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Antibacterial Effect of Bacillus Strains and Partial Characterization of Their Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Author IZ designed the study, Conducted the experiments and wrote the first draft of the manuscript. Authors AH and MI supervised the work. Author SI supplied the necessary equipment for work. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The focus of this study was to isolate and to identify strains with antibacterial activity followed by a partial characterization of their extracts.
Study Design: Screening and identification of bacteria having an anti-mycobacterial effect from soil and water of different biotopes of Fez Morocco were performed and active substances responsible for the biological activity were partially characterized.
Place and Duration of Study: The study was carried out at laboratory of Microbial Biotechnology, Department of Biology, Faculty of Sciences and Technical, University Sidi Mohamed Ben Abdellah, BP 2202, Road of Immouzer, Fez, Morocco, during the period from January 2011 to October 2011.
Methodology: Samples of soil and water of different biotopes of Fez Morocco were explored to isolate compounds-producing microorganisms. The inhibitory spectrum of the isolated bacteria was evaluated against *M. smegmatis, M. aurum, S. aureus, S. haemolyticus, B. subtilis, E. coli DH5α, P. aeruginosa* and *Erwinia chrysanthemi* by using agar well diffusion test and/or a modified spot-on-lawn assay. Identification of strains was executed on the basis of Gram stain, biochemical characteristics and PCR followed by DNA sequencing of 16S ribosomal RNA gene. Crude extracts obtained after precipitation by ammonium sulfate were exposed to proteolytic enzymes (Pepsin and

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proteinase K) and to heat treatment at 100ºC (15 min), 80 ºC (30 min), 37ºC (3h) and 4ºC (1 month). The residual activity after every treatment was assessed by agar well diffusion method.
Results: Six bacteria were isolated from different biotopes of Fez Morocco having an antibacterial effect against *M. smegmatis, M. aurum*, Gram-positive and Gram-negative bacteria. Based on biochemical characterization and 16S rDNA sequence analysis, it was revealed that the isolates belong to the genus Bacillus. The antibacterial activity of three of them was fully affected by proteases and heat treatment at 80ºC and 100ºC but it was stable at 4ºC for a month and 37ºC during 3h.
Conclusion: The lost of the activity suggests a proteinaceous nature of the bio-active compounds, which might be useful in the development of antibacterial agents after their total purification in further work against bacterial infections.

Keywords: Bacterial resistance; antibacterial compounds; Bacillus.

ABBREVIATIONS

B: Bacillus, M: Mycobacterium, E: Escherichia, LB: Luria-Bertani, S: Staphylococcus, P: Pseudomonas.

1. INTRODUCTION

The dissemination of multidrug-resistant pathogenic bacteria represents a serious public health problem worldwide [1]. In nowadays, more than 70% of the species of the bacteria which cause infections are resistant at least to one of the antibiotics commonly used on therapeutics [2]. Thus, the development of alternative antibiotics is urgently needed to improve the management of bacterial infections [1]. For this reason, over than 1000 different bacteria (including actinobacteria), fungi and algae have been investigated [3]. The results of this extensive screening have been the discovery of about 4000 antibiotic substances, many of which have found applications in human and veterinary medicine [3].

Moroccan biotopes are known by its biological diversity but there are only few studies on the microbial diversity of this country [4-7]. This is why we present here antibacterial compounds producing bacteria isolated from different ecological zones of Fez (Morocco) belonging to *Bacillus* which is an interesting genus to investigate for antimicrobial activity since *Bacillus* species produce a large number of antibiotics [1]. The best-characterized are subtilin of *B. subtilis*, megacin of *B. megaterium,* gramicidin of *B. brevis,* circulin of *B. circulans,* laterosporin of *B. laterosporus,* bacitracin of *B. licheniformis* and polymyxin of *B. polymyxa* [8-12]. *Bacillus* antibiotics differ in their structures, as well as spectrum of activity. Some strains of *Bacillus* synthesize bacteriocines, which are only effective against bacteria of the same genus, others produce antibiotics against Gram-negative bacteria and still other strains have a wide spectrum of antibiotic activity [13].

Thus, the objectives of this work include: (a) Screening for antibacterial compounds-producing bacteria, (b) Evaluating antimicrobial activity of the isolated bacteria against Gram positive and negative bacteria, (c) Identifying strains on the basis of Gram stain, biochemical characteristics and PCR followed by DNA sequencing of 16S ribosomal RNA gene and (d) Partially characterizing the secreted substances.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Media

Mycobacterium smegmatis MC² 155 and *M. aurum* A+ are non-pathogenic atypical bacteria [14]. These strains were used as a model to evaluate the effect of active substances on the growth of mycobacteria [15]. They were kindly provided by Dr. Suzana David (Centro de Tuberculose e Micobactérias Instituto Nacional de Saúde Dr. Ricardo Jorge Delegação do Porto, Portugal).

