and dried over anhydrous Na_2SO_4. After removing the solvent by a rotary evaporator, the residue was purified by CC using silica gel eluted with Cyclohexane / Ethyl acetate (8: 2) to give pure ether derivatives (compounds 4 and 5).

A. 2-(2-(2-isopropyl-5-methylphenoxy)ethoxy)ethanol (Product 4): colorless oily liquid, Cyclohexane / EtOAc 8:2, Rf 0.58, Yield 94 %, ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.20 (d, J = 7.5 Hz, 1H,Ar-H), 6.95 (s, 1H, Ar-H), 6.73 (d, J = 8.0 Hz, 1H, Ar-H), 4.25 (t, 2H, CH_2), 3.70 (t, 2H, CH_2), 3.53 (t, 2H, CH_2), 3.40 (t, 2H, CH_2), 3.12 (m, 1H, CH), 2.25 (s, Hz, 3H, CH_3), 1.20 (d, J = 6.5 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz, δ, ppm): 158.30 (CAr), 150.81 (CAr), 135.42 (CAr),125.42 (CAr), 119.41 (CHAr), 108.32 (CHAr), 72.34-70.30 (3XCH_2), 64.23 (CH_2),34.51 (CH), 24.21 (2xCH_3), 17.23 (CH_3).

B. 2-isopropoxy-1-isopropyl-4-methylbenzene (Product 5): Colorless oily liquid, Cyclohexane / EtOAc 8:2, Rf 0.72, Yield 93.5 %, ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.21 (d, J = 7.5 Hz, 1H,Ar-H), 6.90 (d, J = 8.0 Hz, 1H, Ar-H), 6.75 (s, 1H, Ar-H), 4.78-4.26 (m, 1H, CH), 3.13-3.04 (m, 1H, CH), 1.13 (s , 3H, CH_3), 1.21 (d, J = 6.5 Hz, 6H, CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz, δ, ppm): 157.20 (CAr), 138.32 (CAr), 134.30 (CAr), 126.42 (CAr), 121.20 (CHAr), 118.31 (CHAr), 80.40 (CH), 28.36 (CH), 24.53 (2xCH_3), 23.42 (2xCH_3) 22.30 (CH_3).

2.2. Antibacterial assays

2.2.1. Bacterial strains:

Five referenced bacterial strains conserved aseptically at (-24 °C) in Muller Hinton broth supplemented with glycerol were used to perform these tests. These strains are four gram negative bacteria: *Escherichia coli* (E-coli) a CIP 54127, *Salmonella typhimurium* (Sal Typ.) an ATCC 133115; *Pseudomonas aeruginosa* (Ps.a) an ATCC 15442 and *Klebsiella pneumonia* (Kl.P) a CIP 104216 and one gram positive bacteria *Staphylococcus aureus* (Sa) a CIP 4.83.

2.2.2. Determination of minimal inhibitory concentrations and minimal bactericidal concentrations:

The minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of Thymol and its synthesized derivatives were determined in duplicate by a referenced broth dilution method with some modifications [8-9]. Briefly, the tested compounds (Thymol and products 1 to 5) were first dissolved in a minimal quantity of ethanol not exceeding 2,5 % and finally in Muller-Hinton Broth (M-H B) to give one milliliter of an initial concentration of 50 mg/mL for each product. Two-fold successive dilutions were made in Muller-Hinton broth to obtain final concentration of each product ranging from 25 mg/mL to 0,05 mg/mL. Finally 980 µL of each of the previous concentration was completed by 20 µL of an initial bacterial inoculum adjusted by densitometry to 10^8 CFU/mL in Muller-Hinton broth from an 18 hour young bacterial culture [6]. After homogenization, the different concentrations were incubated aseptically in 37 °C for 24 hour. The control test consisted to check the bacterial growth in the presence and absence of ethanol in M-H Broth medium at the same concentration used to solubilize the products. The minimal inhibitory concentration was taken as the minimum concentration of each compound inhibiting

visible growth of bacteria after 24 h of incubation. The minimal bactericidal concentration (MBC) specific to each bacterial strain corresponds to the minimal concentration of a product giving no visible growth of bacteria after transfer to normal nutrient agar medium. It was determined by spreading 100 µL from the different concentrations that not show any visible growth to freshly prepared agar nutrient plate after an incubation time of 48 hour.

