British Microbiology Research Journal
4(8): 905-917, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Tolerance Tests of *Alcaligenes faecalis* BW1 Extract

Ilham Zahir[1*] Abdellah Houari[1], Mohammed Iraqui[1] and Saad Ibnsouda[1]

[1]Laboratory of Microbial Biotechnology, Department of Biology, Faculty of Sciences and Technical, University Sidi Mohamed Ben Abdellah, BP 2202, Road of Immouzer, Fez, Morocco.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Short Research Article

Received 24th February 2014
Accepted 13th April 2014
Published 6th May 2014

ABSTRACT

Aims: To highlight whether metabolites of *Alcaligenes faecalis* BW1 extract can be administered orally for their possible antimycobacterial effects.
Study Design: Study of the influence of certain parameters on the extract of *Alcaligenes faecalis* by using either discs or well diffusion methods against *M. smegmatis*.
Place and duration of study: Laboratory of Microbial Biotechnology, Department of Biology, Faculty of Sciences and Technical, University Sidi Mohamed Ben Abdellah, BP 2202, Road of Immouzer, Fez, Morocco. From April to August, 2012.
Methodology: The impact of acidic pH of gastric juice, bile, hydrogen peroxide, pancreatic enzymes and lysozyme on the antimycobacterial activity of *Alcaligenes faecalis* BW1 extract was evaluated by agar diffusion method. Detection whether or not antibacterial metabolites having a synergistic effect with rifampicin against *M. smegmatis* was also explored.
Results: Antibacterial metabolites of *Alcaligenes faecalis* BW1 extract resist to the action of gastric pH, gallbladder bile and hydrogen peroxide. In addition, they are not affected by pancreatic enzymes and lysozyme. Moreover, they have a synergistic effect with rifampicin against *M. smegmatis*.
Conclusion: Anti-mycobacterial metabolites of *Alcaligenes faecalis* BW1 extract are compatible with rifampicin and could be administered orally as antitubercular agents after their purification, identification in further work.

**Corresponding author: Email: ilham_biologie@hotmail.fr;*

Keywords: Alcaligenes faecalis BW1 extract; tolerance tests; antimycobacterial effect.

ABBREVIATIONS

A: Alcaligenes; H_2O_2: hydrogen peroxide; LB: Luria-Bertani; M: Mycobacterium.

1. INTRODUCTION

Bacterial resistance to different antibiotics currently available has become a major health problem across the globe. This burden has caused the re-emergence of many diseases such as tuberculosis. This disease remains among the curable infections, however being responsible for the death of 1.3 million people worldwide in 2012 according to the latest WHO report [1]. In addition, very few antibiotics are active against *Mycobacterium tuberculosis.* Treatments are long and are accompanied by a plethora of side effects, particularly in patients with HIV [2]. Consequently, it is imperative to use a new alternative to overcome the current impasse and find new molecules with anti- mycobacterial effect with reduced toxicity and be compatible with existing anti-tubercular drugs. In this context that, several paths have been exploited for the discovery of new anti-tuberculosis agents such as the synthesis of chemical molecules, the study of bioactive substances synthesized by plants, invertebrates and microorganisms [3].

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 following heat treatment and proteolytic enzymes (pepsin, proteinase K) that indicated the non-proteinaceous nature of the active agents [4].

Most currently used anti-tubercular drugs are administered orally [5]. Therefore, molecules with anti-mycobacterial effect recently discovered must resist to the action of digestive enzymes before being absorbed from the gut and metabolized by the liver. Otherwise, these substances will be administered parenterally. Thus, before proceeding to the structural characterization of *A. faeclais* BW1 extract's molecules, tolerance tests were performed to different agents. Herein, this investigation is a continuation of our precedent work which aims to investigate the effect of some factors on metabolites synthesized by *A. faecalis* BW1 e.g., acidic pH of gastric juice, gallbladder bile, hydrogen peroxide, pancreatic enzymes, lysozyme and to detect whether or not antibacterial metabolites have a synergistic effect with rifampicin against *M. smegmatis*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Media

Mycobacterium smegmatis MC²155 is a non pathogenic atypical strain with a generation time of approximately 3h [6]. This mycobacterim 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 a group of Gram-negative and positive bacteria [4].

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

2.2 Ethyl Acetate Extraction of the Antimycobacterial Substances

A liquid-liquid extraction was done by using ethyl acetate. An inoculum of *Alcaligenes faecalis* at $OD_{595\ nm}$ = 0.3 was cultivated under agitation on LB medium at 37ºC for 48h. A volume of 100ml of the bacterial culture was centrifuged for 10min at 6000g. Then, the supernatant was recovered, sterilized by filtration and added to 100ml of ethyl acetate. After agitation for one hour at room temperature, the organic extract obtained was evaporated under vacuum at 37ºC. The dry residue was taken up in 1ml of sterile distilled water. The obtained solutions will be referred to as "crude extract" [4].