Staphylococcus aureus [16]; *Staphylococcus haemolyticus* [4], *Bacillus subtilis* ILP 142B [16]; *Pseudomonas aeruginosa* [16]; *Escherichia coli* DH5α (Microbial biotechnology laboratory of Techniques and Sciences Faculty, Fez Morocco); *Erwinia chrysanthemi* 3937 [17]. This bacterium was friendly provided by Dr. Hassouni

(LCB-CNRS-Marseille). These strains were propagated in Luria-Bertani (LB) at 37ºC or at 30ºC for *Erwinia chrysanthemi*.

Bacteria were stored at -70ºC in Luria-Bertani (LB) broth supplemented with 25% glycerol. Throughout the experiments, they were cultured every week on LB agar (10g of peptone, 5g of yeast extract, 10g of NaCl, 15g of agar per liter of distilled water, pH 7) and held at 4ºC [18].

Different media in broth or on agar plates were used including respectively LB medium and YPG medium containing 20g of peptone, 10g of yeast extract, 20g glucose, 60µg/ml of ampicillin and 30µg/ml of kanamycin per liter of distilled water [18].

2.2 Screening and Isolation of Microorganisms

Different samples of water and soil were collected from ecological areas of Fez Morocco and treated independently according to the method followed by Hassi et al. [4]. Colonies that showed clear zones of inhibition against *M. smegmatis* were picked up and transferred to LB agar plates. These were incubated at 37ºC and stored at 4ºC for later assays [4].

2.3 Anti-Mycobacterial Activity Assay

Anti-mycobacterial activity was performed by two different methods, that is, (a) agar well diffusion assay as was led by Muriana and Klaenhammer [19]. In this method, inhibition zone around wells of each isolate prepared in LB broth at $OD_{595\ nm}$ = 0.3 was evaluated by measuring its diameter on sterile Petri dishes containing LB medium and pre-inoculated with a broth culture of the indicator microorganism *M. smegmatis* at $OD_{595\ nm}$ = 0.3. (b) A modified spot-on-lawn assay as was described in Zahir et al. study [7], where a colony of the isolated strain was spotted onto the surface of LB agar plates which had been already spread with 0.1mL of overnight-cultured *M. smegmatis* in LB broth [7]. In both cases, plates were incubated at 37ºC for 24h and the anti-mycobacterial activity was detected by the observation of inhibition area surrounding the test strain. These assays were done three times and they were also carried out to evaluate the inhibitory activity of *E. coli* DH5α used as a control.

2.4 Inhibitory Spectrum of the Isolated Strains

Spot on lawn assay was used to evaluate the inhibitory spectrum of the isolated bacteria. Gram-positive and negative bacteria were assayed comprising *M. aurum, S. aureus*, *S. haemolyticus, B. subtilis*, *E. coli* DH5α, *P. aeruginosa* and *Erwinia chrysanthemi*. The assay was repeated three times.

2.5 Identification of Antibacterial Compounds-Producing Strains

Antibacterial compounds producing bacteria were examined for cellular morphology and Gram characteristics. The biochemical identification was also performed according to Bergey's Manual of Determinative Bacteriology [20].

Furthermore, the isolates were identified by molecular methods. These comprise 16S ribosomal RNA gene amplification by PCR and sequencing. The PCR amplification was performed with universal primers RS16 (5' TACGGCTACCTTGTTACGACTT 3') and fD1 (5' AGAGTTTGATCCTGGCTCAG 3') targeted against conserved regions of 16S rDNA [21]. The amplification protocol was led as described by Zahir et al. [6]. PCR amplicons were purified and sequenced using the Big Dye Terminator with primers (reverse and forward) while automated sequencing of both strands of the PCR products was done on a BIOSYSTEME 3130 automated gene sequencer [22].

Identification analysis was realized by an alignment of consensus sequence of the 16S rDNA genes collected in an international database (Genebank) present at the NCBI website located at http:// www.ncbi.nlm.nih.gov/BLAST. The results were then expressed in percentage of homology between each submitted sequence and the sequences resulting from the database.