3. Results and Discussions

3.1. Chemistry

We used simple condensation reactions for the synthesis of five different Thymol derivatives by targeting its PHG. We obtained respectively compounds 1; 2; 3; 4 and 5 (Figure 1).

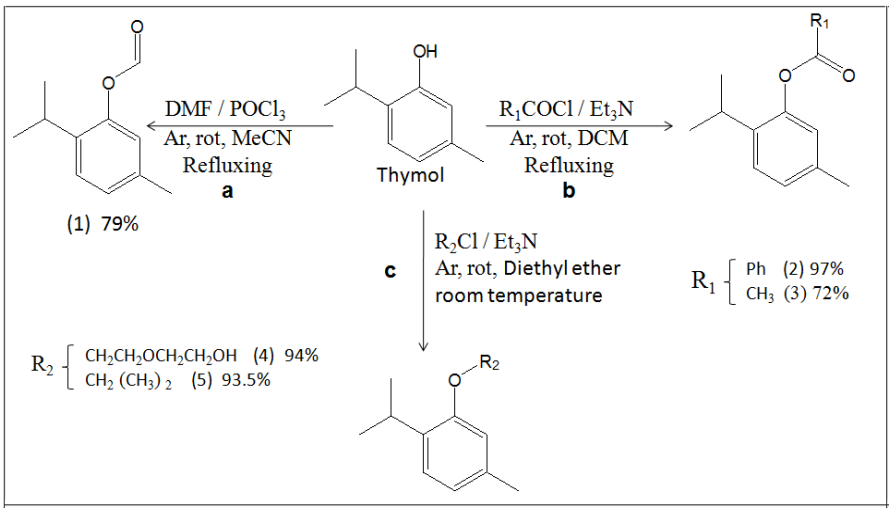

Figure1. Synthesis of 2-Isopropyl-5-methylphenyformate (1), esters (2; 3) and ethers (4; 5) from thymol

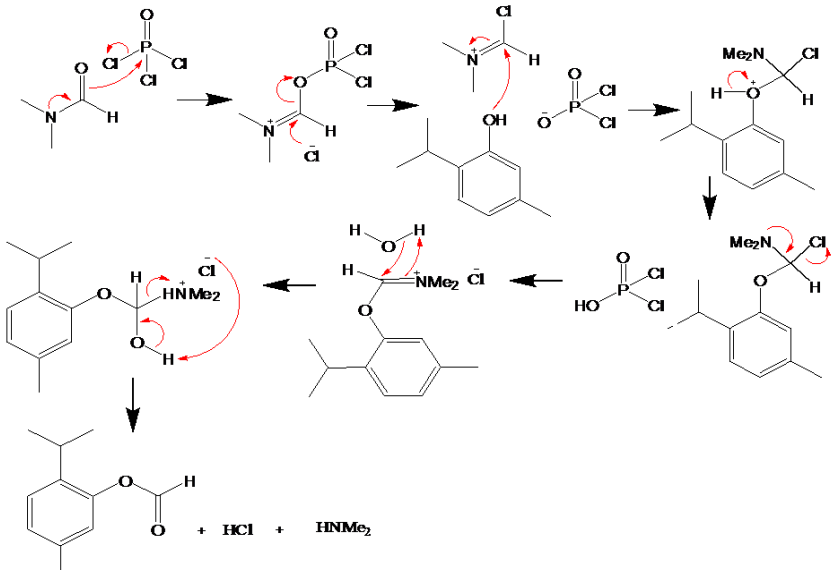

Figure2. Proposed reaction mechanism for compound 1

Primarily (route: a- Figure 1), as part of one pot reaction, we initially selected the formylation reaction of thymol under Vilsmeier-Haack conditions without protection of the PHG of Thymol. Firstly, the Vilsmeier-Haack reagent (V-H R) was obtained by addition of DMF on phosphoryl trichloride, which is an electrophilic attractor. In second step, to Thymol previously dissolved in Acetonitrile, the V-H R was added. We thought to achieve an ortho-formylation