2.3 Impact of Lysozyme and Pancreatic Enzymes

To investigate the impact of lysozyme and some pancreatic enzymes on bioactive substances, the crude extract of *A faecalis* was individually treated with enzyme solutions prepared in phosphate buffer (50 mM, pH 7) at a final concentration 1mg/ml of lysozyme, trypsin, chymotrypsin, amylase and lipase. The mixtures were then thoroughly homogenized and incubated at 37ºC for 2h. The antimycobacterial effect of these preparations was investigated using the method of agar disc diffusion against *M. smegmatis* as the indicator strain. The Petri dishes containing LB medium were incubated at 37ºC for 48h. Controls corresponded to pure extract used alone and to the solutions of enzymes employed at the same used final concentration [8,9].

2.4 Effect of pH

To assess the sensitivity of the bioactive substances at different pH, a cell-free supernatant was obtained after centrifugation at 6000rpm for 15min at 4ºC in a culture of *A. faecalis* for 48h. The pH effect was detected by adjusting the pH of the supernatant to values ranged from 2 to 12 using 10M NaOH or 10M HCl. After 2h of incubation at 37ºC, the residual antimycobacterial activity against *M. smegmatis* was tested by agar well diffusion method [10]. The untreated supernatant was used as a control (pH 7). This test was repeated twice.

2.5 Effect of Bile

To test the influence of bile on *A. faecalis* extract precipitated with ethyl acetate, filtered sheep bile was added to metabolites of *A. faecalis* at a final concentration of 0.3% and 50%. The antimycobacterial effect of the mixture was then tested by the agar disc method after 2h of incubation at 37ºC for 48h [11,12]. The control corresponded to the study of the antimycobacterial activity of the extract alone at different concentrations (100%, 50% and 99.7%) and the bile alone (100%, 50% and 0.3%). This test has been duplicated and statistically evaluated by Student's test with alpha=0.05.

2.6 Influence of Hydrogen Peroxide

To highlight the effect of hydrogen peroxide (H_2O_2) on the bioactive metabolites, the extract of *A. faecalis* was subjected to the action of H_2O_2 at final concentrations of 6% (1.8M), 3% (0.9M), 1% (0.3M), 0.3% (100 mM) and 0.03% (10mM) from a stock solution with 30% of purity. The mixtures were then thoroughly homogenized and incubated at 37°C for 2h. The antimycobacterial effect of these preparations was investigated using the discs method against *M. smegmatis*. The Petri dishes containing LB medium were incubated at 37ºC for 48h. Non-treated extract, different dilutions prepared from the stock solution of oxygenated water and combinations in which H_2O_2 was replaced by sterile distilled water were used as controls [13,14]. This test was repeated twice and statistically evaluated by Student's test with alpha = 0.05.

2.7 Interaction with Rifampicin

For an eventual use of bioactive substances synthesized by *A. faecalis*, the effectiveness of their antimycobacterial effect in the presence of rifampicin, an antitubercular drug able to penetrate inside the macrophages [15], was studied using a modified protocol of Ahmed et al. [16]. Solutions of antibiotic alone or in combination with the extract (precipitated by ethyl acetate) were prepared to final concentrations of rifampicin ranged from 200mg/ml to 0.1mg/ml. Solutions were incubated for 5h at 37ºC. Thereafter, 6mm discs impregnated in each solution were then transferred to sterile Petri dishes containing LB medium and freshly inoculated with a culture of *M. smegmatis*. After incubation at 37ºC for 48h, the absence or presence of zones of inhibition was examined and the diameters of inhibition halos were measured in three different directions. Controls corresponded to extract of *A. faecalis* diluted with sterile distilled water, the antibiotic at different dilutions. This test was repeated twice and statistically evaluated by Student's test with alpha equals 0.05.

To confirm the results, firstly, the inhibitory effect of the tested substances was determined individually by putting on Petri dishes, previously spread by a pure culture of *M. smegmatis,* discs containing 25µl of rifampicin (200mg/ml) and discs containing 25µl of *A. faecalis* extract. Then, the combined effect of these molecules was evaluated by placing the impregnated disks on agar Petri dishes inoculated with the same sensitive bacterium so that the zones of inhibition following incubation for 48h at 37°C would be affected tangentially. The clear zones of inhibition around each disc produced after individual or mutual effect were measured in three different directions to obtain the mean value of each diameter. The occurrence of mutual influence / interference when the molecules were used in combination was evaluated as (i) indifference, when the two tangent circles of inhibition were not affected, (ii) antagonism when the circles were decreased and reniform was assumed, (iii) synergism when the circles were expanded. The increase or decrease of the mean surface of the inhibition's zone (πr^2) due to a particular combination has been calculated on the basis of average diameter (2r) and the percentage increase was estimated as (B-A)/Ax100. A is the surface due to the individual effect and B is the surface due to the combined effect. The results were evaluated by the Student test for their significance levels with alpha =0.05 [17,18].

