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Synthesis, Physicochemical Characterization and Study of the Antimicrobial Activity of Chlorobutanol

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***Abstract*—**Introduction and objectives: Chlorobutanol is a raw material, mainly used as an antiseptic and antimicrobial preservative in injectable and ophthalmic preparations. The main objective of our study was the synthesis and evaluation of the antimicrobial activity of chlorobutanol hemihydrates. Material and methods: Chlorobutanol was synthesized according to the nucleophilic addition reaction of chloroform to acetone, identified by an infrared absorption using Spectrum One FTIR spectrometer, melting point, Scanning electron microscopy and colorimetric reactions. The dosage of Carvedilol active substance was carried out by assaying the degradation products of chlorobutanol in a basic solution. The chlorobutanol obtained was subjected to bacteriological tests in order to study its antimicrobial activity. The antibacterial activity was evaluated against strains such as *Escherichia coli* (ATCC 25 922), Staphylococcus aureus (ATCC 25 923) and *Pseudomonas aeroginosa* (ATCC = American type culture collection). The antifungal activity was evaluated against human pathogenic fungal strains, such as *Candida albicans* and *Aspergillus niger* provided by the parasitology laboratory of the Hospital of Tizi-Ouzou, Algeria. Results and discussion: Chlorobutanol was obtained in an acceptable yield. The characterization tests of the product obtained showed a white and crystalline appearance (confirmed by scanning electron microscopy), solubilities (in water, ethanol and glycerol), and a melting temperature in accordance with the requirements of the European pharmacopoeia. The colorimetric reactions were directed towards the presence of a trihalogenated carbon and an alcohol function. The spectral identification (IR) showed the presence of characteristic chlorobutanol peaks and confirmed the structure of the latter. The microbiological study revealed an antimicrobial effect on all strains tested (*Sataphylococcus aureus* (MIC = 1250 µg/ml), *E. coli* (MIC = 1250 µg/ml), *Pseudomonas aeroginosa* (MIC = 1250 µg/ml), *Candida albicans* (MIC =2500 µg/ml), *Aspergillus niger* (MIC =2500 µg/ml)) with MIC values close to literature data. Conclusion: Thus, on the whole, the synthesized chlorobutanol satisfied the requirements of the European Pharmacopoeia, and possesses antibacterial and antifungal activity; nevertheless it is necessary to insist on the purification step of the product in order to eliminate the maximum impurities.

***Keywords*—**Antimicrobial agent, bacterial and fungal strains, chlorobutanol, MIC.

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I. Introduction

EXCIPIENTS play a vital role in the design, manufacture and preservation of drugs. The addition of an antimicrobial agents is of great interest in the case where the pharmaceutical preparations do not have adequate antimicrobial properties, (especially aqueous preparations), to prevent proliferation or limit microbial contamination [1], [2]. Chlorobutanol is a trihalogen alcohol with bacteriostatic and fungistatic activity. The main objective of our work was to obtain chlorobutanol hemihydrate by chemical synthesis, its purification and characterization as well as the study of its antimicrobial activity.

II. Experimental

A. Chemistry

Chlorobutanol is obtained by the nucleophilic attack of carbanion formed from choroform in the alkaline medium (sodium hydroxide) on the partially positively charged carbon of acetone [3]. The product obtained is characterized by colorimetric reactions (reaction with pyridine, reaction with ammonium nitrate and reaction with iodized potassium iodide), by chromatographic (IR) techniques, scanning electron microscopy crystal analysis and melting point determination were also performed. The technique used for the determination of chlorobutanol is based on the release of chloride ions after treatment with an alkaline solution, these chloride ions are subsequently titrated by argentimetry (method of Charpentier Volard) [4].

B. Antimicrobial Activity

We evaluated the antimicrobial activity of chlorobutanol hemihydrate synthesized by the liquid-based macro-dilution method to determine the minimum inhibitory concentration (MIC) [5].

1. The Microbial Strains Tested

We tested the microbiological activity of chlorobutanol hemihydrate synthesized on the microbial strains recommended by the European Pharmacopoeia 8th edition, for the evaluation of the antimicrobial conservation efficacy of pharmaceutical preparations (Table I).

