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# Antibacterial Effect of *Pseudomonas aeruginosa* Isolated from a Moroccan Hot Spring Discharge and Partial Purification of its Extract

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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. Author AH supervised the work. Author SI supplied the necessary equipment for work. 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 microorganisms possessing an antibacterial activity followed by a partial purification of their extracts.
**Study Design:** Screening and identification of bacteria with an antibacterial effect were performed and active substances responsible for the biological activity were localized and partially purified.

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**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 February 2010 to August 2010.
**Methodology:** Samples of a hot spring discharge localized in the city Fez Morocco were explored to isolate compounds-producing microorganisms. The inhibitory spectrum of the isolate was evaluated against *M. smegmatis, M. aurum, S. aureus*, *S. haemolyticus, B. subtilis*, *B. licheniformis, B. amyloliquefaciens, E. coli* DH5α and *Erwinia chrysanthemi* by using agar well diffusion test and/or a modified spot-on-lawn assay. Identification of strain was executed on the basis of Gram stain, biochemical characteristics and PCR followed by DNA sequencing of 16S ribosomal RNA gene. Crude extract of the isolate was obtained by using ethyl acetate and was exposed to proteolytic enzymes (pepsin and proteinase K) and to heat treatment at 121°C (60 min), 100°C (20 min), 80°C (30 min), 37°C (3h) and kept at 4°C (six months). The antimycobacterial effect before and after every treatment was assessed by agar well diffusion method. Synthesis of antibacterial compounds was monitored during the isolate growth cycle. The extract was then fractionated by thin layer chromatography and the bioactivity was investigated with a bioautography technique followed by spots elution test.
**Results:** One bacterium was isolated having a broad antagonistic effect against all the tested bacteria. Based on biochemical characterization and 16S rDNA sequence analysis, the strain was identified as *Pseudomonas aeruginosa*. The antibacterial compounds were synthesized during the exponential growth phase and were not affected following heat treatment and proteases that indicated the non-proteinaceous nature of the active agents. The crude extract developed in chloroform: acetone (9:1) showed metabolite (s) at Rf = 0.68 which it may be pyocyanin, inhibiting the growth of *M. smegamtis*.
**Conclusion:** Metabolites of *P. aeruginosa* responsible for the sought effect were localized and characterized. These compounds might provide an alternative bio-resource for the bio-control of plant pathogens after their total purification in further investigation.

*Keywords: Bio-control of phytopathogens; Pseudomonas aeruginosa; thin layer chromatography*.

## ABBREVIATIONS

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

## 1. INTRODUCTION

Plants diseases caused by soil-borne as well as seed borne pathogens account for major crop losses worldwide [1,2]. Among them, *Erwinia amylovora,* the causative agent of fire blight on pome fruits and many ornamental plants from *Rosaceae* family [3], *Erwinia carotovora* identified as the responsible agent of stem rot and vascular wilt symptoms of hydroponically grown plants [4], *Erwinia chrysanthemi* associated with tuber soft rot and blackleg [5]. *Erwinia* spp rely mainly on the production of large quantities of extracellular cell wall degrading enzymes to cause diseases [5]. Additional bacteria have been involved in plant pathology. For instance, *Bacillus subtilis* causing soft rot of Mango [6], *Bacillus* sp. generating pistachio dieback [7] and *Mycobacterium* sp. was linked with Alfalfa bacterial wilt disease [8].

In Morocco, plant diseases caused by phytopathogenic bacteria are a major problem that impacts many valuable agricultural crops, causing damage in yield potential each year and significant economic losses [9]. For example, in 2006, the fire blight was first observed in pear in Ain orma, region of Meknes, Morocco. Since then, the disease has progressed most of rosaceous region affecting a total area of about 4000 ha causing serious economic losses menacing the national production of rosaceous plant [3].

At present, chemical control remains the main measure for the treatment of infected crops [1,10]. However, irrational and excessive use of agrochemicals has led to deteriorating human health, environmental pollution, and development of pathogen resistance to bactericides [10,11]. On account of these problems, significant advances in control plant diseases have been achieved both in research and application by the use of biological control [10] which has drawn much attention in recent years [1]. In fact, in the last decade, many bacteria such as *Bacillus* and *Pseudomonas* spp. were reported to induce systemic resistance against a broad spectrum of soil-borne and foliar pathogens [2,10,12]. The plant and bacterial interactions in the rhizophere are important for plant health and resistance to diseases in an eco-friendly way [12]. Recently, *Pseudomonas* species have played a major role in plant disease suppression [10]. Interestingly, the antimicrobial activity of Pseudomonads (e.g. *P. aeruginosa, P. aureofaciens, P. fluorescens, P. putida* and *P. cepacia*) has been attributed to diverse secondary metabolites such as phenazines, iron chelators, lytic enzymes, hydrogen cyanide and non-nitrogen-containing compounds [1,13,14,15]. Phenazines are a large family of colorful, nitrogen-containing tricyclic molecules [14]. They include phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), oxychloraphine, pyocyanin and 1-hydroxy-phenazine [14-17]. Numerous recent studies have reported that these bioactive compounds have contributed to inhibit the growth of many phytopathogenic fungi and bacteria [1,2,14-17].