reaction by nucleophilic attack of Thymol on the carbon of the iminium group which should lead to ortho-formylation of thymol. But in our case, and as confirmed by spectral NMR analysis, we obtained a thymyl formate (product 2) which indicate a formylation of the PHG of thymol instead of ortho-position. To our knowledge, the PHG formylation reaction as produced in our conditions has not been described in the bibliography. To explain the formation of product 1, the proposed reaction mechanism is shown in Figure 2. We suggest that it is the PHG of Thymol that attack the carbon of iminum leading to the condensation of the Vilsmeier reagent to Thymol. By elimination of Cl^- ions, the obtained product, a chlorinated amine, reorganizes into iminium ions. By adding water, a hydrolysis is carried out resulting in the departure of the iminium group which leads to the formation of Thymyl formate, a colorless oily product (product 2) in a good yield (79 %) In the second route (b-Figure 1), the esterification reaction between the acyl chloride and Thymol is carried out using triethylamine as a catalyst. Indeed, as shown in Fig 3, the nucleophilic attack of Thymol PHG on the carbonyl of acyl chloride leads to the condensation of these two compounds and to the release of HCl in the medium. Triethylamine, by its presence, acts as a trap for HCl and fixes it, resulting in the formation of a salt complex: triethylamine hydrochloride. This effectively promotes condensation between thymol and acyl chloride. The mechanism of this synthesis can be summarized in two steps: nucleophilic addition of Thymol to acyl chloride and departure of HCl to finally give compounds 2 and 3 in good yield as a colorless oily liquid. Other methods of synthesizing these alternative types have been described previously [23-25]. In our case, by comparison the precisions methods, we have used simple and different method and gives a good performance with less than two hours of reaction.

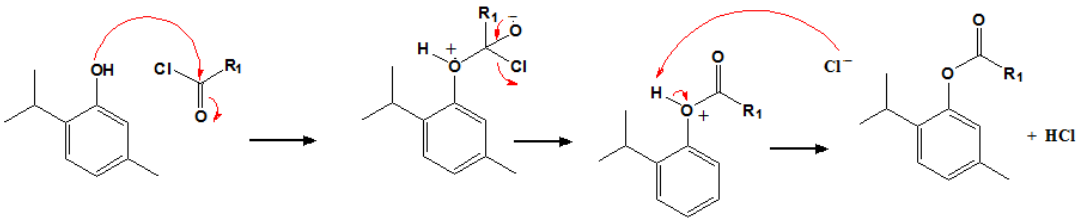

Figure3. Proposed reaction mechanism for esterification

In the third route (c-Figure 1), the etherification reaction of the same process as that used in route (b), except for the change of acyl chloride by alkyl chloride and the reaction temperature. The mechanism of this synthesis can be summarized in two steps as shown in Fig 4. A nucleophilic attack of PHG of thymol on the carbon of the chlorine-bound alkyl leads to the condensation of these two reagents and to the release of HCl in the medium, Finally, the two compounds 4 and 5 are obtained in good yield as oily liquids.

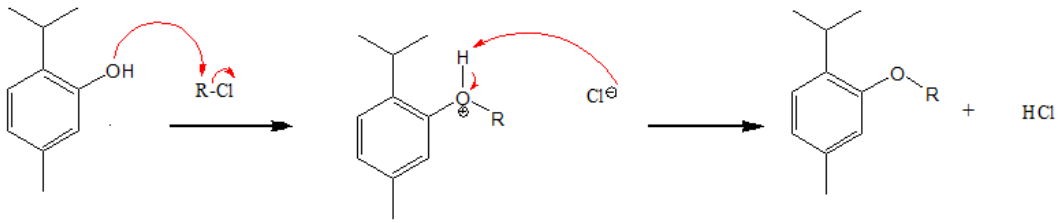

Figure4. Proposed reaction mechanism for etherification

3.2. Antibacterial assays

To study the antibacterial activity of the synthesized compounds in comparison to Thymol, we used a referenced broth dilution method, [26-27]. These consist to prepare ranges of concentrations of each compound by solubilization and incorporation in Müller-Hinton broth medium. We used also five referenced bacterial strains to assess this

antimicrobial activity: (E-coli), (Sal.Typ), (Sa); (Ps.a) and (Kl.P). These allow as broadening the scope of our antibacterial study and confirming if there will be indeed an improvement or a decrease of the antibacterial activity of the synthesized molecules. The antibacterial activity of a product is better when its MIC and MBC are small reflecting the product's ability to exert the antibacterial activity at low dose. Obtained results are summarized in table 1.

