British Microbiology Research Journal
4(11): 1178-1188, 2014

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Partial Purification and Antimycobacterial Screening of the Ethyl Acetate Extract of *Alcaligenes faecalis* BW1

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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.

Original Research Article

Received 1st April 2014
Accepted 9th June 2014
Published 1st July 2014

ABSTRACT

Aims: The focus of this study was to evaluate the antimycobacterial activity of *Alcaligenes faecalis* BW1 extract and to purify it partially.
Study design: Partial purification of *A. faecalis* BW1 extract was performed by using thin layer chromatography and active substances responsible for the biological activity were localized.
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 July 2011.
Methodology: Crude extract of *A. faecalis* BW1 was obtained by using ethyl acetate as an organic solvent and its antimycobacterial effect was investigated by agar discs diffusion method. The extract was then fractionated by thin layer chromatography and the bioactivity was assessed with a bioautography technique followed by spots elution tests.
Results: The results showed that *A. faecalis* BW1 produced compounds with antimycobacterial activity. All the detected spots by thin layer chromatography inhibited the growth of *M. smegamtis*.

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Conclusion: Various metabolites of *A. faecalis* BW1 are responsible for the sought effect or they could act synergistically to inhibit mycobacterial growth. These compounds could be used after their total purification in further work against mycobacterial infections.

Keywords: Alcaligenes faecalis; BW1 extract; thin layer chromatography; bioautography.

ABBREVIATIONS

LB, Luria-Bertani; TLC, Thin layer chromatography.

1. INTRODUCTION

Mycobacteria are ubiquitous in nature and can survive in harsh conditions i.e., low nutrients, low pH and hypoxia [1]. These microorganisms are difficult to eradicate with common decontamination practices and are relatively resistant to standard disinfectants [1,2]. In addition, antibiotic resistance in mycobacteria reaches a crucial point in healthcare because of the emergence of an increasing number of multidrug-resistant strains. Thus, it is not surprising to see an array of species responsible for infections [1] e.g., tuberculosis caused by the complex *Mycobacterium* including *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii* and *M. pinnipedii* [3]; skin lesions due to *M. marinum, M. ulcerans*; catheter infections attributable to *M. kansasii, M. fortuitum*, *M. chelonae* [4].

Furthermore, the acquired immunodeficiency syndrome (AIDS) is responsible for increased frequency of mycobacterial infections making them a major concern e.g., pulmonary infection due to *Mycobacterium abscessus*, which is difficult to cure [5,1]. Consequently, new drug candidates with enhanced activity against multiple drug-resistant mycobacteria are needed [1]. For this reason, several studies have led to find antibiotics by exploring multiple tracks such as the synthesis of chemical molecules, the study of bioactive substances synthesized by plants, invertebrates and microorganisms [6].

Previously, we have found that, the bacterium *Alcaligenes faecalis* BW1 possess an antibacterial effect against a group of Gram-negative and positive bacteria especially *Mycobacterium smegmatis*. The antibacterial compounds were not affected after being treated by heat and proteolytic enzymes (pepsin, proteinase K) that indicated the non-protein nature of the active agents [7].

Herein, this investigation is a continuation of our previous work [7], which aims to purify partially the *A. faecalis* BW1 extract by using thin layer chromatography technique and to detect spot(s) responsible for antimycobacterial effect.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Media

Mycobacterium smegmatis MC² 155 is a non-pathogenic atypical strain with a generation time of approximately 3 h [8]. This strain was used as a model to evaluate the effect of active substances on the growth of mycobacteria [9,10,11]. This mycobacterium was kindly provided by Dr. Suzana David (Centro de Tuberculose e Micobactérias Instituto Nacional de Saúde Dr. Ricardo Jorge Delegação do Porto, Portugal).

Alcaligenes faecalis BW1 (accession number HG737341) was originally isolated from a Moroccan tannery waste. It has a broad antagonistic effect against various Gram-negative and positive bacteria [7].

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 (10 g of peptone, 5 g of yeast extract, 10 g of NaCl, 15 g of agar per liter of distilled water, pH 7) and held at 4ºC [12].