Although bacterial phytopathogens pose serious problems in the cultivation of economically important plants in Morocco, so far few studies have been carried out regarding the biological control. In this concern, the ongoing work was undertaken to identify an antibacterial compounds producing bacterium isolated from a hot spring discharge in the region Moulay Yakoub of Fez (Morocco), which is responsible for the inhibition of pathogenic bacteria and to partially purify its bioactive substances.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial Strains and Media

*Mycobacterium smegmatis* MC² 155 and *M. aurum* A+ are non-pathogenic atypical bacteria [18]. These strains were used as a model to evaluate the effect of active substances on the growth of mycobacteria [19]. 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* [20]; *S. haemolyticus* [21], *Bacillus subtilis* ILP 142B [20]; *B. licheniformis* [22]*; B. amyloliquefaciens* [22]*; Escherichia coli* DH5α (Microbial biotechnology laboratory of Techniques and Sciences Faculty, Fez Morocco); *Erwinia chrysanthemi* 3937 [23]. This bacterium was friendly provided by Dr. Hassouni (LCB-CNRS-Marseille). These strains were propagated in Luria-Bertani (LB) at 37°C or at 30°C for *Erwinia chrysanthemi*.

Bacteria were stored at -70°C 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 4°C [24].

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 [24].

## 2.2 Screening and Isolation of Microorganisms

Different samples of water and soil were collected from a hot spring discharge in Moulay Yakoub region of Fez Morocco and treated independently according to the method followed by Hassi et al. [21]. Colonies that showed clear zones of inhibition against *M. smegmatis* were picked up and transferred to LB agar plates. These were incubated at 37°C and stored at 4°C for later assays [21].

## 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 [25]. 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 (2013) [26], 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 [26]. In both cases, plates were incubated at 37°C 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 Strain

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*, *B. licheniformis, B. amyloliquefaciens, E. coli* DH5α and *Erwinia chrysanthemi*. The assay was repeated three times [26].

## 2.5 Identification of Antibacterial Compounds-producing Strain

Antibacterial compounds producing bacterium was characterized by using the API 20 E multi-test system (BioMerieux, France) complemented with cellular morphology, microscopic observation and biochemical tests performed according to Bergey's Manual of Determinative Bacteriology [27]. In order to confirm biochemical identification, the isolate was 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 [28]. The amplification protocol was led as was described by Zahir et al. [29]. 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 [30]. Databases (GenBank) were used for sequence similarity comparison with the obtained 16S rDNA sequence.

## 2.6 Ethyl Acetate Extraction and Activity Assay of the Antimycobacterial Substances

The bioactive substances were extracted by ethyl acetate as was described by Zahir et al. [31]. An inoculum of the strain at $OD_{595\ nm}$ = 0.3 was cultivated under rotary agitation at 150 rpm at 37°C for 48h in 250ml Erlenmeyer flask containing 100 ml of LB broth. After incubation, the bacterial culture was centrifuged for 10 min at 6000 rpm at 4°C. Then, the supernatant was recovered, sterilized by filtration and added to 100ml of ethyl acetate (A Fisher Scientific International Company, 99% of purity). After agitation for one hour at room temperature, the obtained organic extract was evaporated under vacuum at 37°C. The dry residue was taken up in 1 ml of sterile distilled water. The obtained solution was referred as "crude extract" and its antibacterial well-diffusion assay was performed on LB agar plates already inoculated by *M. smegmatis* at 37°C. The antibiosis effect was determined by measuring the size of inhibition halos formed around the wells. Extraction of *E. coli* DH5α culture by ethyl acetate was used as a negative control. This test was performed in triplicate [31].

## 2.7 Determination of the kinetics

Synthesis of antibacterial compounds was monitored during the growth cycle by growing the culture of the producer strain on LB broth under shaking condition for 48h at 37°C. After every 2h, optical density at $595_{nm}$ was recorded. The cells were removed by centrifugation at 6000 rpm for 15 min and bioactive supernatants were then extracted with an equal volume ethyl acetate [32]. The crude extract was tested for antimycobacterial activity using well-diffusion assay on LB agar plates previously inoculated with the same indicator strain suspension at 37°C. The assay was performed two times and the average of the inhibition zone diameter was calculated.

