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Study of the antibacterial effect of 5-(4-chlorophenyl)-1H-tetrazole and its oxime precursor against strains isolated from the hospital environment

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ABSTRACT

The compounds used in this study, TET and OXM, were tested in vitro for their antibacterial activities against isolated strains from the hospital environment (S. aureus H, E. coli H and K. pneumoniae H) and reference strains (S. aureus A, E. coli A, B. subtilis A and P. aeruginosa A). The calculation of the MBC/minimum inhibitory concentration ratio of the products TET and OXM showed that the two products have a bactericidal effect on the strains tested.

1. Introduction

The hospital environment is largely contaminated by microorganisms of human or environmental origin.[1] This contamination varies qualitatively and quantitatively in time, from one establishment to another and within the same establishment in terms of the services, patients, care and practised techniques. The microorganisms that colonise the hospital environment are numerous; they constitute true ecological niches.[2] The contamination by these microorganisms is diffuse, and its control involves constraining, complex and expensive procedures. Despite the absence of an indicator allowing us to measure the effect of the hospital environment in the occurrence of hospital infections, it is established that bio-contamination in hospitals constitutes a major risk for fragile patients; for the caring staff and for certain places where invasive care or acts are practised.

Microorganisms can develop resistance to various antimicrobial agents, described as acquired resistance. Bacterial resistance to antibiotics and cleaning products is a major problem of public health. The dissemination of the multiresistant bacteria between hospitalised often fragile patients is at the origin of a considerable increase of mortality and morbidity.[3-4] Faced with this problem, it is necessary to find alternative solutions with the use of antibiotics. Chemical synthesis of new molecules or the search for natural substances, particularly essential oils, with antibacterial properties will be of a great contribution and interest. Within this framework, the antibacterial effects of two synthetic chemical products derived from tetrazole on strains isolated from the hospital environment were studied. The tetrazole derivatives have attracted much attention because of their various applications in several fields.[5] We were interested to study the antibacterial activity of two synthesised compounds derived from tetrazole against strains isolated from the hospital environment and identified by our team [2-6], namely, Escherichia coli (E. coli H), Staphylococcus aureus (S. aureus H) and Klebsiella pneumoniae (K. pneumoniae H).

2. Result and discussion

2.1. Demonstration of the antibacterial activity

The antibacterial activity of the tetrazole derivatives (by disc diffusion method) was evaluated on the seven strains tested. The diameters of the aureoles of inhibition were measured in millimetre (mm). The results obtained are shown in **Table 1**.

Table 1 Diameters of the zones of inhibition of the Tetrazole derivatives in (mm)

	TET	OXM	Positive Witness
***S. aureus* H**	<8	8 ± 0	26
***S. aureus* A**	<8	9 ± 0,6	26
***E. coli* H**	<8	11 ± 0	25
***E. coli* A**	<8	15 ± 0	27
***K. pneumoniae* H**	<8	9,66 ± 0,6	27
***B. subtilis* A**	<8	9,33 ± 0,6	24
***P. aeruginosa* A**	9±0	12,33 ± 0,6	25

Strains A: ATCC strains; Strains H: strains isolated from the hospital environment; Positive Control: Chloramphenicol (30 µg/mL).

The OXM product has an inhibiting activity against all of the strains studied, with an inhibition diameter ranging from 8 to 15 mm: Escherichia coli A. (15 mm), Pseudomonas aeruginosa A. (12.33 mm), Escherichia coli H. (11 mm), Bacillus subtilis A. (9.33 mm), Klebsiella pneumoniae H. (9.66 mm), Staphylococcus aureus A (9 mm) and Staphylococcus aureus H (8 mm). The TET product showed no activity against all strains tested, except for the Pseudomonas aeruginosa A strain which was found to be not very sensitive with a diameter of 9 mm (**Table 2**). The results of the antibiogram showed that all strains tested are sensitive to Chloramphenicol, with inhibition diameters ranging from 24 to 27 mm.