2.2 Ethyl Acetate Extraction and Activity Assay of the Antimycobacterial Substances

A liquid-liquid extraction was done by using ethyl acetate. An inoculum of *A. faecalis* at $OD_{595\ nm}$ = 0.3 was cultivated under rotary agitation at 150 rpm at 37ºC for 48 h in 250 ml Erlenmeyer flask containing 100 ml of LB broth. After incubation, the bacterial culture was centrifuged for 10 min at 6000 rpm at 4ºC. Then, the supernatant was recovered, sterilized by filtration and added to 100 ml 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 37ºC. The dry residue was taken up in 1 ml of sterile distilled water. The obtained solution was referred as "crude extract" [7]. Thereafter, a 6 mm disc (Whatman paper) impregnated in 25 µl of the extract was transferred to a sterile Petri dish containing LB medium and pre-inoculated with the target bacterium by spreading 0.1 mL of overnight-cultured *M. smegmatis* at $OD_{595\ nm}$ = 0.3 in LB broth. The antimycobacterial activity was evaluated after incubation at 37ºC for 48h. Extraction of *E. coli* culture by ethyl acetate was used as a negative control. This assay was performed in triplicate.

2.3 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 12 cm long, was used as the adsorbent (stationary phase). The eluent (mobile phase) consisted of the organic solvents ethanol / ammonia / water (8: 1: 1, v/v/v), which were selected from previously tested mixtures for optimal separation of the components. For TLC analysis, 25 µl of *A. faecalis* BW1 extract were applied to silica gel plate. After migration in a closed container for 80 min, the plate was air-dried and the separation of compounds (spots) was visualized under ultraviolet light (wavelength, 366 $_{nm}$). The retention factors (Rf) of the separated components were then determined [9]. This test was repeated four times.

2.4 Identification of the Fractions Responsible for Antimycobacterial Activity

2.4.1 Bioautography

In order to detect molecule(s) in *A. faecalis* BW1 extract responsible for the antimycobacterial activity, a bioautographic method was undertaken [13,14]. 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 72 h of incubation at 37ºC, the inhibition zone was detected. A TLC done without addition of the extract was included as a blank control. The experiment was repeated twice.

2.4.2 Spots elution test

This experiment allows detecting precisely spot(s) responsible for the antimycobacterial activity. Thus, after migration of the metabolites of the extract on three silica plates, the obtained spots were drawn by a pencil. They were scraped and the obtained powder from each spot was placed in a glass tube wherein a volume of 2 ml of ethyl acetate was added and then incubated for 30 min. The tubes were centrifuged for 10 min at 2000 rpm under room temperature. Each supernatant was collected and evaporated to dryness in a rotary evaporator and the obtained residue was dissolved in 280 µl of sterile distilled water. A volume of 25 µl of each eluant was deposited on a sterile 6 mm diameter paper disc placed at the center of LB agar plates 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 repeated three times [15].

2.4.3 Second purification by TLC and elution test

This test is to further purify the spot(s) responsible for the antimycobacterial activity. Thus, the eluate from each target spot underwent another migration on silica plate by using the same solvents. Possibly received spots were detected under UV light and the elution test was redone as it was described in the previous paragraph. The eluates were tested against *M. smegmatis* by using the disc agar diffusion method [16,17]. This assay was done in duplicate.

3. RESULTS AND DISCUSSION

3.1 Ethyl Acetate Extraction and Activity Assay of the Antimycobacterial Substances

Ethyl acetate is an organic solvent, which has been widely employed to extract antibacterial metabolites produced by microorganisms [3,7,9,14,18]. The crude extract of *A. faecalis* BW1 was tested against *M. smegmatis*. The antimycobacterial assay showed an inhibition zone with a diameter value of 17.00±0.05 mm. This result indicates that *A. faecalis* acts by substance(s) secreted in the medium and which are soluble in ethyl acetate, while the crude extract of *E. coli* used as control did not exhibit any inhibitory activity against the indicator strain.

3.2 Thin Layer Chromatography Fractionation of the Extract

The purpose of this assay was to locate the molecules, on the silica gel plate, presenting biological activity against *M. smegmatis*. Under UV radiation, four distinct spots were detected having different Rf values between 0.7 and 0.92 in *A. faecalis* BW1 extract (Fig. 1).