## 2.8 Physico-chemical Characterization

### 2.8.1 Heat treatment

Thermal stability of the antibacterial activity was evaluated after incubation of the strain acetyl ethyl extract at different temperatures: 121°C (60 min), 100°C (20 min), 80°C (30 min), 37°C (3h) and kept at 4°C (six months). The residual inhibitory activity was checked by agar well diffusion assay against *M. smegmatis*. This test was triplicate and the average of the inhibition zone diameter was calculated [33,34].

### 2.8.2 Effect of proteolysis activity on the crude extract

Pepsin (Sigma) and proteinase K (Sigma) were tested for their proteolysis activity on the antibacterial compounds contained in the strain ethyl acetate extract. The assay was performed at a final concentration of 1mg/ml, respectively at pH 3 and 7 [35,36]. Samples with and without enzymes were held at 37°C 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.

## 2.9 Detection of the Metabolites Responsible for the Antimycobacterial Activity

### 2.9.1 Thin layer chromatography fractionation of the extract

The thin layer chromatography (TLC) is a physical method of separating mixtures into their constituents. Silica gel (Sigma-Aldrich®), on plates 1.5 cm wide and 12cm long, was used as the adsorbent (stationary phase). The eluent (mobile phase) consisted of the organic solvents chloroform / acetone (9: 1, v/v) [37], which were selected from previously tested mixtures for optimal separation of the components. For TLC analysis, 25µl of the strain ethyl acetate extract were applied to silica gel plate. After migration in a closed container for 60 min, the plate was air-dried and the separation of compounds (spots) was visualized under ultraviolet (UV) light (wavelength, 366 $_{nm}$). The retention factors (Rf) of the separated components were then determined [31]. This test was repeated four times.

### 2.9.2 Bioautography

This technique was undertaken to determine active compounds found in the strain ethyl acetate extract as previously described [31]. After determination of the spots localization under UV light, the chromatogram was transferred aseptically into a Petri dish. Thereafter, it was flooded by semisolid LB medium with 2% (w/v) agar mixed with *M. smegmatis* culture (OD 595 $_{nm}$ = 0.3). After 72h of incubation at 37ºC, the inhibition zone was detected. A bioautography done without addition of the extract was included as a blank control. The experiment was repeated twice.

### 2.9.3 Spots elution test

In order to confirm the results from bioautography, the silica gel around the inhibition zone was eluted using ethyl acetate. After evaporation, the product was recuperated in sterile distilled water and a volume of 25µl of each eluant was deposited onto a sterile 6 mm diameter paper disc placed at the center of LB agar plate previously inoculated with a liquid culture of *M. smegmatis.* The control corresponded to a plate prepared by using identical migration conditions, but without extract deposition. Each test was done twice [38].

# 3. RESULTS AND DISCUSSION

## 3.1 Screening, Isolation of Microorganisms and Anti-mycobacterial Activity Assay

The screening of bacteria collected from water and soil of a thermal source waste in Moulay Yakoub region of Fez Morocco allowed the selection of one isolate, ZI4, 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 method showing a diameter of inhibition zone about 12±1 mm (Fig. 1A).

## 3.2 Inhibitory Spectrum of the Isolated Bacterium

The isolate ZI 4 exhibited a broad antagonistic activity spectrum against all the tested bacteria by the agar spot-on-lawn assay (Fig. 1). These results demonstrated that the microorganism inhibited the growth of the indicator strains through the diffusion of antibacterial compounds into the medium. Furthermore, the broad spectrum of activity of the isolate suggests that its target is highly conserved; this finding prompted us to focus on the compounds mode of action in later work.