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Table 2 Minimum inhibitory concentration of the TET product

Concentration (mg/mL)	2,5	1,25	0,62	0,31	0,15	0,07	0,03	0,01	Witness
Staphylococcus aureus A	-	-	+	+	+	+	+	+	+
Staphylococcus aureus H	-	+	+	+	+	+	+	+	+
Escherichia coli A	-	-	+	+	+	+	+	+	+
Escherichia coli H	-	+	+	+	+	+	+	+	+
Klebsiella pneumoniae H	-	+	+	+	+	+	+	+	+
Bacillus subtilis A	-	-	+	+	+	+	+	+	+
Pseudomonas aeruginosa A	-	-	+	+	+	+	+	+	+

Strains H: strains isolated from the hospital environment; Strains A: ATCC strains.

Table 2 Minimal inhibitory concentrations of the **OXM** product

Concentration (mg/mL)	2	1	0.5	0.25	0,12	0,06	0,03	0,01	Witness
Staphylococcus aureus A	-	-	+	+	+	+	+	+	+
Staphylococcus aureus H	-	-	+	+	+	+	+	+	+
Escherichia coli A	-	-	-	+	+	+	+	+	+
Escherichia coli H	-	-	+	+	+	+	+	+	+
Klebsiella pneumoniae H	-	-	+	+	+	+	+	+	+
Bacillus subtilis A	-	-	-	-	+	+	+	+	+
Pseudomonas aeruginosa A	-	-	-	-	+	+	+	+	+

Strains H: strains isolated from the hospital environment; Strains A: ATCC strains.

It was noted that the two products TET and OXM have showed a weak antibacterial activity compared to the antibiotic tested (Table 1).

The results of the inhibiting activity of the synthetic chemical product OXM corroborate with the work of Dhayanithi et al.[7] Indeed, these authors have shown that some derived products of tetrazole substituted by the benzyl group have an inhibiting activity against strains of E. coli (going from 10 to 11 mm), S. aureus (from 11 to 13 mm) and B. subtilis (from 11 to 12 mm). Other tetrazole derivatives gave important antibacterial activities against the strains tested: E. coli (ranging from 22 to 24 mm), B. subtilis (from 21 to 23 mm) and S. aureus (from 15 to 20 mm), with the exception of K. pneumoniae and P. aeruginosa (less than 10 mm).

The results of the inhibiting activity of chemical product TET show that the majority of strains tested appeared resistant to chemical product TET. These results are in agreement with works of Dhayanithi, which showed that chemical products derived from tetrazole do not have any antibacterial activity or have a weak inhibiting activity (≤10 mm) against the strains studied.[7] Thus, one could deduce that the antibacterial activity of the products that we tested varies according to the substituents or the radicals related to the cycle tetrazole which can modify its chemical properties (hydrophobicity…) while influencing its mode of action.

2.2. Determination of minimum inhibitory concentrations

The results of the MIC of the TET and OXM tetrazole derivatives are shown in Tables 2 and 3. The MIC is the lowest concentration that showed no bacterial growth. The results in Table 2 show that the TET product has a low inhibitory activity on strains of S. aureus A, E. coli A, B. subtilis A and P. aeruginosa A, with a MIC of about 1,25 mg/mL. The strains isolated from the hospital environment, namely E. coli H, S. aureus H and K. pneumoniae H, showed greater resistance to TET, with a MIC of 2.5 mg/mL (Table 2).

Table 5 shows that the MBCs of product OXM vary from 0.5 mg/mL against P. aeruginosa A to 1 mg/mL against E. coli A and B. subtilis A. The strains S. aureus A, E. coli H and K. pneumoniae showed resistance opposite the OXM, with an MBC of 2 mg/mL. The strain S. aureus H showed strong resistance, with an MBC higher than 2 mg/mL (Table 5).