In the chromatogram, the spots of the compounds are separated by differences in polarity and they are localized far from the original point of extract deposition. In fact, the mixture ethanol / ammonia / water (8: 1: 1, v / v / v) has an eluting power due to different polarities of its constituents which lead to a better resolution of compounds than systems consisting of one or two solvents [19]. In addition, water is among the most strongly adsorbed solvents, the presence of a little water in a solvent can greatly increase its eluting power. Thus, these results suggest that the extract could possibly contain polar molecules.

Previously, it was found that a fraction eluted from the most polar spot of *Desulfovibrio desulfuricans* extract on the TLC plate by the solvent mixture CH_2Cl_2/MeOH (v/v, 1:1) has a remarkable antibacterial effect towards Gram positive and Gram negative bacteria [20]. According to another investigation, it was also reported that the most active *Streptomyces* of four strains isolated from Ezzmoul saltpans produces polar bioactive molecules showing an antibacterial activity against antibiotic resistant bacteria [21].

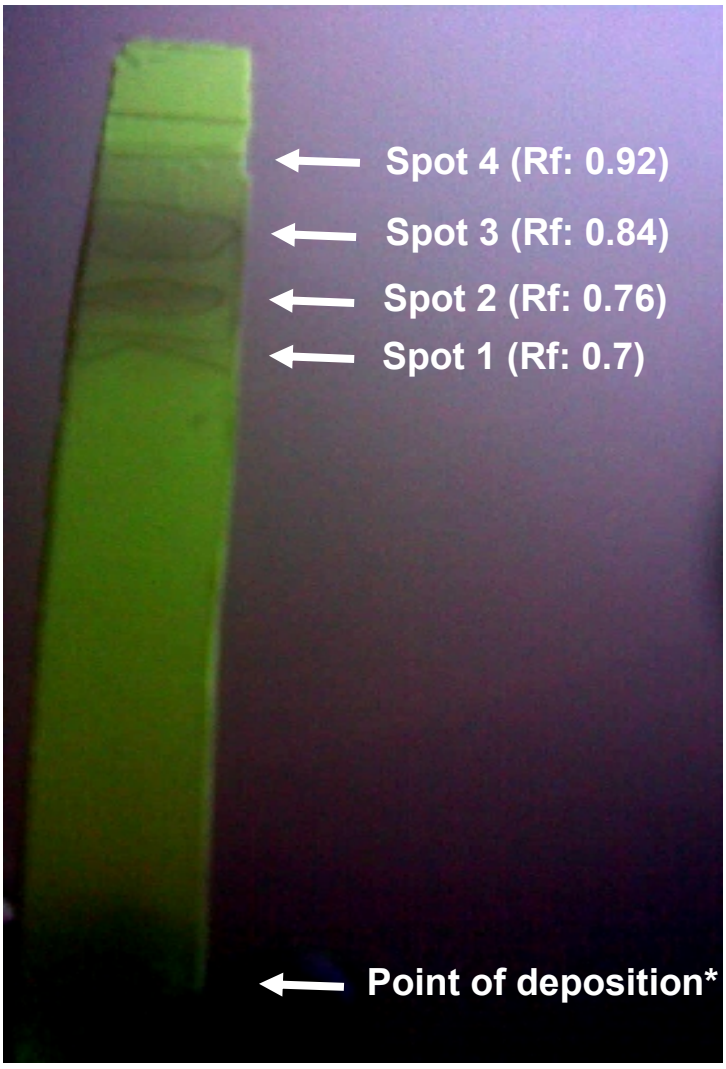

Fig. 1. Thin layer chromatography fractionation of *Alcaligenes faecalis* BW1 extract
At the point of deposition, a volume of 25 µl of the extract was put on the silica gel plate then it was migrated for 80 min in the mixture ethanol / ammonia / water (8: 1: 1, v / v / v). After separation, spots on plate were visualized under UV light at 366 nm

3.3 Identification of the Fractions Responsible for Antimycobacterial Activity

3.3.1 Bioautography

The bioautography of *A. faecalis* extract showed a halo of inhibition against *M. smegmatis* distributed over the entire area of appearance of the four detected spots (Fig. 2), while control test exhibited no inhibitory activity against the same indicator strain.