2.6 Characterization of the Nature of the Antibacterial Metabolites

2.6.1 Precipitation of the antibacterial substances

The bioactive substances of three isolates were precipitated by using ammonium sulfate (80% ammonium sulfate) as was described by Hassi et

al. [5]. The antibacterial activity was evaluated by agar well diffusion assay which was triplicate by using *M. smegmatis* as an indicator strain. It was also carried out to evaluate the inhibitory activity of ammonium sulfate crude extract of *E. coli* DH5α which was used as a negative control [5].

2.6.2 Physico-chemical characterization

2.6.2.1 Thermostability

To check the thermal stability, strains ammonium sulfate extracts were exposed to 100ºC (15 min), 80ºC (30 min), 37ºC (3h) and kept at 4ºC (1 month). The residual activity was checked by agar well diffusion assay which was done three times by using *M. smegmatis* as an indicator strain. The average of the inhibition zone diameter was calculated [23].

2.6.2.2 Effect of proteolysis activity on the crude extract

Pepsin (Sigma) and proteinase K (Sigma) were tested for their proteolysis activity on the crude ammonium sulfate extracts of the antibacterial compounds from the bacterial strains. The assay was performed at a final concentration of 1mg/ml, respectively at pH 3 and 7 [24,25]. Samples with and without enzymes were held at 37ºC for 3h and the remaining activity was determined by agar well diffusion assay by using *M. smegmatis* as an indicator strain. The assay was done three times and the average was calculated. Extracts not treated by proteases or by heat were used as controls.

3. RESULTS AND DISCUSSION

3.1 Screening, Isolation of Microorganisms and Anti-Mycobacterial Activity Assay

The screening of bacteria isolated from water and soil of different ecological areas of Fez morocco showed six isolates having inhibitory properties by agar diffusible metabolites against *M. smegmatis*. After that, this anti-mycobacterial activity was confirmed by both spot-on lawn assay and agar well diffusion assay. Between the isolates, C alpha 1 and ZI 9 were the bacteria that showed the largest diameters of inhibition of about 34±1mm and 28±0.5 mm, respectively (Table 1).

Table 1. Anti-mycobacterial activity assay of the isolates

Isolate	Inhibition by spot on lawn assay	Diameter of inhibition zone by agar well diffusion assay (mm)
Bêta	+	12±0.1
C alpha 1	**+**	34±1
L4	+	14±1
ZH	+	8±1
H	+	8±0.5
ZI 9	+	28±0.5
E. coli DH5α (control)	-	0

Relative activity of the isolated strains was measured by both agar well diffusion test and spot on lawn assay against M. smegmatis. (-): no inhibition; (+): inhibition

3.2 Inhibitory Spectrum of the Isolated Strains

Spot-on-lawn assay was performed to assess the antagonistic activity of the isolates against indicator strains including *M. aurum, E. coli* DH5α, *S. haemolyticus, S. aureus*, *B. subtilis*, *P. aeruginosa* and *Erwinia chrysanthemi*. The antibacterial effect differed from an isolate to another. In fact, L4 showed a broad spectrum against all the indicator bacteria, while the isolates Bêta and H acted only against *M. aurum* and *M. smegmatis* which may indicate that their bio-actives substances are specific to mycobacteria. It is also noted that none of the studied bacteria had inhibited the growth of *Staphylococcus* sp. and *P. aeruginosa* with the exception of L4. However, all the isolates had an anti-mycobacterial effect (Table 2 and Fig. 1).

3.3 Identification of Antibacterial Compounds-Producing Strains

After PCR amplification of the 16S rRNA gene and DNA sequencing, sequences obtained with RS16 and fD1 primers had different sizes, between 371 and 630bp. In literature, some researchers have made identification using sequences of about 500bp [26] or even less than 200bp [27].

According to the criteria defined by Drancourt and collaborators [28], BlastN search showed that the partial sequences of 16SrRNA gene of the isolated strains belong to the genus *Bacillus* (Table 3).

Moreover, all the strains were Gram positive bacilli, motile, spore forming organisms and able to grow at 50ºC on LB agar which confirms partial sequence alignment of 16S rDNA data. However, in order to determine whether L4 and H belong to *B. subtilis* or *B. amyloliquefaciens*, biochemical characteristics were examined according to Bergey's manual of determinative bacteriology [20]. Thus, these two bacteria were catalase positive and able to hydrolyze starch, pectins and urea. Acetoine was produced from glucose and citrate was metabolized as sole source of carbon. NaCl was tolerated at a concentration of 6.5% but growth didn't occur at 55ºC. Whereas, the species *B. amyloliquefaciens* which related to *B. subtilis* is unable to hydrolyze pectins and to split urea [29] (Table 4).