2. Cultivation of the Strains

a. Activation of Reference Bacterial Strains

We perform streak isolation (four-quadrant method) directly from the lyophilisates of the reference strains on selective media with each bacterium (Fig. 1):

- Milieu Chapman for *Staphylococcus aureus*
- Milieu Hektoen for *pseudomonas aeruginosa* and *E. coli.*

TABLE I
STRAINS OF MICROORGANISMS TESTED

Microorganisms	Strains tested	Species tested	Origin
bacteria	Gram -	*Escherichia coli* (ATCC 25 922)	Reference strains American type culture collection (ATCC)
		Pseudomonas aeruginosa (ATCC 27 853)	
	Gram +	*Staphylococcus aureus* (ATCC 25 923)	
fungal species		*Candida albicans*	Parasitology laboratory of Tizi-Ouzou hospital
		Aspergillus niger	

b. Fungal Strains

The fungal strains tested are 15-day cultures provided by the parasitology laboratory of the University Hospital Center of Tizi-Ouzou on tube agar medium (Fig. 1):

- Sabouraud-chloramphenicol-actidione medium for *Candida albicans*
- Sabouraud-chloramphenicol medium for *Aspergillus niger*.

Fig. 1 Microbial strains used in the control of the antimicrobial activity of synthesized chlorobutanol hemihydrate

3. Preparation of the Dilution Range of Synthesized Chlorobutanol

a. Preparation of the Stock Solution at 5000 µg/ml (0.5%)

We weigh with an analytical balance 58.82 mg of chlorobutanol hemihydrate synthesized which corresponds to 50 mg of pure chlorobutanol (test portion corrected for the content of active ingredient which is 85%), solubilized with ultrasound in 10 ml buffered glucose broth or nutrient broth.

b. Preparation of Dilutions

- We perform semi-logarithmic dilutions of half to half of the chlorobutanol hemihydrate synthesized for a concentration range from 5000 µg/ml up to 312.5 µg/ml) (Table II)
- We prepare a dilution range for each strain tested, i.e. *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus niger*
- We provide negative control tubes for each dilution series (without microbial inoculum).
- *Preparation of the Microbial Inoculums:* The microbial stock suspension should have a turbidity of 0.5Mac Farland which corresponds to 1.108 CFU/ml. In our work, in the absence of densitometer, we have estimated this density to 1 or 2 identical and well isolated colonies which would cause a slight disturbance observed with the naked eye under the white light of the day.

TABLE II
DILUTION RANGE OF CHLOROBUTANOL

Dilution ratio	1/2	1/4	1/8	1/16	1/32
[ClbH] %	0.5	0.25	0.125	0.0625	0.03125
[ClbH] in µg/ml	5000	2500	1250	625	312.5

[ClbH]: concentration of chlorobutanol hemihydrate.

c. Preparation of the Bacterial Suspension

- From a young culture of 24 hours and using a platinum loop, we take one to two bacterial colonies well isolated and identical;
- We introduce the collected colonies in about 5 to 10 ml sterile physiological saline 0.9% NaCl, we homogenize vortex and check for the appearance of a slight disorder;
- We take 100 µl of the bacterial inoculum and introduce it into 9.9 ml of sterile physiological saline to obtain a 1/100 dilution.

d. Preparation of the Fungal Suspension

- Using a sterile loop, we take 1 to 2 fungal colonies;
- We Immerse the collected colonies in about 5 to 10 ml sterile physiological saline, we homogenize and check for the appearance of a slight disorder;
- We take 100 µl of the fungal inoculum and introduce it into 9.9 ml of sterile physiological saline;

e. Distribution of the Microbial Inoculums

- The distribution of the inoculum must be done within 15 minutes of its preparation. We incorporate 100 µl of microbial suspension into each tube of the dilution range;
- We proceed from the lowest concentration to the highest concentration
- We prepare a positive control tube; by incorporating 100 µl of the inoculum into a tube containing 5 ml of BGT without chlorobutanol (Fig. 2).
- We prepare a control box (control box) by spreading with a rake 100 µl of each suspension on a nutrient agar for bacteria and Sabouraud medium without antibiotic for yeast and mold, this control allows us to check the purity of each strain and estimate the microbial density (Fig. 3).

f. Incubation

- We incubate the broths and the controls, in incubators programmed according to the conditions mentioned in Table III.

g. Reading the MIC

- The reading of the MIC is done visually, by observing the presence or absence of turbidity in incubated broth;
- The MIC value is the lowest concentration of the antimicrobial that inhibits any visible bacterial growth with the naked eye, which is the concentration of the first clear tube in the dilution range.