3. RESULTS AND DISCUSSION

3.1 Impact of Lysozyme and Pancreatic Enzymes

A new anti-tubercular drug administered orally must resist the action of digestive enzymes before being metabolized by the liver. These enzymes include lysozyme which is a hydrolase found in a number of secretions (mucus, saliva, etc.) [19] and pancreatic juice secreted by exocrine portion of the pancreas into the duodenum during the digestion. This juice contains four distinct classes of enzymes divided according to their substrate specificity into; amylolytic for digestion of carbohydrates, proteolytic for hydrolysis of proteins, lipolytic for cleavage of fats and also nucleolytic for digestion of nucleic acids [20].

The inhibitory potency of the extract of *A. faecalis* BW1 against *M. smegmatis* was evaluated after treatment with lysozyme, trypsin, chymotrypsin, amylase and lipase. This anti-mycobacterial effect was not affected since the diameters of inhibition zones remained unchangeable before and after treatments (Table 1), while the solutions of enzymes used as control didn't exhibit any inhibitory activity against the indicator strain. Our results are corroborated with another research that describe the ability to inhibit bacterial growth by bioactive substances synthesized by *A. faecalis* is not reduced after treatment with trypsin [21].

It should be noted that these results have also confirmed those found in previous work [4] that bioactive molecules produced by *A. faecalis* BW1 are not protein in nature. In addition, they emphasized that these molecules are not lipid or carbohydrate in nature.

Table 1. Anti-mycobacterial effect of *A. faecalis* BW1 extract after treatment with lysozyme and pancreatic enzymes

Type of treatment	Diameter of inhibition zone (mm)
Lysozyme	17±0,05
Trypsin	17±0,05
Chymotrypsin	17±0,05
Amylase	17±0,05
Lipase	17±0,05
Non- treated extract of *A. faecalis* BW1 (control)	17±0,05

Ethyl acetate crude extract treated by lysozyme and pancreatic enzymes was tested against M. smegmatis by discs method

3.2 Effect of pH

Susceptibility of metabolites of *A. faecalis* BW1 was studied at pH in the range of 2 to 12. Thus, we found that the residual anti-mycobacterial activity was not affected at acidic to slightly alkaline pH (Fig. 1).

These results are not in agreement with those of Bacic and Yoch [21], reporting that the inhibitor capacity of the antibiotic produced by *A. faecalis* M3A is not affected by pH changes, nor with those of Thangam and Rajkumar [22], demonstrating the existence of an extracellular protease synthesized by the same bacteria and which is stable at high alkaline pHs. Nevertheless, our data reveal that bioactive substances can withstand the acidity of the

stomach where the pH is between 1.35 and 3.5 [23] and that of the surrounding inside macrophages having a pH equaling approximately 5 [24,25].

It should be noted that previously, we also found that *A. faecalis* BW1 extract was not degraded by pepsin [4], a gastric protease responsible for the degradation of proteins [26].

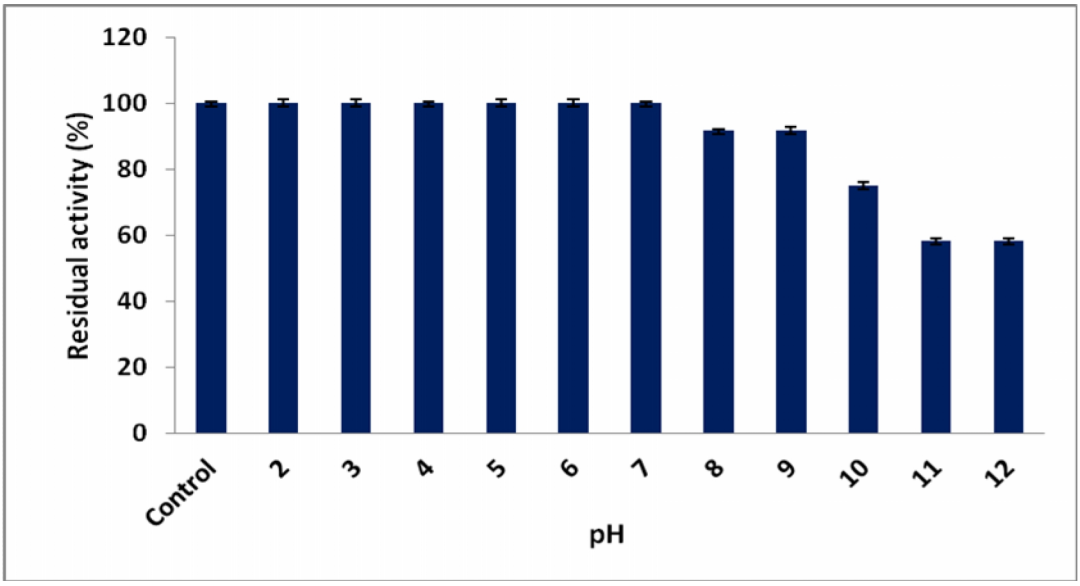

Fig. 1. Residual activity percentage of *A. faecalis* BW1 extract against *M. smegmatis* at different pH

A. faecalis cell-free supernatant was treated by adjusting the pH to values in a range of 2 to 12 and tested against *M. smegmatis* by agar well diffusion method. The residual activity was measured.