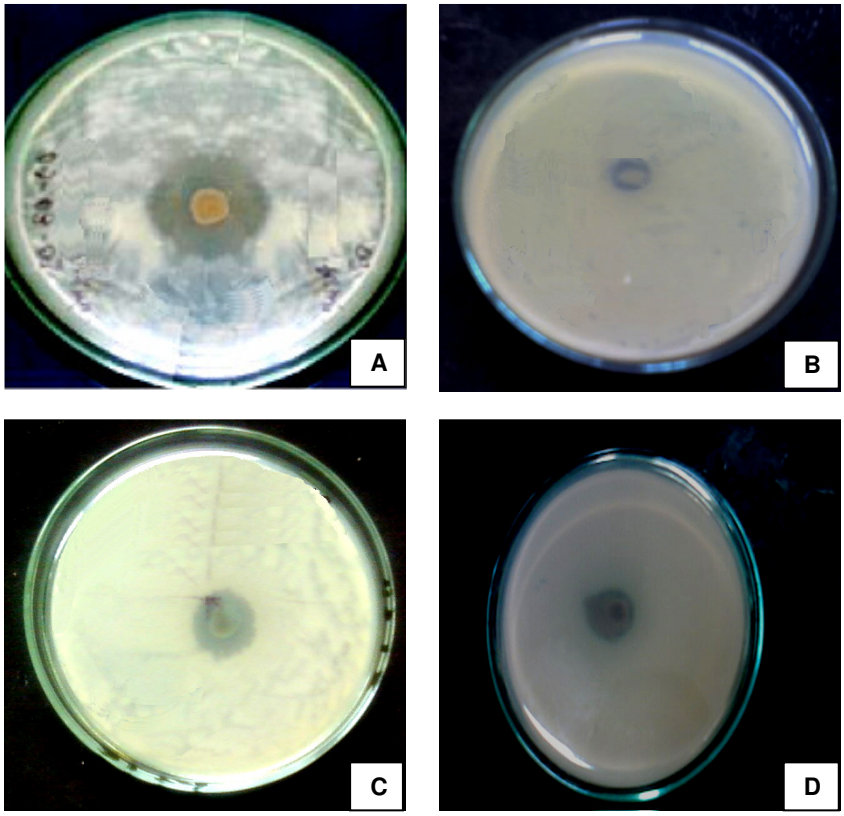


**Fig. 1. Antagonistic activity of the isolate ZI 4 on *M. smegmatis* (A); *Erwinia chrysanthemi* (B); *B. subtilis* (C) and *B. amyloliquefaciens* (D), respectively, evaluated by spot on lawn assay. After incubation, the antibacterial activity was detected by the observation of an inhibition area**

## 3.3 Identification of Antibacterial Compounds-producing Strain

A preliminary identification of ZI 4, through phenotypical and biochemical characteristics revealed that this isolate belongs to *Pseudomonas* genus and the use of API 20E kit indicated identity with *P. aeruginosa*. Indeed, morphologically, it was a Gram-negative bacillus, motile and arranged alone or in pairs. The research of catalase was positive and the culture had an aromatic smell of mock orange. The bacterium was not able to ferment lactose but attacked glucose by oxidative pathway without $H_2S$ production. It was found, in addition, that it can assimilate many carbon substrates as a sole source of carbon and

energy as citrate, melibiose, arginine while mannitol, arabinose, amygdalin, sucrose, rhamnose, sorbitol, inositol were not utilized. Negative reactions were visualized from ornithine decarboxylase, lysine decarboxylase, tryptophan deaminase, beta-galactosidase and the production of acetoin and indole. However, gelatin was liquefied and nitrate was reduced. Moreover, the synthesis of pigments pyocyanin and pyoverdine was highlighted on King A and King B media, respectively, such a feature is characteristic of this bacterial species [15,16,17,39]. These results collectively satisfy the criteria for *P. aeruginosa* given by Bergey's manual of determinative bacteriology [27] and corroborate with the researches of other studies [40-45].

In the other hand, PCR amplification of the 16S rRNA gene with the fD1 and RS16 primers allowed the amplification of a DNA fragment of approximately 1.5 kb, as previously reported in the literature [28]. BlastN search showed that the partial sequence of 16S rRNA gene of the isolated strain ZI 4 had a homology of 97% to that of *P. aeruginosa*. According to the criteria defined by Drancourt and collaborators [46], the bacterial strain ZI 4 belonged to *P. aeruginosa* strain.

Regardless of its morphology, cultural appearance and biochemical characteristics mentioned above, together with the phylogenetic analysis, the strain ZI4 was identified as *P. aeruginosa*. Based on these data, we assigned our strain as *P. aeruginosa* strain ZI 4.

*P. aeruginosa* is a ubiquitous bacterium which colonized the soil and plant roots as well as the various types of water from all sources namely river water, sewer, pool, sea, mineral water and hot springs [47]. This explains its isolation from a discharge of warm water localized in Fez Morocco. Nevertheless, to date, no described antibacterial actives substances producer bacterium has been related to *P. aeruginosa* from a Moroccan hot spring waste.

This bacterium belongs to one of the most remarkable genera which contain species having antimicrobial activities explored to control plant diseases through various mechanisms [12,37,47-55]. Certainly, it was highlighted that the membrane vesicles, containing peptidoglycanes hydrolases naturally produced by *P. aeruginosa*, lyse the peptidoglycane layer of several Gram-negative and Gram-positive bacteria [56]. Other studies described that among the microorganisms inhibited by this bacteria were *E. coli* [13,39,57-60].; *S. aureus*, *S. haemotylicus* [14,13,39,58,59,61,62], *B. licheniformis* [39], *B. subtilis* [39,58], *B. amyloliquefaciens* [13] and *M. aurum* [63]. This is in perfect correlation with our achievements. However, to the best of our knowledge, this is the first report of isolation of *P. aeruginosa* showing an inhibitory effect against *M. smegmatis* and *Erwinia chrysanthemi* which is associated with potatoes causing tuber soft rot and blackleg [5].