In accordance with the literature, these results suggest that these two bacteria belong to the species *B. subtilis* [8,11,20,30,31]. Therefore, based on the morphology, cultural and biochemical characteristics described above, together with phylogenetic analysis, the bacteria L4 and H have been classified as a member of *B. subtilis*.

The majority of natural antibacterial agents are produced by Gram positive bacteria such as *Actinomycetes* and *Bacillus*. In fact, approximately 85% of bioactive substances are synthesized by *Actinomycetes*, 11% by fungi and 4% by bacteria, namely *Bacillus* [32,33].

The distribution of *Bacillus* is ubiquitous due to their high resistance in the external environment. Their main biotope is the soil but they can also exist in fresh water and plants [34], which justify their isolation, from different ecological zones of Fez Morocco (water and soil).

Four bacteria from the isolated *Bacillus* belong to *B. subtilis*, a remarkable species deemed by its broad antimicrobial spectrum due to its ability to produce different antibiotics [8,10,12]. Only the isolate L4 exhibited activity against *P. aeruginosa.* The reason for this includes, mainly, the external membrane presence in this bacterium that delays the antibiotic entrance in cell [2]. However, it was previously demonstrated that *B. subtilis* inhibited not only the growth of *P. aeruginosa* [1,35], but also the growth of several bacterial species such as *E. coli* [1,35], *Erwinia* sp. [35], *S. aureus* [1,13,36], *S. haemolyticus* [1] and *M. smegmatis* [36] which emphasizes deeply our findings.

The isolates C alpha1, L4 and ZH prevented also the growth of *B. subtilis*. It's known that some substances produced by *Bacillus* sp. are active against the same or closely related species [1,13].

In the other hand, various investigations had shown that *B. licheniformis* was able to inhibit the growth of mycobacteria such as *M. phlei* and *M. tuberculosis* [37]. However, it didn't act against the bacteria Gram-negative according to the study of Hussein and AL-Janabi [33] highlighting that *B. licheniformis* was unable to inhibit *E. coli*. These results corroborate with our outcomes which had shown more its inability to inhibit *Erwinia chrysanthemi* and *P. aeruginosa*.

It is noteworthy that the mycobacteria show a resistance to bacitracin produced by *B. subtilis* and *B. licheniformis*. For this reason, this antibiotic is not used to treat tuberculosis or other mycobacterial infections. Therefore, we could say that our isolates possess bio-active substances having mode(s) of action different from that of bacitracin.

Moreover*, B. amyloliquefaciens* is known by its use as a factor of bio control of phyto-pathogenic microorganisms [9]. In fact, Ryu and his collaborators [38] had observed that the butanediol, produced by *B. amyloliquefaciens* IN937, had fallen significantly the impact of the disease caused by *Erwinia carotovora* on the seeds of *Arabidopsis*. Similarly, Lisboa et al. [9] had also shown the inhibitory activity of a bacteriocin produced by this microorganism toward multiple bacteria including *B. subtilis* ATCC 9372, *E. coli* and *Erwinia carotovora* [9], but not against *Staphylococcus* spp. [9] and *P. aeruginosa* [39] which joins deeply our data. Nevertheless, to the best of our knowledge, this is the first report of isolation of *B. amyloliquefaciens* showing an inhibitory effect against mycobacteria.

Table 2. Activity spectrum of the isolates

Isolate	Inhibitory effect against:							
	M. smegmatis	***M. aurum A+***	***E. coli* DH5α**	***S. haemolyticus***	***S. aureus***	***B. subtilis***	***P. aeruginosa***	***Erwinia chrysanthemi***
Bêta	+	+	-	-	-	-	-	-
C alpha 1	+	+	-	-	-	+	-	-
L4	+	+	+	+	+	+	+	+
ZH	+	+	+	-	-	+	-	+
H	+	+	-	-	-	-	-	-
ZI 9	+	+	+	-	-	+	-	+

Inhibitory spectrum of the isolated bacteria was investigated by using spot on lawn assay on LB agar. Inhibition was scored based on an abstract scale as follows :(-), no inhibition; (+), presence of inhibition zone