Table1. Minimal inhibitory and bactericidal concentration (mg/mL) of the synthesized products and Thymol

	Bacterial strains									
	E-coli		*Sal. Typ*		*Sa*		*Ps.a*		*Kl.p*	
Products	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Thymol	0.78	3.13	0.78	3.13	1.56	6.25	3.13	12.50	0.78	3.13
1	25	ND	25	ND	> 25	ND	> 25	ND	12.50	> 25
2	25	ND	25	ND	> 25	ND	> 25	ND	12.50	> 25
3	0.78	3.13	0.78	3.13	1.56	6.25	3.13	12.50	0.78	3.13
4	6.25	25	6.25	25	12.5	> 25	25	ND	3.13	12.50
5	0.10	0.78	0.10	0.78	0.20	1.51	1.51	6.25	0.10	0.78

These results were presented in the form of histograms as shown in Figures 5 and 6 below :

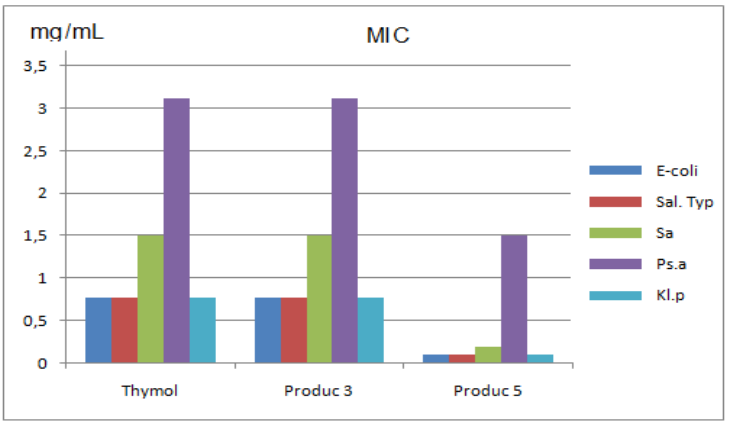

Figure5. Minimal inhibitory concentrations of products 3, 5 and Thymol

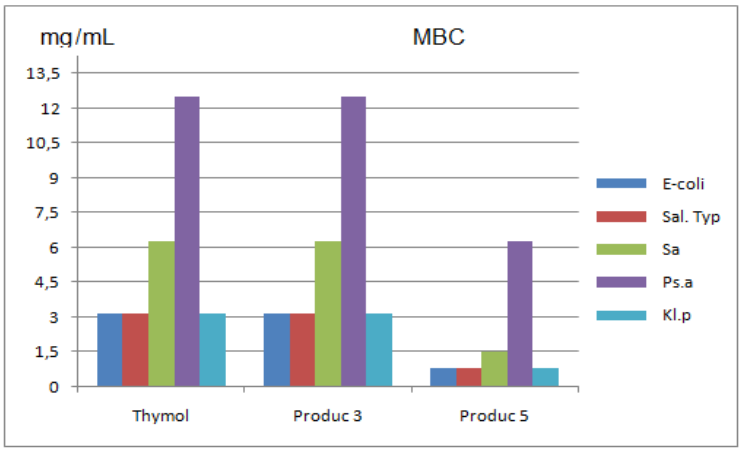

Figure5. Minimal bactericidal concentration of products 3, 5 and Thymol

As shown in table 1 and Figures 5 and 6, if we look at the activity of the different molecules, it is easy to distinguish products 3 and 5 exhibiting antibacterial activity higher or equal to thymol and products 1, 2 and 4 with lower activity