## 3.4 Ethyl Acetate Extraction and Activity Assay of the Antimycobacterial Substances

Organic solvents have been employed to extract antibacterial, antifungal, anti-malarial and antiviral substances produced by microorganisms. Among the organic solvents, ethyl acetate has been widely employed [2,53,59,64]. The crude extract of the antibacterial substances prepared from *P. aeruginosa* was tested against *M. smegmatis*. The antibacterial assay showed an inhibition zone of which the diameter was 24±-1 mm which indicates that *P. aeruginosa* acted by substance(s) secreted in the medium and soluble in ethyl acetate.

Meanwhile, the ethyl acetate crude extract of *E. coli* DH5α used as control did not exhibit any inhibitory activity against the same indicator strain.

Our results agree with the data concerning metabolites synthesized by *P. aeruginosa* such as pyocyanin, a blue green phenazine pigment specific to this species [15,16,17,39]. It turned out not only soluble in water [15,16,39,65,66] but also it was soluble in several solvents like ether, chloroform, benzene, ammonia, methanol, acetone and ethyl acetate [15,16,17,37,40,49,66]. Moreover, it was produced in both solid and liquid culture media. [15,16,65]. Similarly, it had been published in various studies that other phenazines produced by the same bacterium as PCA and PCN were also soluble in organic solvents [47,55,58,67,68].

## 3.5 Determination of the Kinetics

*P. aeruginosa* was oxically incubated in LB medium at 37°C in a rotary shaker and the compounds production was evaluated every two hours by the well-diffusion assay. Monitoring the growth of this strain showed that the production of the bio-active substances started at the beginning of the growth exponential phase (4h) and reached its maximum during the stationary phase (Fig. 2). These outcomes are comparable to those found in the research led by Saha and collaborators [45] demonstrating that the appearance in the culture medium of pyocyanin and pyorubrine began after 6 h of growth. This synthesis increased, then, throughout the incubation period by being perfectly correlated with the biomass and stabilized toward the end of growth [45]. Our results are also substantiated El-Shouny et al. report [16] and the first studies aiming the production of pyocyanin which started during the first 24 hours of growth and achieved at the end of incubation at 35°C [40,69,70]. This proves, therefore, that the production of these metabolites is associated with the bacterial growth [58,65,71]. In fact, all the investigations about the phenazines synthesis by *P. aeruginosa* revealed that it is low during the logarithmic phase and this is due to catabolite repression by sources of carbon and nitrogen. An increase of production is then seen at the end of the exponential and the stationary phases as a result of the depletion of nutrients, oxygen limitation as well as its activation by the quorum sensing system [16,54,67,72].

## 3.6 Physico-chemical Characterization

Antibacterial activity was tested for its sensitivity towards heat and proteolytic enzymes by measuring residual activity against *M. smegmatis*. Metabolites of *P. aeruginosa* were not affected by proteases and their activities were also stable after heat treatments at 37, 80, 100 and 121°C while the storage of the bioactive substances at 4°C for six months did not influence the activity (Table 1). Conservation of this anti-mycobacterial effect after both heat evaluation and proteolysis suggests that active compounds do not require a peptide moiety for their biological activity.

Previously, heat stability and proteolysis resistance properties were observed in other antimicrobial metabolites produced by *P. aeruginosa* [1,45,61,67]. It had been reported that pyocyanin and pyorubrine showed a resistance to boiling without losing their antibacterial effect [45] and it had been published as well that the antimicrobial activity of PCA and PCN was remarkable at higher temperatures [16,67].