3.3 Effect of Bile

Bile is a green yellow aqueous solution produced by the liver and stored in the gallbladder which concentrated it. It plays a role in the dissolution and emulsification of ingested fats. The major components are the bile salts, cholesterol, phospholipids, ions biliverdin pigments and bile acids namely cholic acid, deoxycholic acid, chenodeoxycholic acid and lithocholic acid [27].

The results of the bile's influence on *A. faecalis* BW1 extract showed that bioactive substances were not affected by the bile at the final concentrations 50% and 0.3%. Moreover, it was demonstrated that bile had an inhibitory effect against *M. smegmatis* at concentrations 50% and 100%.

It was also noted that the diameters of inhibition zones increased when the bile and the extract were combined to a final concentration 50% (Fig. 2). The differences observed between the combination and control (extract at the concentration 50%) were significant after statistical evaluation by Student's test for a value of alpha equals to 0.05. Therefore, gallbladder juice may potentiate the activity of anti-mycobacterial metabolites contained in *A.*

faecalis BW1 extract. To ensure the validity of this hypothesis, the impact of bile acids on *A. faecalis* extract should be sought as a perspective of the present work.

Previously, it was reported that bile causing oxidative stress and DNA damage in various bacteria. Thus, according to the study by Kristoffersen et al. [27], it was found that bile salts (0.01%) inhibit the growth of *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus megaterium* AH1388, AH1391 *Azotobacter vinelandii* and *Arthrobacter crystallopoietes*. Furthermore, our study is the first to show that bile has anti-mycobacterial effect beyond 0.3%, as comparable to that found in the small intestine concentration [27]. Other enteric pathogens such as *Listeria monocytogenes*, *Enterococcus faecalis* and *Salmonella* sp. can also survive at the same concentration [27,28].

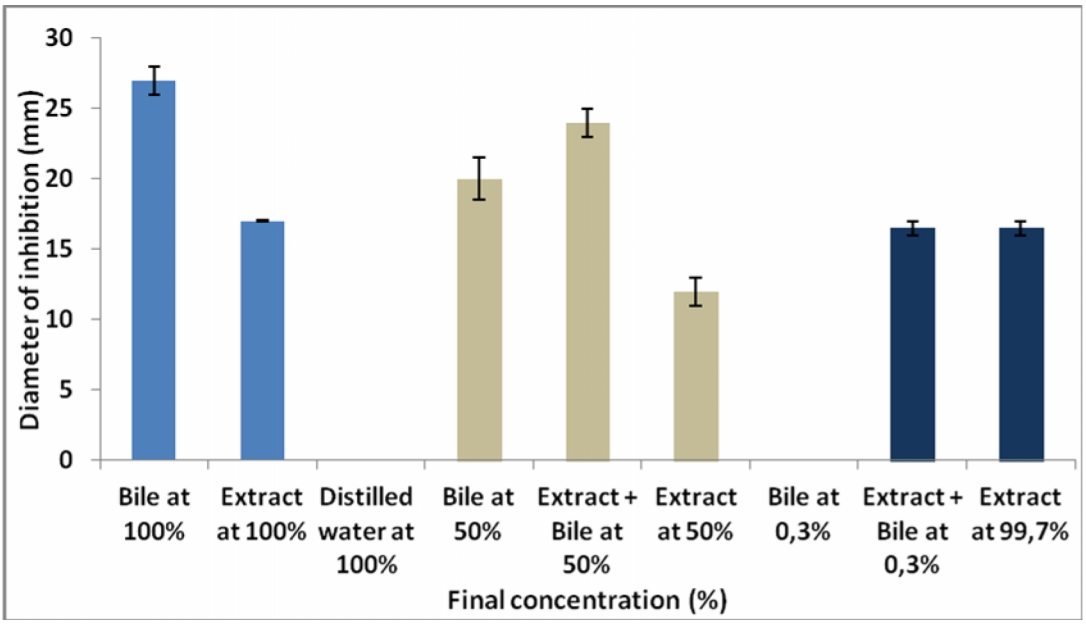

Fig. 2. Effect of *A. faecalis* BW1 extract treated with bile at different concentrations against *M. smegmatis*

The impact of filtered sheep bile on A. faecalis extract was evaluated at final concentrations of 0.3% and 50%. The anti-mycobacterial effect of mixtures was tested by the discs method. Controls corresponded to the extract alone at different concentrations (100%, 50% and 99.7%) and bile alone (100%, 50% and 0.3%). Results were statistically significant by Student's test for a value of alpha equals to 0.05

3.4 Influence of Hydrogen Peroxide

To be a good anti-tubercular drug, a bioactive substance must diffuse inside macrophages and must resist the action of H_2O_2 generated by these phagocytic cells [29].

Results showed that anti-mycobacterial effect of *A. faecalis* BW1 extract precipitated by ethyl acetate persisted after treatment with H_2O_2. In addition, this effect was further potentiated by increasing concentrations of H_2O_2 (Figs. 3 and 4). The differences observed between the various combinations used and controls were significant after statistical evaluation by Student's test for a value of alpha equals to 0.05.