than Thymol. The MIC values indeed reflect the strength of the antimicrobial activity of a molecule by considering that it is more active when it's MIC and MBC are lowest. This results show that only products 3 and more interestingly product 5 exhibit enhanced antibacterial activity compared to Thymol. Basing on previous study and bibliographic data, the terpenic phenolic compounds of essential oils such as Thymol, carvacrol and Eugenol are among the most active terpenic compounds. They exhibits their antibacterial and bactericidal activity due to their ability to permeabilize, damage and disturb the structure of the cytoplasmic membrane of bacteria leading to the release of their cytoplasmic material, [28-29]. Indeed, according to several and recent studies, the action of phenolic compounds on bacteria is dose dependent but too complex and varied because it concerns several parts and structures of the bacterium [1]. Comparing to Thymol, we suggest that the enhanced antibacterial activity of product 3 and 5 can be interpreted by electronic effects on and around the double bond of oxygen of the phenolic hydroxyl group of thymol. In the case of product 3 (Thymyl acetate), the electronic doublet of the oxygen is relatively available by the presence of the methyl of acetate which exerts a donor electron effect contributing to compensation of the attracting effect of the ester bond and to stabilize the electronic doublet of oxygen of the phenolic hydroxy group. In the case of product (1), the donor effect of H is too low that the electronic effect of the ester bond can be compensated. In the case of product 2, the benzene nucleus of the benzoate has an attracting mesomeric effect, which makes the electronic doublet of oxygen even less available, thereby contributing to minimizing the activity of the product 2. In the case of product 5, by the donor effect of isopropyl on the ether bond, the electronic doublet of the Oxygen is more available which give best activity to this product. Comparing to product 5, product 4 is also characterized by the steric hindrance as product 2 but also there is the enticing effect of the oxygen and the hydroxyl of the radical connected to the phenolic hydroxyl group of thymol which also makes its electronic doublet less available. It appears so clearly that the product 5 is the most active molecule and therefore the most powerful compound among the molecules studied. Indeed, despite the fact that the compounds 2, 3 and 4 are liquid, they are relatively less polar and practically insoluble in water. But if we examine the antimicrobial activity of phenolic compounds, Thymol is especially well studied and referenced by several bibliographical data [23-25], [30-31]. It is also possible to say that the size of the molecules and the steric encumbrance of the molecules condensed with thymol have an influence on the antibacterial activity. This may be explained in part by protein-ligand interactions. Admitting the existence of receptor bacterial membrane structures of Thymol and its derivatives, only the less bulky derivatives can interact with the receptor part of these structures, as in the case of enzyme-substrate interactions. It should be finally mentioned that other studies have been carried out on thymol in order to improve its antimicrobial activity but these works lacks precision concerning the antibacterial tests carried out and it would be almost impossible to compare the results between several studies [23-25], [32]. Some authors, for example, use the disc method, which is incompatible with organic products because of their insolubility in an aqueous medium and non-homogeneous diffusion in agar medium. Other authors use the well method [33] making comparison between results very difficult. In our study, we used a standardized method of dilution in broth according to the NCCLS recommendations (guidelines M7-A4) [9], [34]. This technique makes it possible to determine the MIC and in the same time the MBC for a better evaluation of the antibacterial activity of the synthesized products. Finally, we note that this important result will allow us to better target our next synthesis work to the most interesting parts likely to give compounds with greater antibacterial activity. We therefore envisage in our perspectives to target this pathway and to broaden our study to antifungal and anti-candida activity.

4. Conclusion

In our work, five different products were synthesized with acceptable yields using simple reactions targeting the PHG of Thymol. We managed to modify the physical and chemical properties of thymol and in the same time to improve its

antibacterial activity. Unfortunately, this antibacterial activity decreased for products (1, 2 and 4) but increased for products (3 and 5). This confirmed that the PHG is effectively involved in the antibacterial activity of Thymol. Basing on these results, we may suggest that the electronic doublet of oxygen of the PHG should be free and more available to establish interactions with active bacterial structures such as for example membrane proteins which can lead to their inactivation. This result will be taken into account in our future synthesis work for enhanced antibacterial activity.

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