The works of Rao et al. [8] showed that the MBCs of tetrazole derivatives ranged from 0.5 to 1 mg/mL against S. aureus and B. subtilis strains [9], the MBC against E. coli is on the order of 6.25 mg/mL.

Table 4 Minimum bactericidal concentration of the **TET** product

Concentration (mg/mL)	2.5	1.25
Staphylococcus aureus A	-	+
Staphylococcus aureus H	+	+
Escherichia coli A	-	+
Escherichia coli H	+	+
Klebsiella pneumoniae H	+	+
Bacillus subtilis A	-	+
Pseudomonas aeruginosa A	-	+

Strains H: strains isolated from the hospital environment; Strains A: ATCC.

Table 5 Minimum bactericidal concentration of the **OXM** product

Concentration (mg/mL)	2	1	0.5	0.25
Staphylococcus aureus A	-	+	+	+
Staphylococcus aureus H	+	+	+	+
Escherichia coli A	-	-	+	+
Escherichia coli H	-	+	+	+
Klebsiella pneumoniae H	-	+	+	+
Bacillus subtilis A	-	-	+	+
Pseudomonas aeruginosa A	-	-	-	+

Strains H: strains isolated from the hospital environment; Strains A: ATCC.

The calculation of the MBC/MIC ratio of the products TET and OXM showed that the two products have a bactericidal effect on the strains tested.

3. Conclusion

The aim of this work was to study the antibacterial activity of two tetrazole derivatives, TET and OXM, against isolated strains of the hospital environment and reference strains. The evaluation of the antibacterial activity of the OXM product using the disk diffusion method showed an inhibitory activity against all of the studied strains: Escherichia coli A (15 mm), P. aeruginosa A (12.33 mm), E. coli H (11 mm), B. subtilis A (9.33 mm), K. pneumoniae H (9.66 mm), S. aureus A (9 mm) and S. aureus H (8 mm). Indeed, the OXM product has an important activity against strains tested, with MIC ranging from 0.25 to 2 mg/mL. The TET product showed no antibacterial activity against the strains studied, except for P. aeruginosa which showed a low sensitivity of 9 mm. The MICs of the TET product range from 1.25 to 2.5 mg/mL. Moreover, the results of MBC of the products tested showed that the two products have a bactericidal effect against the strains tested. Finally, as a perspective of this work, the products derived from tetrazole can be modified chemically or combined with other molecule credits in order to increase their spectrum of activity or to test their effects on cell cultures.

4. Materials and methods

4.1. Chemistry

These compounds being studied are tetrazole substituted in position 5 by an electron-withdrawing group 5-(4-chloro-phenyl)-1*H*-tetrazole (**TET**) and its precursor 4-chlorobenzaldehyde oxime (**OXM**) [7].

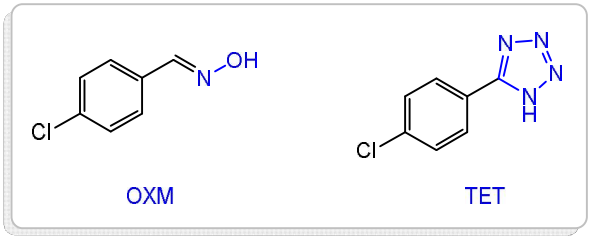

Fig 1. Compounds used in antibacterial study

4.2. Biology

This study was conducted in the Biotechnology Laboratory at the Faculty of Sciences, Sidi Mohamed Ben Abdellah University–Fez, from January 18 to May 20, 2016. We chose strains isolated from the hospital environment and identified by our team [2-6]:

- Escherichia coli **(**E. coli H)
- Staphylococcus aureus **(**S. aureus H**)**
- Klebsiella pneumoniae **(**K. pneumoniae H)

These strains were isolated, purified and stored at −80°C in the Regional Laboratory for Epidemiological Diagnosis and Environmental Health (LRDEHM) of the Fez-Boulmane region in Fez.