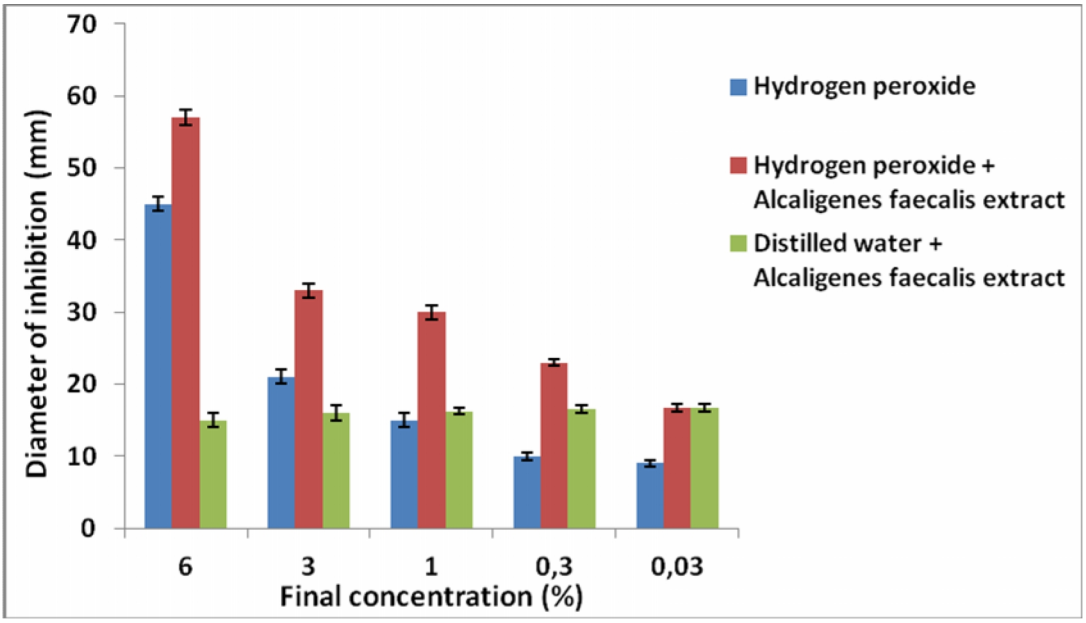

Fig. 3. Effect of *A. faecalis* BW1 extract treated with different concentrations of hydrogen peroxide against *M. smegmatis*

The effect of hydrogen peroxide (H_2O_2) on A. faecalis extract was examined at final concentrations 6%, 3%, 1%, 0.3% and 0.03% from a stock solution with 30% of purity. Combinations were tested against M. smegmatis by using the discs method. Results were statistically the same font

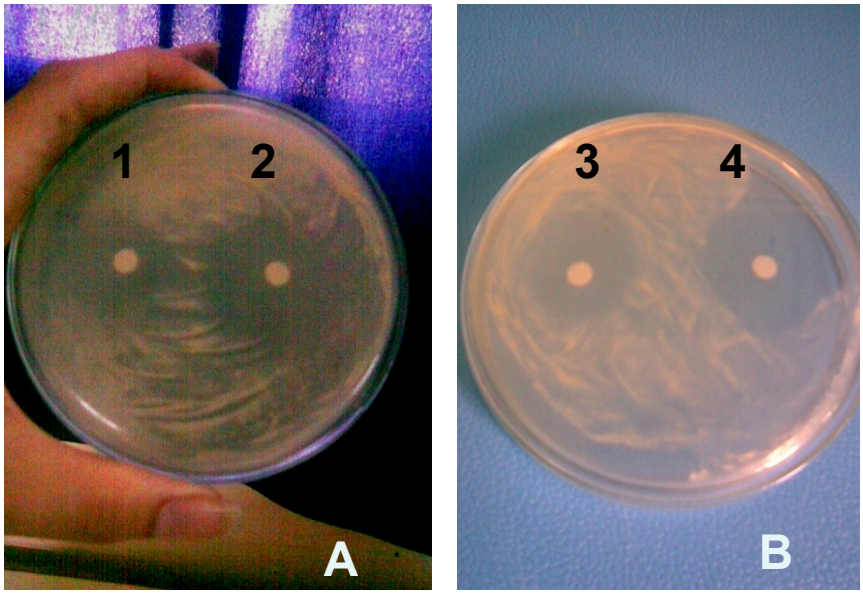

Fig. 4. Photos showing the effect of hydrogen peroxide on *A. faecalis* BW1 extract at final concentrations of 1% (A) and 3% (B)

1: H_2O_2 at the final concentration 1% (control); 2: H_2O_2+ extract of *A. faecalis* (combination at 1%);
3: H_2O_2 at the final concentration 3% (control); 4: H_2O_2 + extract of *A. faecalis* (combination at 3%)

Moreover, the stock solution of H_2O_2 with 30% purity stopped the growth of *M. smegmatis*. This correlates with the literature reporting that this molecule is an active agent that affects a wide range of organisms such as bacteria (*Mycobacterium sp*. *Streptococci sp*...), yeasts, fungi, viruses, and spores [30,31,13,14,32,33,34]. Indeed, the antibacterial action of H_2O_2 involves hydroxyl radicals. These are powerful antioxidants which can easily react with macromolecules namely membrane lipids and DNA thereby causing bacterial death [34].