This approach has wide application in the search for new antibiotics because it allows an easy detection of activity of antimicrobial compounds obtained from secondary metabolites of microorganisms and plants on a chromatogram [13,14]. Indeed, two recent studies made by EL guendouzi et al. [9] and Haouat et al. [10] revealed that the spot inhibiting the growth of *M. smegmatis* was exactly highlighted in bio-autographies of extracts of *Staphylococcus epidermidis* and of the plant *Populus alba*, respectively. Whereas in another investigation, the bioautography of *Streptomyces* ethyl acetate extract allowed localizing of two active spots against *Staphylococcus aureus* [22]. However, in the present study, this technique was not able to determine precisely the spot(s) responsible for the desired activity or maybe all the spots presented inhibition zones that were mixed, hence it was necessary to make spots elution test.

The results support those described by the study of Suleiman et al. in 2010 [23], that antimicrobial activity of many plants such as *Loxostylis alata, Combretum vendee, Protorhus longifolia* is due to multiple spots shown by bioautography. This suggests that the bioactivity is a result of the interaction of many compounds and not of a single one. Likewise, it was revealed by bioautography that the bacterium *Desulfovibrio desulfuricans* produced at least eight antimicrobial components [20].

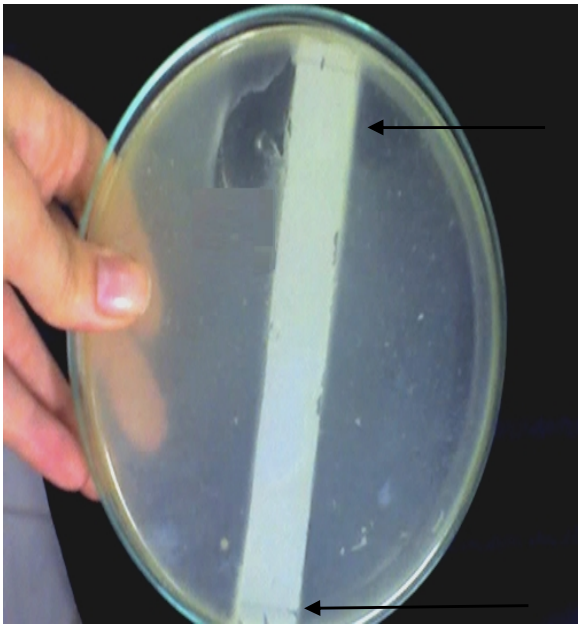

Fig. 2. Bioautography of *Alcaligenes faecalis* BW1 extract

**At the point of deposition, a volume of 75 µl of the extract was put on the silica gel plate. This was covered with a thin layer of LB agar mixed with a liquid culture of M. smegmatis. The Petri dish was incubated for 72 h at 37°C. Spots were colored only under UV light, thus they were invisible in the chromatogram.*

3.3.2 Spots elution test

The different spots were eluted and tested against *M. smegmatis*. Thus, we found that all spots previously detected showed an inhibitory effect (Fig. 3).

Whereas in the study of Li et al. [18] conducted on the extract of *A. faecalis* A72, it was shown that a single fraction from six others has an antibacterial effect against *Staphylococcus aureus* through the implementation of the TLC followed by the spots elution method in the solvent system CH_2Cl_2/MeOH (1:1 v / v). This fraction was subjected to silica gel column chromatography and elution with n-hexane/acetone (4:1 v / v) which led to the purification of two compounds. One of them was the molecule responsible for the bioactivity. Later, this compound was recognized as Cyclo (L-Pro-L-Phe), a diketopiperazine [18].