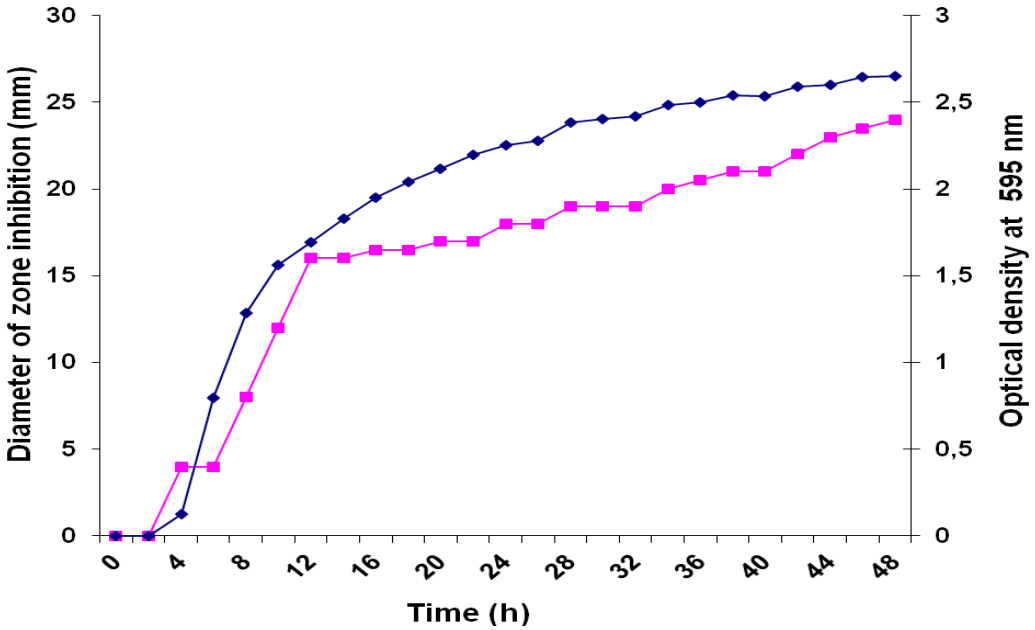


**Fig. 2. Growth kinetic and antibacterial agent production by *P. aeruginosa*. (♦) growth kinetic, (■) antibacterial production. *P. aeruginosa* cells were cultured in LB broth and were incubated for 48 h at 37°C. The OD and antibacterial activity were measured.**

**Table 1. Influence of temperature and proteases on the activity of *P. aeruginosa* ZI 4 extract**

| Treatment | Antibacterial activity of C ZI 4 (%) |
|---|---|
| **Enzymatic treatments** | |
| Proteinase K | 100 |
| Pepsin | 100 |
| None (positive control) | 100 |
| *E. coli* DH5α (negative control) | 0 |
| **Heath treatment** | |
| 4°C for six months | 100 |
| 37°C for 3h | 100 |
| 80°C for 30 min | 100 |
| 100°C for 20 min | 100 |
| 121°C for 60 min | 100 |
| None (positive control) | 100 |
| *E. coli* DH5α (negative control) | 0 |

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

The bacterium *P. aeruginosa* secretes several extracellular molecules, for instance, rhamnolipids, proteases and phenazines [41,73-75]. These latter represent a large family of nitrogenous heterocyclic molecules intensely pigmented. They are synthesized from shikimic acid and have a broad-spectrum antimicrobial action [45,52]. Effectively, their antibiotic, anti-parasitic, antifungal, anti-malarial and antitumor activities were highlighted [14,47,55]. Furthermore, they play a role in the microbial competitiveness and the suppression of

phytopathogens [47,52,58,76]. Hence, the antibacterial compounds of *P. aeruginosa* ZI4 may be organic compounds in the form of phenazines. Further study is required to examine the exact nature of the antibacterial components.

## 3.7 Detection of the Metabolites Responsible for Antimycobacterial Activity

### 3.7.1 Thin layer chromatography fractionation of the extract

The purpose of this assay was to locate the molecules which could present biological activity against *M. smegmatis* on the silica gel plate. Under UV radiation, distinct spots were detected having different Rf values between 0.09 and 0.86 in *P. aeruginosa* ZI4 extract (Fig. 3). In the chromatogram, the spots of the compounds are separated by differences in polarity.