3.5 Interaction with Rifampicin

To prevent the emergence of bacterial resistance in tuberculosis cases, combination therapy is a necessity during treatment because the drug combination often has a synergistic effect that exceeds the performance of a single antibiotic [16]. It is in this context that, the inhibitory effect of *A. faecalis* BW1 extract in the presence of rifampicin was investigated. Thus, for example the final concentration 200mg/ml of rifampicin, when used alone, presented a zone of inhibition of 29.3mm, while the extract, when employed alone, formed an inhibition area of 17mm. But, when they were combined, the diameter of inhibition zone exceeded these two values and reached 32mm in comparison with the control (extract with distilled water) showing only an area of 9.25mm (Fig. 5).

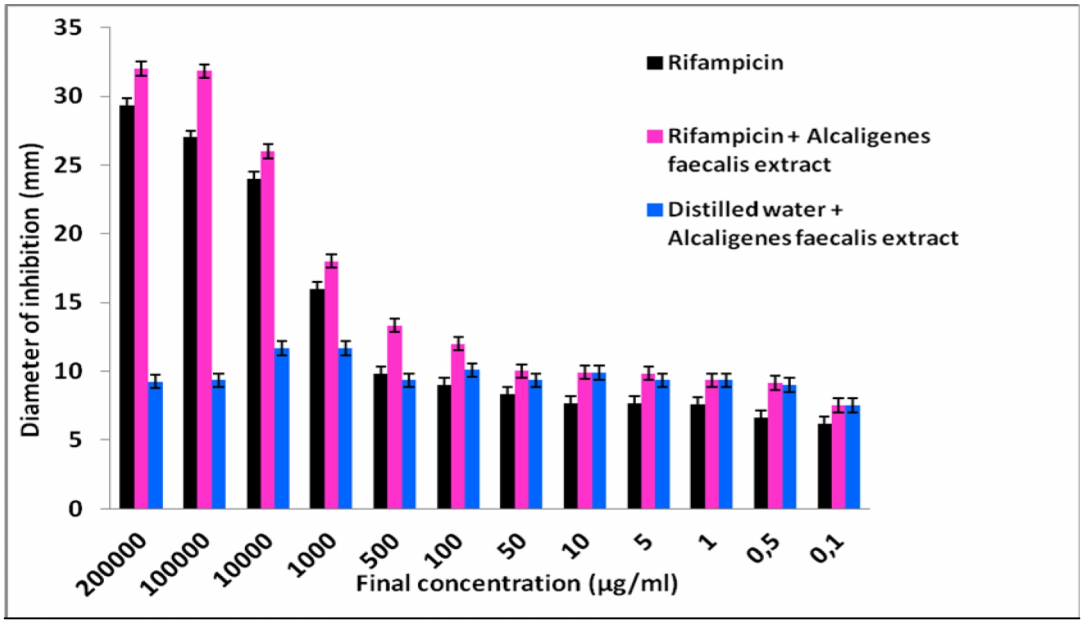

Fig. 5. Antimycobacterial activity of *A. faecalis* BW1 extract combined with rifampicin at different concentrations against *M. smegmatis*

Solutions were prepared of rifampicin alone or in combination with the extract precipitated by ethyl acetate to final concentrations ranged from 200mg/ml to 0.1mg/ml. These preparations were tested against *M. smegmatis* by using discs method. Non- treated extract of *A. faecalis*,

combinations including the antibiotic which was replaced with sterile distilled and different dilutions prepared from 300mg/ml of rifampicin were used as controls. Results were statistically the same font.

For other different tested concentrations, increased diameters of the inhibition zones of bioactive substances combined with rifampicin were also observed (Fig. 6). This increase was statistically significant by Student's test for a value alpha equals to 0.05. This increase in the zone of inhibition surrounding the discs tests compared with controls could be considered as a synergism [35,36].