ATCC reference strains:

- Staphylococcus aureus ATCC 29213 (S. aureus A)
- Escherichia coli ATCC 25922 (E. coli A)
- Bacillus subtilis ATCC 3366 (B. subtilis A)
- Pseudomonas aeruginosa ATCC 27853 (P. aeruginosa A)

These strains were subcultured by exhaustion on Luria Bertani medium (LB) and incubated at 37°C for 24 h.

➢ ***Highlighting of antibacterial activity: antibiogram***

The antibiogram or the diffusion method is one of the oldest approaches for determining the susceptibility of bacteria to antibiotics and remains one of the most commonly used methods in medical analysis laboratories. It is recommended by the European Committee for Antibacterial Susceptibility Testing [10] and the National Committee for Clinical Laboratory Standards.[11]

4.2. 1. Principle

This method consists in depositing a disk impregnated with the antibacterial agent in a Petri dish previously seeded by specific bacterial species. The agent will diffuse into the agar, creating a halo of growth inhibition of the bacteria around the disc. Antibacterial activity by disk diffusion of the synthetic chemicals tested, TET and OXM, was performed according to

the protocol recommended by EUCAST and CLSI, with some modifications.[10-11]

➢ ***Preparation of solutions of derivatives of tetrazole***

10 mg samples of the chemicals, TET and OXM, were dissolved in 400 µL of DMSO to obtain a final concentration of 25 mg/mL.

➢ ***Preparation of the inoculum***

From a pure bacterial culture of 24 h on LB medium, three to four colonies were removed with the aid of a loop and then transferred into 4 mL of sterile physiological saline (0.9%). The bacterial suspension was homogenised. The turbidity of the bacterial suspension was adjusted to that of the McFarland 0.5 standard, which corresponds to an inoculum of a bacterial load of approximately 1×10^8 CFU/mL for Escherichia coli.[11]

➢ ***Inoculation of boxes***

Inoculation was performed on Mueller Hinton medium (Appendix 4) by swabbing. A sterile cotton swab was plunged in the bacterial suspension. Excess liquid was removed by rotating the swab on the walls of the tube. Spreading out on limps is performed by tight streaks on the agar surface in three directions.

➢ ***Deposit of the discs***

Discs of paper Whatman, N°1 with a diameter of 6 mm were prepared and autoclaved for 20 min at 121°C. The discs were then soaked in 10 µL of TET or OXM solutions. They were then firmly deposited on the surface of the dishes inoculated with the bacterial strains tested (S. aureus H, E. coli H, K. pneumoniae H, S aureus A, E. coli A, B. subtilis A and P. aeruginosa A). The discs soaked in Chloramphenicol (30 µg/mL) were used as positive witnesses. The negative witness is a disc containing 10 µL of DMSO. The dishes are incubated at 37°C for 24 h. Each test was carried out in three repetitions.

After 24 h of incubation, the product endowed with an antibacterial activity formed a halo of growth inhibition of the bacteria around the disc. Inhibition diameters were measured in (mm) disc inclusive.

4.3. Determination of the minimum inhibitory concentration in liquid medium

The minimum inhibitory concentration (MIC) is defined according to the Committee of Antibiogram of the French company of Microbiology (CA-SFM) as being the weakest concentration which involves the inhibition of the visible bacterial growth.[10]

4.3.1. Principle

The determination of the minimum inhibitory concentration was carried out by the preparation of a series of dilutions of ½ of the antimicrobial agent to be tested on solid or liquid medium. The MIC is the lowest concentration of the antibacterial agent present in the tube, well or can which shows no visible bacterial growth.[11]

4.3.2. Protocol

4.3.2.1. Preparation of the solutions of the stock of the chemical products

- 32 mg of OXM was dissolved completely in 1 mL of DMSO. Then, 3 mL of medium BHI was added to obtain a final concentration of 8 mg/mL.
- 40 mg of TET was dissolved completely in 1 mL of DMSO. Then, 3 mL of medium BHI was added in order to obtain a final concentration of 10 mg/mL.