Table 3. Identification of the isolates

Isolates under study	Bacterial species showing a high degree of sequence similarity with the isolates under study	Size of the sequenced fragment using primer RS16 (bp)	% of similarity obtained using primer RS16	Size of the sequenced fragment using primer fD1 (bp)	% of similarity obtained using primer fD1
Bêta	*Bacillus licheniformis* (EU445292.1)*	394	100	371	98
C alpha 1	*Bacillus subtilis* (EU334513.1)*	530	99	550	99
L4	*Bacillus subtilis* (EU723210.1)*		99		99
	Bacillus amyloliquefaciens (EU359773.1)*	609	99	590	99
ZH	*Bacillus subtilis* (EU266071.1)*	599	99	630	99
H	*Bacillus subtilis* (EU240965.1)*		100		99
	Bacillus amyloliquefaciens (EU334107.1)*	618	100	612	99
ZI 9	*Bacillus amyloliquefaciens* (JN661699.1)*	589	99	620	99

**Access number National Center for Biotechnology Information (NCBI); bp: base pairs*

3.4 Characterization of the Nature of the Antibacterial Metabolites

3.4.1 Precipitation of the antibacterial substances

Since the isolates C alpha 1, L4 and ZI 9 showed the highest activity against *M. smegmatis,* they were selected for further work. The extraction was done by ammonium sulfate which is widely used to extract antimicrobial compounds from Gram positive and negative bacteria [5,9,19,40]. The crude extracts of the antimycobacterial substances prepared from C alpha 1, L4 and ZI 9 were tested against *M. smegmatis*. Thus, the antimycobacterial assays showed inhibition zones in which the diameters were 32±1, 16±0.5 and 34±1mm, respectively (Fig. 2). While the crude extract of *E. coli* DH5α used as control didn't exhibit any inhibitory activity against the indicator strain, which suggested that C alpha 1, L4 and ZI 9 acted by substances secreted in the medium.

3.4.2 Physico-chemical characterization

The antibacterial activity was tested for its sensitivity towards proteases and heat treatment (Table 5). The anti-mycobacterial effect was conserved at 4ºC and 37ºC but it was severely altered at temperature higher than 80ºC and after proteolysis, suggesting that a peptide moiety is associated with the activity of the strains compounds.

These results are in accordance with previous studies mentioning that *B. subtilis* species are well reputed as producers of a great number of antimicrobial compounds with different structures in which peptide antibiotics represent the predominant class [1]. Effectively, it was underlined in the investigation of Stöver and Driks, in 1999 [35] that *B. subtilis* PY79 secreted a protein named Tas-A, which inhibited the growth of several bacterial species such as *E. coli, S. epidermis*; *Enterobacter* sp. *P. aeruginosa* and *Erwinia* sp. [35]. It was shown elsewhere that *B. subtilis* C1 synthesized a lipopeptide N1 possessing an activity against *M. smegmatis* and *S. aureus* [36]. Furthermore, *B. subtilis* produced also many bacteriocins, namely, subtilosin A which was considered as a good candidate in food preservation, as it showed a strong antimicrobial activity against food-borne pathogens [39,41,42].

Table 4. Results of biochemical tests of the isolates L4 and H compared to those of the literature of *B. subtilis* and *B. amyloliquefaciens* [8,29,32]

Biochemical characteristic	Results for:			
	L4	H	*B. subtilis*	*B. amyloliquefaciens*
Catalase test	+	+	+	+
Citrate test	+	+	+	+
Voges-Proskaue test	+	+	+	V
Hydrolysis of starch	+	+	+	+
Hydrolysis of pectins	+	+	+	-
Urea splitting	+	+	+	-

+, Positive reaction; -, negative reaction

Besides, it had reported that *B. amyloliquefaciens* is known by its production of several antimicrobial peptides whose action is abolished under the effect of proteases and after heat treatment [9,32,42-44]. For instance, the bacilysin formed of two amino acids with a wide antibacterial activity [45] and the subtilosin which is a bacteriocin of 35 amino acids having an anti-bacterial effect by disrupting the lipid bi-layer of the cell membrane and causing intracellular damage possibly leading to cell death [39,42,46]. The cyclic lipopeptides are also figured among bio-actives compounds synthesized by *B. amyloliquefaciens* including fengycin formed of ten amino acids and a lipid attached to the N-terminal end of the molecule [32,47] and iturin group formed of seven α-amino acids and one β-amino fatty acid [9]. These substances have been used as biological control agents to suppress fungal plant pathogens [9,32,47]. The surfactin is also a cyclic lipopeptide which had been demonstrated to exhibit antimicrobial, antiviral, antifungal, and hemolytic properties by modifying the integrity of membranes [32,47]. Until now, among this list of substances, the surfactin is the only molecule whose the anti-mycobacterial effect has been proven with a CMI ranging from 5 to 25 µg/ml against *M. smegmatis*, *M. avium* and *M. phlei* [36,48,49]. Consequently, the metabolites secreted by *B. amyloliquefaciens* highlighted during this present work might correspond to surfactin or to its combination with the other molecules mentioned above, or even to a new peptide.