Fig. 2 Distribution of microbial inocula

Fig. 3 Control boxes

TABLE III
INCUBATION CONDITIONS OF THE MICROORGANISMS TESTED

Microorganism	Incubation temperature	Incubation time
Escherichia coli	37 °C	24 hours
Staphylococcus aureus	37°C	24 hours
Pseudomonas aeruginosa	37°C	24 hours
Candida albicans	37°C	48 hours
Aspergillus niger	25°C	48 hours

- The MIC value characterizes the bacteriostatic and fungistatic effect of an antimicrobial [4].

III. RESULTS AND DISCUSSION

A. Chemistry

Chlorobutanol is obtained with a yield of 54%, it is in the form of white crystalline powder with a very strong camphor flavor (Figs. 4 and 5), slightly soluble in water, freely soluble in ethanol at 96 percent and soluble in glycerol at 85 percent. The melting point obtained with the synthesized chlorobutanol is 78.0 °C.

Fig. 4 Synthesized chlorobutanol

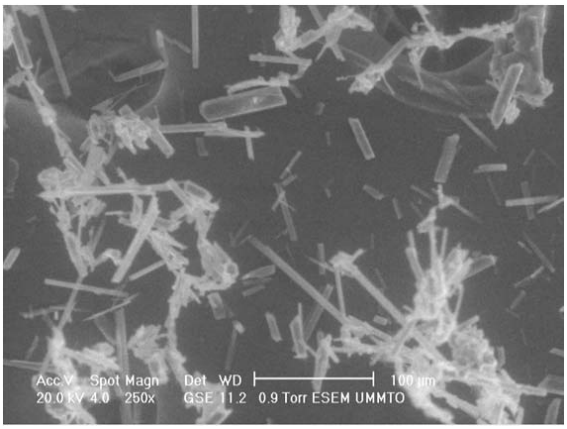

Fig. 5 Result of the analysis by electron microscopy

The reaction of pyridine with our product gave a red layer on the surface after heating. This red layer corresponds to the Fujiwara-Ross reaction which directs towards the trichlorinated carbon of chlorobutanol hemihydrate.

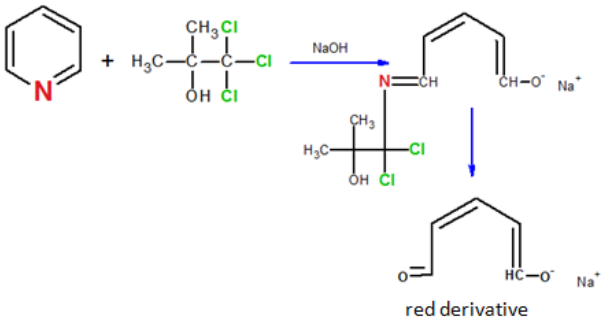

Fig. 6 Fujiwara-Ross reaction between chlorobutanol and pyridine

The reaction of chlorobutanol with ammoniacal silver nitrate gave a black precipitate. This precipitate corresponds to the silver hydroxide obtained in alkaline medium from AgCl; product of the reaction between chloride ions (released after degradation of chlorobutanol) with silver nitrate (Fig. 7).

The reaction of chlorobtanol with potassium iodide gave a yellowish precipitate. This precipitate corresponds to the formation of iodoform from potassium iodide and acetone resulting from the degradation of the alcohol function of chlorobutanol in alkaline medium (Fig. 7).

Absorption spectra in IR of synthesized chlorobutanol: The spectra absorption obtained with synthesized chlorobutanol is shown in Fig. 8.

To interpret the infrared absorption spectrum of synthesized chlorobutanol, we divide it into two main regions:

- The functional group region of 4000 cm -1 to 1500 cm $^{-1:}$

In this region we find the main functional groups of Chlorobutanol namely: The alcohol group (OH) which is characterized by a band at 3369.88 cm- due to the elongation of the free OH bond, and the methyl groups CH3 characterized by bands at 2995.99 cm- and 2945.02 cm- due to the symmetrical elongations CH bonds.