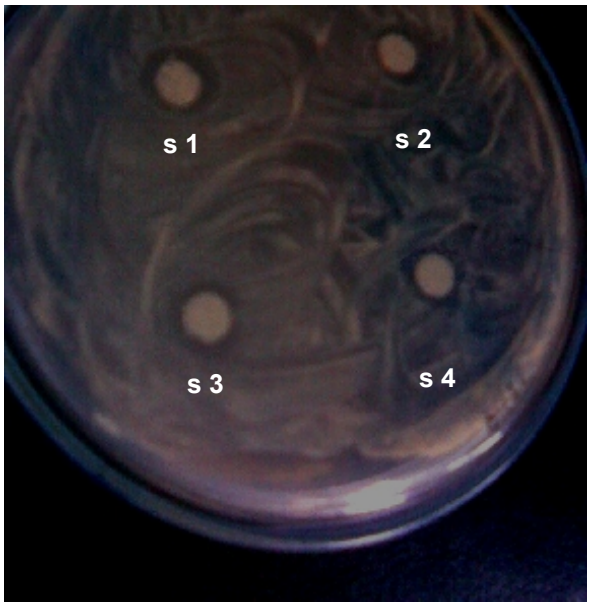

Fig. 3. Antimycobacterial activity of eluted spots from the TLC of *Alcaligenes faecalis* BW1 extract, show as such s1, s2, s3 and s4 are respectively the spots at Rf 0.7, 0.76, 0.84 and 0.92 separated from the solvents system of ethanol, ammonia and water (8:1:1, v/v/v)

It is also noted that the diameter of inhibition zone of each of these four spots (Table 1) is less than that of the crude extract after using agar disc diffusion method. This could be explained by the fact that compounds of spots could work synergistically to reach the anti-mycobacterial effect.

This finding was supported by the study of Eloff et al. [24], which indicated that when all Combretaceae compounds isolated with bioautography having an antimicrobial activity, they had a much lower activity than could be expected, indicating the presence of a synergy.

Table 1. Antimycobacterial activity of spots eluted from the TLC of *Alcaligenes faecalis* BW1 extract against *M. smegmatis*

TLC fraction	Retention factor (Rf)	Diameter of inhibition zone (mm)
Spot 1	0.70	9.5 ± 1.5
Spot 2	0.76	7.0 ± 0.8
Spot 3	0.84	8.0 ± 1.0
Spot 4	0.92	7.0 ± 0.5

This table elucidates that the four observed spots have inhibited the mycobacterial growth after elution. The negative control did not exhibit any inhibitory activity against *M. smegmatis*.

3.3.3 Second purification by TLC and elution test

This assay was done to verify the purity of antimycobacterial metabolites, thus a second thin layer chromatography was made for each target spot. We found that, the spots at Rf 0.76 and 0.92 are pure. Nevertheless, the second purification showed the presence of two spots for each of the two spots at Rf = 0.7 and Rf = 0.84, respectively (Fig. 4A). Inhibition zones against the indicator bacterium were detected from the four spots (a, b, c and d) obtained after elution (Fig. 4B). The appearance of additional metabolites during the second purification using the same mixture of solvents can be explained by a stack of products in the first purification. The second TLC has permitted a better separation of metabolites bonded to each other in the same fraction.

Spots 1 and 3 having Rf 0.7 and 0.84 in the first TLC, respectively, were subject to a second chromatography (A) which revealed the presence of two bands for spot 1 (a and b) and the same finding was showed for spot 3 (c and d). The four obtained spots inhibited the growth of *M. smegmatis* after their elution (B).

It should also be noted once again that the inhibition zones observed after completion of the second elution are smaller than those presented by the crude extract. This finding can be interpreted by the fact that various metabolites in the extract are responsible for the sought effect or they would work synergistically to inhibit the mycobacterial growth and might have different modes of action. In the other hand, the reduction of the diameter of inhibition zone could be explained that a part of the antimicrobial materials has been possibly lost during the recovering procedure because of an incomplete extraction or dissolution.

These outcomes corroborate with those published by Santos et al. [14] by investigating the antifungal effect of *A. faecalis*. Effectively, the bioautography of the bacterial extract, separated in the solvent system ethyl acetate / hexane (7:3), revealed that all five spots detected with Rf values of 0.043, 0.085, 0.4; 0.430 and 0.97 showed inhibition zones against *Stachytarpheta crassifolia* [14]. In contrast, in the study of Li et al. [18] focusing on the antibacterial effect of *A. faecalis* A72 extract against *Staphylococcus aureus*, only one compound was detected. It was responsible for the desired activity and was then recognized as Cyclo (L-Pro-L-Phe) after purification. However, until now, there are no other reports demonstrating that *A. faecalis* synthesizes several metabolites inhibiting the growth of *M. smegmatis*.