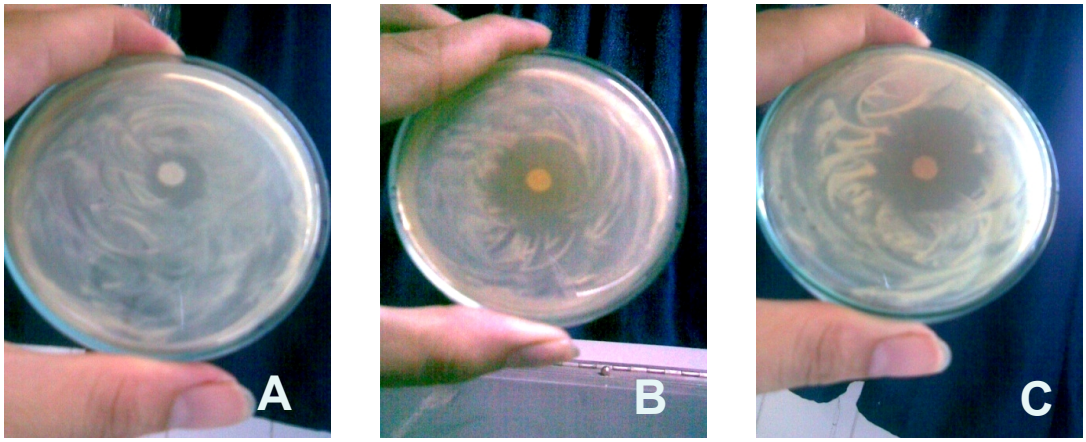

Fig. 6. Photos showing the inhibition zones against *M. smegmatis*. (A), effect of *A. faecalis* BW1 extract; (B), effect of rifampicin (C), effect of combination between *A. faecalis* BW1 extract and rifampicin at the final concentration 100 mg/ml

To confirm our results, the determination of synergism between rifampicin and *A. faecalis* BW1 extract was carried out by disc diffusion method. The results showed that the average diameter of inhibition zone was respectively 29.33mm and 17mm for rifampicin and extract. While their combined activity was synergistic, the inhibition of rifampicin area increased by 2.67mm while that of bioactive substances increased by 3mm (Fig. 7). The percent increase in surface area of the inhibition zones was 19.03% and 38.4% respectively for rifampicin and extract (Table 2). Statistical analysis of these values by the Student's test showed the results were significant (alpha=0.05). Therefore, it can be said that these molecules act synergistically.

Table 2. Individual and combined effect of rifampicin and *A. faecalis* BW1 extract on *M. smegmatis*

Diameter of the inhibition zones (mm)					
Individual effect (A)		**Combined effect (B)**		**Percentage increase based on πr^2 (%)***	
Rifampicin	**Extract**	**Rifampicin**	**Extract**	**Rifampicin**	**Extract**
29.33	17	32	20	19.03	38.4

** Average area of inhibition zones (mm^2) was calculated on the basis of πr^2 average diameter (2r) and the percentage increase was estimated as (B - A) / A x 100. A is the surface due to the individual effect and B is the surface due to the combined effect. Results were statistically significant (alpha = 0.05)*

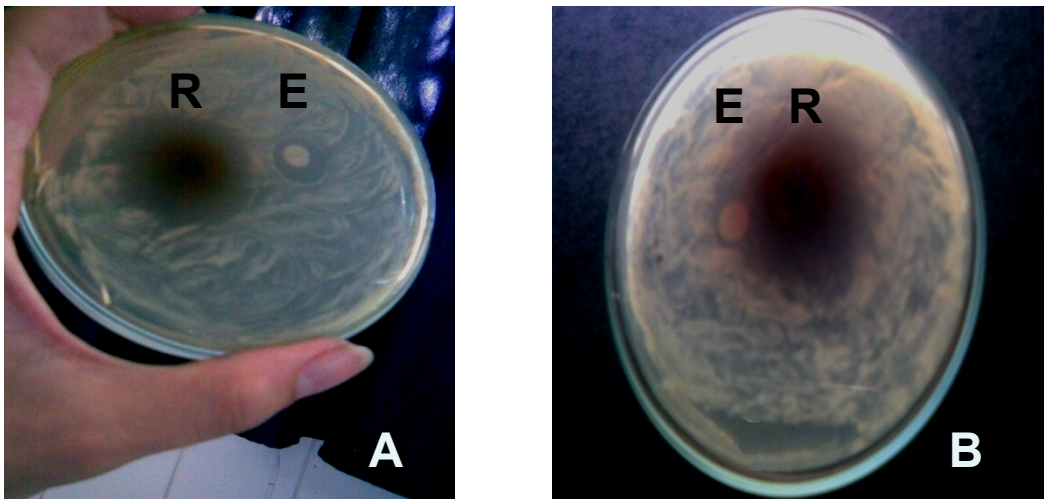

Fig. 7. Pictures of synergism between rifampicin (R) and extract of *A. faecalis* BW1 (E) against *M. smegmatis*. (A) individual effect, (B) combined effect