The MIC was determined by the method of dilution in liquid medium by macrodilution and microdilution using medium BHI.[12]

4.3.2.2. Determination of the MIC by dilution in liquid medium: macrodilution

The macrodilution method consists of preparing dilution series in test tubes with a minimum final volume of 1 mL.[11] Determination of the MIC of synthetic TET and OXM by macrodilution is performed according to the protocol recommended by CLSI [11], with some modifications.

4.3.2.2.1. Preparation of the series of dilution of products TET and OXM

500 µL of medium BHI was distributed in sterile test tubes. Then, 500 µL of the solution of the stock of TET and OXM was added in the first tube. After agitation, a series of dilution in cascade was carried out by the addition of 500 µL of the solution of the first tube to the second and so on to the last tube where 500 µL were eliminated to have same volume in all tubes. The concentrations obtained reduced from 4 to 0.031 mg/mL for OXM and from 5 to 0.039 mg/mL for TET.

4.3.2.2.2. Preparation of the inoculums

From an 18 h old préculture in medium BHI of the stocks tested (S. aureus H, E. coli H, K. pneumoniae H, S. aureus A, E. coli A, B. subtilis A and P. aeruginosa A), a bacterial suspension is prepared in the same medium BHI.

The optical density was adjusted from 0.1 to 600 nm, which corresponds to a bacterial load of 10^8 CFU/mL. Then, 5 mL of the inoculum of the strains tested was prepared by adding 4.950 mL of the BHI medium and 50 µL of the bacterial suspension in order to dilute the bacterial load by 1/100 to have a final concentration of 10^6 CFU/mL. 500 µL of the bacterial inoculum was added in the tubes containing the series of dilution. The final volume in tubes is 1 mL, and the bacterial load is 5×10^5 CFU/mL. The final concentrations reduced from 2 to 0.015 mg/mL for OXM and from 2.5 to 0.019 mg/mL for TET.

A negative control tube was realised; it contains the bacterial inoculum with 5×10^5 CFU/mL. All tubes were incubated at 37°C for 24 h.

The MIC corresponds to the tube containing the weakest concentration of the product tested which did not show any visible bacterial growth.

4.3.2.3. Determination of the MIC by dilution in liquid medium: microdilution

The microdilution method consists of preparing dilution series in a 96-well polypropylene microplate. MIC determination by microdilution was performed according to the protocol recommended by CLSI.[13]

4.3.2.3.1. Realisation of the dilution series

100 µL of BHI medium was distributed in all wells, except those in the first line. Then, 200 µL stock solutions of TET and OXM products were added in the first line. The dilution series was carried out by taking 100 µL of the first well from the first column and adding it to the second well belonging to the same column, until the penultimate well. The same steps were repeated for the other columns.

4.3.2.3.2. Preparation of the inoculum

The preparation of the inoculum was carried out as described above for macrodilution, except that we prepared 2 mL of the inoculum instead of 5 mL. The wells were inoculated with 100 µL of the bacterial suspension with a concentration of 106 CFU/mL. The wells in the last line of the microplate contain only the inoculum (control). The microplate was incubated at 37°C for 24 h.

The MIC corresponds to the well containing the lowest concentration of the product tested, which showed no visible bacterial growth.

4.4. Determination of the minimum bactericidal concentration (MBC) in solid medium

The inhibiting minimal concentration corresponds to the weakest concentration of the product tested able to kill 99.9% of the bacteria and to let push only 0.01%.[14]

4.4.1. Protocol

From the tubes and wells used for the determination of the MIC, 5 µL of each tube or well was deposited on LB medium and then spread by streaks and incubated at 37°C for 24 h. The MBC was considered to be the lowest tested product concentration showing no growth.[13] The calculation of the MBC/MIC ratio makes it possible to evaluate whether an antibacterial agent has a bactericidal (MBC/MIC < 4) or bacteriostatic (MBC/MIC > 4) effect.[15-16]

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