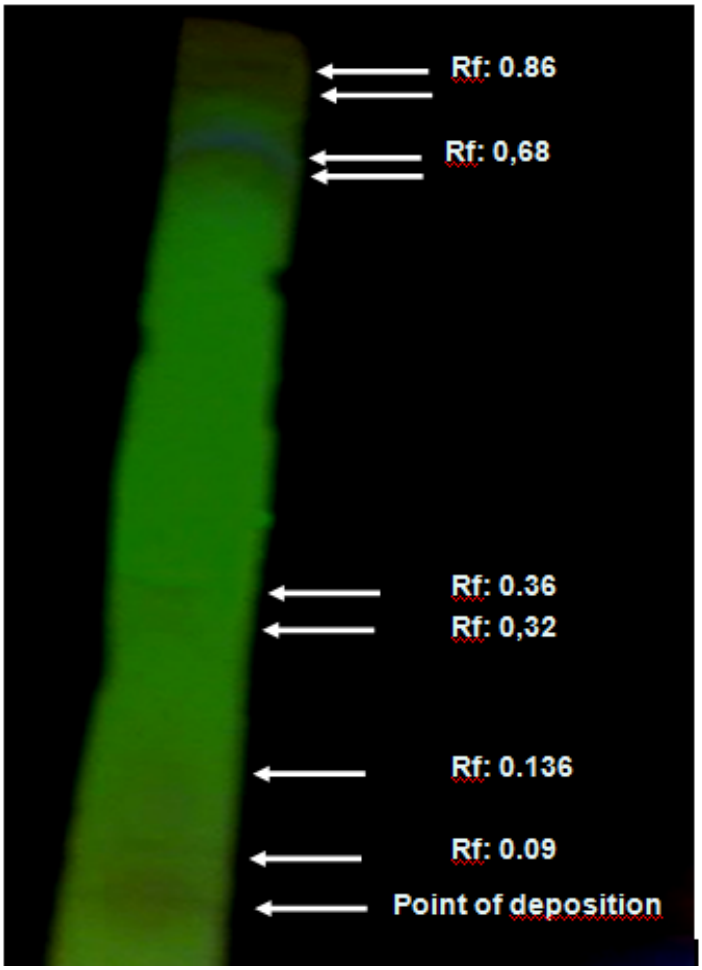


**Fig. 3. Thin layer chromatography fractionation of *P. aeruginosa* ZI4 extract. At the point of deposition, a volume of 25µl of the extract was put on the silica gel plate then it was migrated for 60 min in the mixture chloroform / acetone (9: 1, v/v). After separation, spots on plate were visualized under UV light at 366 $_{nm}$**

### 3.7.2 Bioautography and spots elution test

The bioautography of *P. aeruginosa* extract against *M. smegmatis* showed a halo of inhibition surrounding the components corresponding to spots with Rf 0.66; 068; 0.82 and 0.86 (Fig. 4A), while no inhibitory activity was exhibited by other spots and control test.

Silica gel around spots was separately eluated and evaporated. The resulting products were separately recuperated in sterile distilled water and tested using the disc method. The obtained results revealed that the only active spot was the bleu one with Rf equals to 0.68 (Fig. 4B).

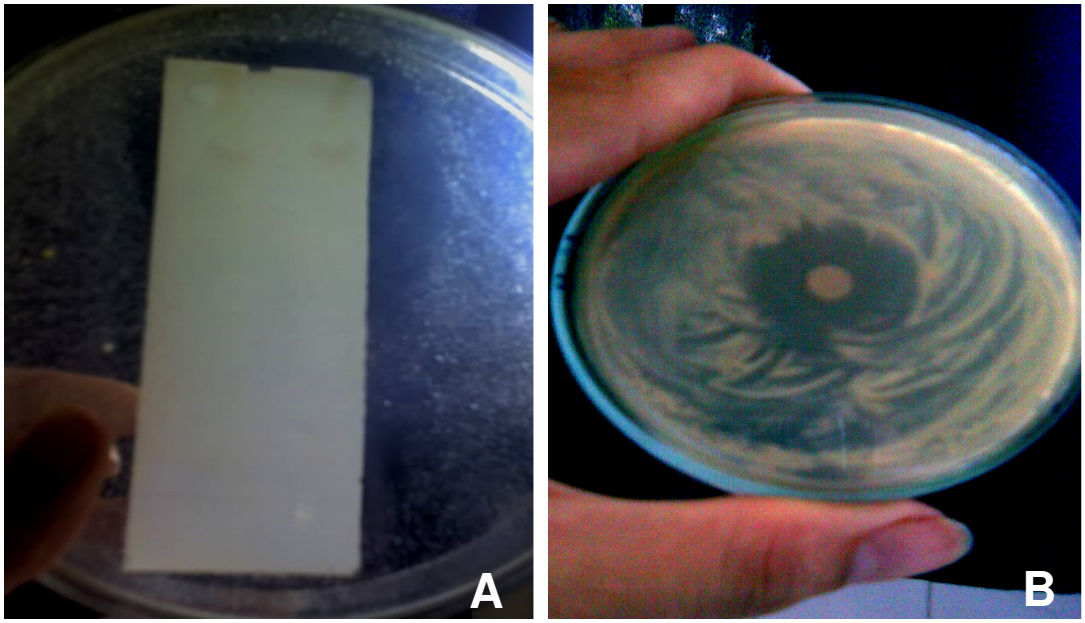


**Fig. 4. Bioautography (A) and antimycobacterial activity of the eluted bleu spot (B) from the thin layer chromatography of *P. aeruginosa* extract. At the point of deposition, two volume of 25 µl of the extract was put on the silica gel plate (A). This was covered with a thin layer of LB agar mixed with a liquid culture of *M. smegmatis*. After detection of an inhibition zone, spots separated from the solvents system chloroform/acetone (9: 1, v/v) were eluated and tested against the same indicator strain (B). Only result of the spot having Rf 0.68 was shown**