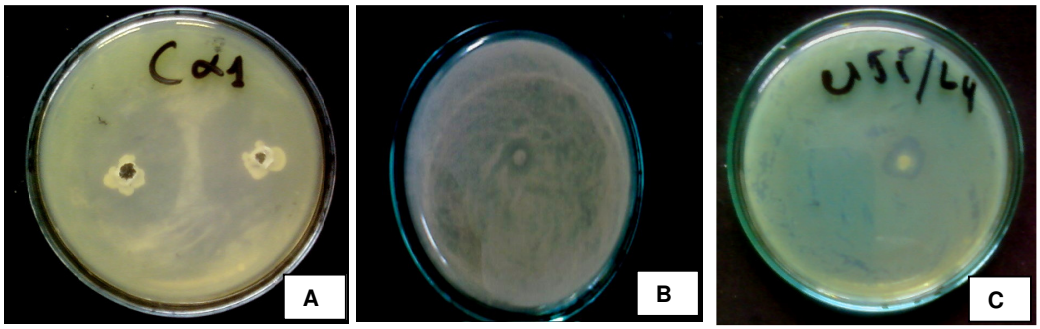

Fig. 1. Antagonistic activity of the isolated bacteria. (A): The effect of isolate C alpha 1 on *M. smegmatis* evaluated by agar well diffusion test; (B) and (C): the effect of isolate L4 against *E. coli* and *P. aeruginosa,* respectively, shown by spot on lawn assay. After incubation, the antibacterial activity was detected by the observation of an inhibition area

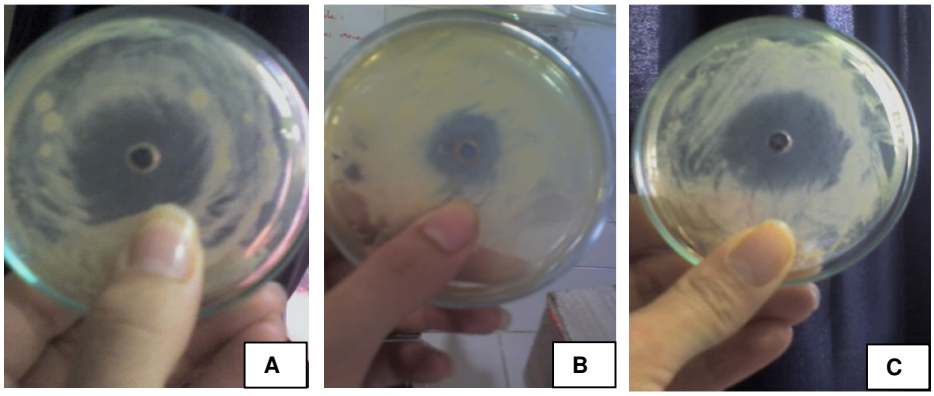

Fig. 2. Photos showing the inhibition zones against *M. smegmatis* of C alpha 1 (A), L4 (B) and ZI9 (C) extracts, respectively. The anti-mycobacterial effect was evaluated by agar well diffusion assay

Table 5. Influence of temperature and proteases on the activity of C alpha 1, L4 and ZI 9 extracts

Treatment	Antibacterial activity of C alpha 1, L4 and ZI 9 (%)
Enzymatic treatments	
Proteinase K	0
Pepsin	0
None (positive control)	100
E. coli DH5α (negative control) Heath treatment	0
4ºC for a month	100
37ºC for 3h	100
80ºC for 30 min	0
100ºC for 15 min	0
None (positive control)	100
E. coli DH5α (negative control)	0

Relative activity was measured by well-diffusion agar test against M. smegmatis

4. CONCLUSION

Six bacteria belonging to *Bacillus* were isolated from soil and water of different biotopes of Fez Morocco. They displayed a spectrum of activity against Gram positive, Gram negative bacteria and/or mycobacteria. Further work is required to purify the bioactive compounds in order to establish their chemical structures, followed by other investigations aiming to seek for the efficacy, safety, toxicology, and the pharmacodynamic properties of these substances *in vivo*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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