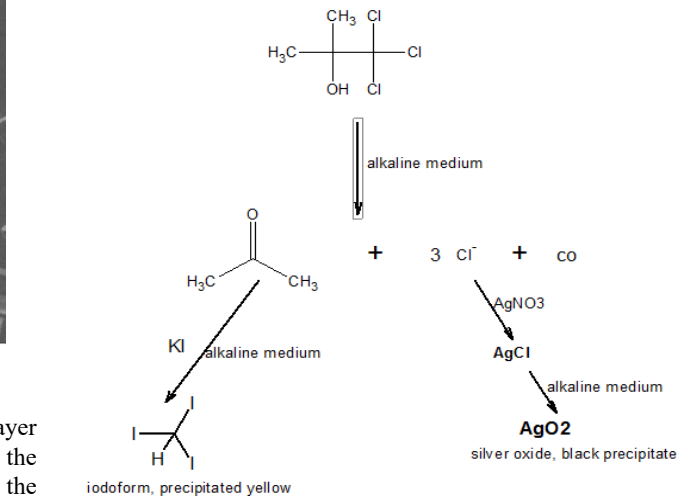

Fig. 7 Reaction of chlorobutanol with potassium iodide and silver nitrate

- The area of the fingerprint from 1500 cm- to 400 cm-:

In this region we find all the characteristic binding bands of chlorobutanol

- ✓ The bands 1459,26cm-; 1440.87cm-; 1385.39 cm -1 and 917.83 cm -1 due to different deformations of the C-H bonds of the methyls;
- ✓ We have been able to confirm with the bands 1370.33cm-, 983.16 cm-, 565.17cm-, 442.3cm- the presence of a tertiary alcohol bonded to an alkane, substituted by methyl radicals in the structure of our product.
- ✓ The bands 791.18cm- and 612.69 cm- confirm the presence of a trichlorinated carbon;
- ✓ In addition, the 833.30 cm-band confirms that tertiary alcohol and trichlorinated carbon belong to the same compound.
- ✓ However, the presence of two strands foreign to the structure of chlorobutanol, namely the 704.05cm- and the 1651.74cm- band, which point to a monosubstituted benzyl, is probably due to the presence of the related chlorobutanol substances. namely phenoxyethanol or phenylethanol.

B. Antimicrobial Activity

The microbiological study revealed an antimicrobial effect on all strains tested, with MIC values close to literature data.

For *Escherichia coli*, we observed a disorder in the positive control tube, the 0.031% dilution and the 0.062% dilution indicating the presence of a bacterial outbreak. However, no disturbances were observed in the negative control tube, the 0.125% dilution, the 0.25% dilution and the 0.5% dilution.