Earlier, it had been reported in the investigation of Lee and Walden [40], that paper chromatography of *P. aeruginosa* extract developed in two solvent systems n-butyl alcohol-acetic acid-water (4:1:5) and n-butyl alcohol-ammonium hydroxide (1:1) revealed a deep blue spot with Rf values were 0.65 and 0.62, respectively. Later, in the same study, infrared, mass spectroscopy and UV spectra analysis had identified the blue pigment as pyocyanin. The purified pigment prevented the growth of *E. coli* and normal flora from the human throat [40]. In another study, this same molecule had been purified represented by a single blue band (Rf=0.53) showed by thin layer chromatography in the system of solvents dichloromethane- methanol (1:1) [77]. Even more, data given by Karpagam et al. [15] had showed that the bluish green of *P. aeruginosa* broth culture indicated pyocyanin production. This was confirmed by UV visible spectra which revealed a maximum absorbance at 278 $_{nm}$,

characteristic of pyocyanin. The molecular weight of the compound was determined as 210.23kDa by GC-MS analysis with a retention time of 11.94min and Nuclear Magnetic Resonance (NMR) study demonstrated the presence of methyl group linked to condensed nitrogen aromatic ring, indicative of phenazine presence [15].

For this reason, the blue color of the detected spot in the present research prompted us to think that the compound responsible for the sought effect could be pyocyanin, a very distinctive water soluble blue pigment produced in large quantities only by the species *P. aeruginosa* [15,16,17,39,67,76,77] and it is soluble in chloroform [15,16,17,41]. Besides, this blue spot cannot be pyoverdine, a fluorescent green pigment, because it is insoluble in chloroform [41].

*P. aeruginosa* synthesizes an arsenal of bioactive substances whose pyocyanin is by far the best known [15,16,17,45,74,77]. This metabolite exhibits not only an antifungal effect against *Septoria tritici* [78], *Rhizoctonia solani* [49] and an anti-parasitic activity against the nematode *Caenorhabditis elegans* [79-81] but also it presents an antibacterial effect vis-à-vis *M. tuberculosis* [48], *S. aureus* [16,17,48,39,61,82], *S. epidermis* [16,82], *Clostridium botiulinum* [16], *Micrococcus luteus*, *B. subtilis* [39, 82], as well as *B. licheniformis* [39]. Prevention of Gram negative bacteria growth by this molecule was also recorded. For example, *E. coli* [16,17,39,57,61], *Enterobacter cloacae* [61], *Serratia marcescens* [48, 61], *Salmonella typhi, Vibrio parahaemolyticus, Proteus mirabilis* [16] *Proteus vulgaris* [17, 39], *Paracoccus denitrificans* [39], *Citrobacter* sp. [45] and *Erwinia carotovora* [55]. It worth noting that the antibacterial effect of pyocyanin has never been elucidated towards *M. smegmatis*, used as a model of mycobacteria, and vis-à-vis *Erwinia chrysanthemi*, a phytopathogenic bacterium. These activities are due to the redox potential of pyocyanin which deviates the normal transport of electrons through the respiratory chain and reduces oxygen to generate, therefore, a large amount of hydrogen peroxide and superoxide radical causing an oxidative stress within bacterial cells [16,52,57,83,84,85].

In addition to the pyocyanin, *P. aeruginosa* produces other molecules known for their antibiotic properties and used as powerful antimicrobial agents [55,58] namely salicylic acid, pseudomonic acid, 1-hydroxyphenazine, PCA and PCN [14,15,45,47,49,54,55,61,67,86]. These substances are valuable tools for biological control of plant pathogens [10,45,47,52,54,55,86]. Consequently, the metabolites secreted by *P. aeruginosa* highlighted during this present investigation might correspond to pyocyanin or to its combination with the other molecules mentioned above. However, verification of the chemical structure of the resulting spot needs other techniques such as high performance liquid chromatography (HPLC), GC-MS and NMR analysis in further work.

## 4. CONCLUSION

*P. aeruginosa* ZI4 isolated from discharge of hot spring water produced antibacterial metabolites with promising features in the bio-control of pathogenic bacteria causing plant diseases. Bioactive substances showed remarkable stability towards proteolytic enzymes and heat treatment. Further work is required to purify compounds of the detected bleu spot responsible for the sought activity in order to establish their exact chemical structures, followed by other investigations having as a goal demonstrating the performance of purified metabolites in the field to define their possible use as bio-pesticide.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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