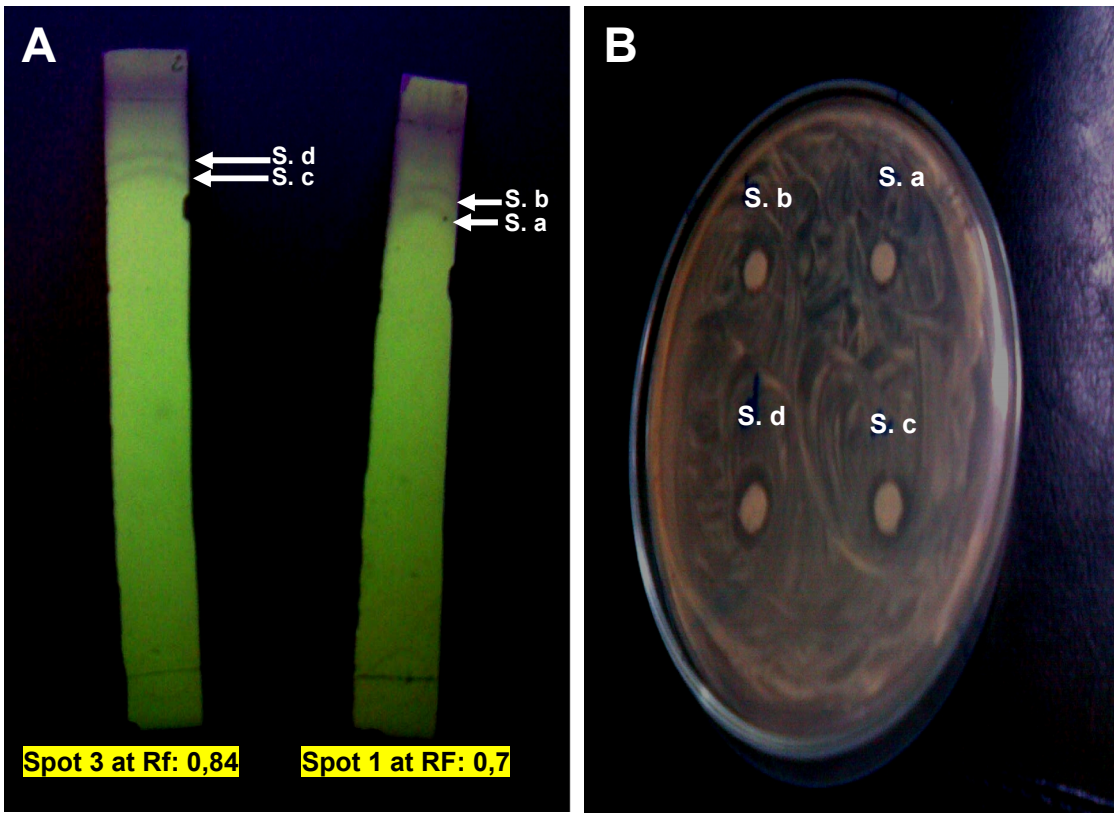

Fig. 4. Photos showing the second TLC of target spots 1 and 3 (A) and their antimycobacterial activity after elution (B)
S: is spot.

4. CONCLUSION

Analysis of *A. faeclais* BW1 extract by thin layer chromatography and bioautography followed by elution spots have allowed to localize the fractions responsible for the antimycobacterial effect, which may suggest that various metabolites in the extract could act synergistically to inhibit the mycobacterial growth.

Nevertheless, it should be noted that the verification of the purity of the resulting spots by the second TLC requires other techniques such as the high performance liquid chromatography (HPLC). In addition, *M. smegmatis* is used as a surrogate model in drug discovery against mycobacteria, but to judge whether metabolites of *A. faeclais* BW1 could be used as antimycobacterial agents, they should be tested towards other mycobacteria such as *M. tuberculosis* and *M. ulcerans*, the real causative agents respectively of tuberculosis and Buruli ulcer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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