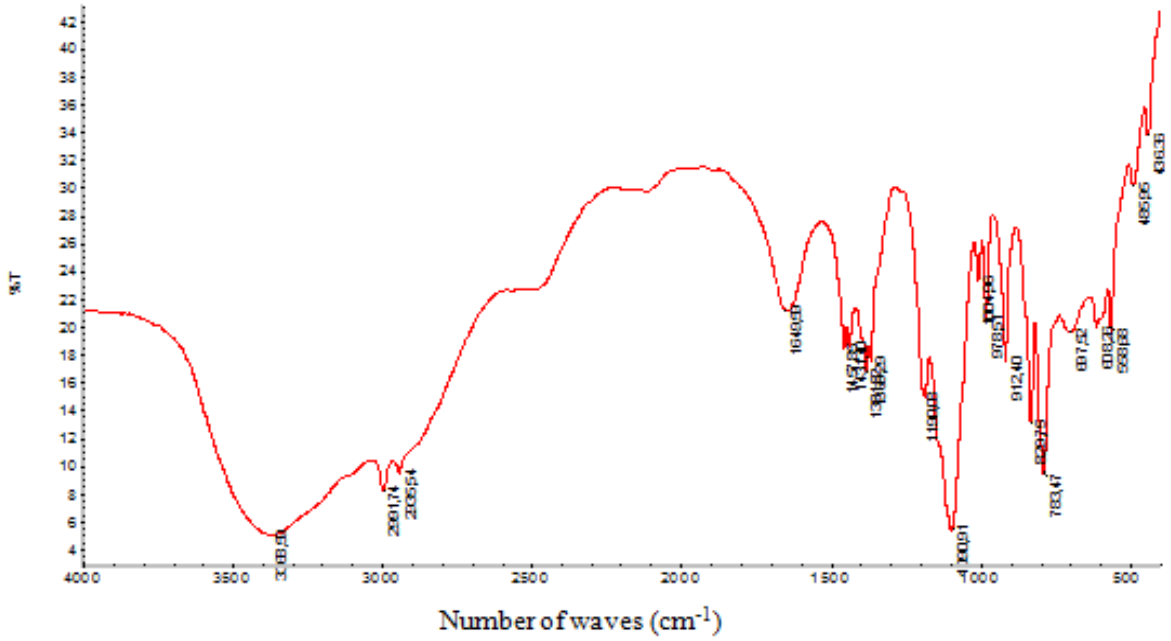

Fig. 8 Absorption spectra in IR of synthesized chlorobutanol

The value of the MIC of the chlorobutanol hemihydrate synthesized on the *Escherichia coli* strain (ATCC 25 922) tested is therefore 0.125% :

$$MIC_{\text{Chlorobutanol -}E.coli\text{ (ATCC 25 922)}} = 1250\ \mu g/ml$$

For *Pseudomonas aeruginosa,* we observed a disorder in the positive control tube, the 0.031% dilution and the 0.062% dilution indicating the presence of a bacterial outbreak. However, no disturbances were observed in the negative control tube, the 0.125% dilution, the 0.25% dilution and the 0.5% dilution.

The MIC value of the chlorobutanol hemihydrate synthesized on the strain of Pseudomonas aeruginosa (ATCC 27 853) tested is therefore 0.125%:

$$MIC_{\text{Chlorobutanol-pseudomonas (ATCC 27 853)}} = 1250\ \mu g/ml$$

For Staphylococcus aureus (ATCC 25,923) we observed a disorder in the positive control tube, the 0.031% dilution and the 0.062% dilution indicating the presence of a bacterial outbreak. However, no disturbances were observed in the negative control tube, the 0.125% dilution, the 0.25% dilution and the 0.5% dilution.

The MIC value of chlorobutanol hemihydrate synthesized on the strain of Staphylococcus aureus (ATCC 25923) tested is 0.125%:

$$MIC_{\text{chlorobutanol-staphylococcus (ATCC 25 923)}} = 1250\ \mu g/ml$$

For Candida albicans, we observed a disorder in: the positive control tube, the 0.031% dilution, the 0.062% dilution and the 0.125% dilution, which indicates the presence of a bacterial outbreak. However, no disturbances were observed in the negative control tube, the 0.25% dilution and the 0.5% dilution.

The value of the MIC of the chlorobutanol hemihydrate synthesized on the candida albicans strain tested is therefore 0.25%:

$$MIC_{\text{Chlorobutanol-candida}} = 2500\ \mu g/ml$$

For *Aspergillus niger*, we observed a disorder in: the positive control tube, the 0.031% dilution, the 0.062% dilution and the 0.125% dilution, which indicates the presence of a bacterial outbreak. However, no disturbances were observed in the negative control tube, dilution at 0.25% and dilution at 0.5%.

The value of the MIC of the chlorobutanol hemihydrate synthesized with respect to the *Aspergillus niger* strain tested is therefore 0.5%:

$$MIC_{\text{chlorobutanol-aspergillus}} = 2500\ \mu g/ml$$

Table VI summarizes the values of the MIC on the strains tested.

IV. Conclusion

The synthesized chlorobutanol drug substance is of good physicochemical quality. It meets the requirements of the European Pharmacopoeia. The microbiological study revealed an antimicrobial effect on all strains tested (*Sataphylococcus aureus*, E. coli, *Pseudomonas aeroginosa*, *Candida albicans*, *Aspergillus niger*) with MIC values close to the data in the literature.

TABLE IV
THE RESULTS OF THE MIC

	312.5	625	1250	2500	5000
E. coli	+	+	- (CMI)	-	-
Pseudomonas aeruginosa	+	+	- (CMI)	-	-
Staphylococcus aureus	+	+	- (CMI)	-	-
Candida albicans	+	+	+	- (CMI)	-
Aspergillus niger	+	+	+	- (CMI)	-

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