4. CONCLUSION

Tolerance tests of *A. faeclais* BW1 extract were performed vis-a-vis different agents. Thus, the bioactive metabolites are found resistant to the action of gastric pH, gallbladder bile and hydrogen peroxide. Moreover, they are not affected neither by pancreatic enzymes nor lysozyme which suggest that these anti-mycobacterial metabolites could be administered orally to cure tuberculosis after their purification, identification and determination of inhibitory minimal concentration against *M. tuberculosis* in further work. In addition, these substances of *A. faeclais* BW1 extract are compatible with rifampicin against *M. smegmatis*. Nevertheless, to contribute efficiently to find new drugs against multidrug resistant *M. tuberculosis*, other investigations are required aiming to look for the efficacy, safety, toxicology, and the pharmacodynamic properties of these substances *In vivo*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. World Health Organization, 2014. Global tuberculosis report 2013. Geneva: WHO, 2013.
   Available: http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf.
2. Baulard AR. Dopage dans le peloton des antituberculeux. Med Sci. 2005;21(4):1-3.
3. Copp BR. Antimycobacterial natural products. Nat Prod Rep. 2003;20:535-557.
4. Zahir I, Houari A, Bahafid W, Iraqui M, Ibnsouda S. A novel *Alcaligenes faecalis* antibacterial-producing strain isolated from a Moroccan tannery waste. Afr J Microb Res. 2013;7(47):5314-5323.
5. Nicolet G, Rochat T, Zellweger JP. Traitement de la tuberculose. Curriculum, Forum Med. Suisse. 2003;22:506-516.

6. Grosset J, Boisvert H, Truffot-Pernot C. Mycobacteria. In: Bactériologie médicale L. Le Minor M. Veron. Ed. Flammarion Paris. 1989;965-1017.
7. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. 2 nd. Cold Spring Harbor laboratory Press, ISBN 0-87969-309-6;1989.
8. Alam SI, Kamrani M, Sohail M, Ahmad A, Khan SA. Partial characterization of bacteriocin like inhibitory substance from *Bacillus subtilis* BS15, a local isolate. Pak. J. Bot. 2011;43(4):2195-2199.
9. Sifour M, Tayeb I, Ouled Haddar H, Namous H, Aissaoui S. Production and characterization of bacteriocin of *Lacotobacillus plantarum* F12 with inhibitory activity against *Listeria monocytogenes*. The online journal of science and technology. 2012;2(1):55-61.
10. Ivanova I, Kabadjova P, Pantev A, Danova S, Dousset X. Detection, purification and partial characterization of a novel bacteriocin substance by *Lactococcus lactis* subsp. *lactis* B14 isolated from boza bulgarian traditional cereal beverage. Biocatalysis-2000: Fundamentals & Applications. 2000;41(6):47-53.
11. Jin LZ, Ho YW, Abdullah N, Jalaludin S. Acid and bile tolerance de *Lactobacillus* isolated from chicken intestine. Lett Appl Microbiol. 1998;27:183-185.
12. Audisio MC, Apella MC. Bacteriocin-like substance produced by *Lactobacillus salivarius* subsp. *Salivarius* CRL1384 with anti *Listeria* and anti *Salmonella* effects. Res J Microbiol. 2006;1(1):61-69.
13. Thomas EL, Milligan TW, Joyner RE, Jefferson MM. Antibacterial activity of hydrogen peroxide and the lactoperoxidase-hydrogen peroxide-thiocyanate system against oral *Streptococci*. Infect Immun. 1994;62(2):529–535.
14. Kniel KE, Sumner SS, Lindsay DS, Hackney CR, Pierson MD, Zajac AM, Golden DA, Fayer R. Effect of organic acids and hydrogen peroxide on *Cryptosporidium parvum* viability in fruit juices. J. Food Protect. 2003;66(9):1650-1657.
15. Veziris N, Cambau E, Sougakoff W, Robert J, Jarlier V. Résistance aux antituberculeux. Arch Pédiatrie. 2005;12:102-109.
16. Ahmed Z, Khan SS, Khan M. Synergistic effect of *Salvadora persica* extracts, tetracycline and penicillin against *Staphylococcus aureus*. Afr J Basic Appl Sciences. 2010;2(1-2):25-29.
17. Dutta NK, Dastidar SG, Kumar A, Mazumdar K, Ray R, Chakrabarty N. Antimycobacterial activity of the anti-inflammatory agent Diclofenac sodium and its synergism with streptomycin. Braz J Microbiol. 2004;35:316-323
18. Kumar KA, Mazumdar K, Dutta NK, Karak P, Dastidar, SG, Ray R. Evaluation of synergism between the Aminoglycoside antibiotic Streptomycin and the cardiovascular agent Amlodipine. Biol Pharm Bull. 2004;27(7):1116—1120.
19. Grunclov L, Fouquier H, Hyps V, Kopásek P. Lysozyme from the gut of the soft tick *Ornithodoros moubata*: the sequence, phylogeny and post-feeding regulation. Dev & Comp Immunol. 2003;27(8):651-660.
20. Ganong WF. Review of medical physiology. New York: McGraw-Hill Medical. 2005;912.
21. Bacic MK, Yoch DC. Antibiotic composition from *Alcaligenes* species and method for making and using the same. U.S. Patent. 2001;(6)224:863.
22. Thangam EB, Rajkumar GS. Studies on the production of extracellular protease by *Alcaligenes faecalis*. World J Microbiol Biotechnol. 2006;16(7):663-666
23. Schwalfenberg GK. The Alkaline Diet: Is There Evidence That an Alkaline pH Diet Benefits Health?. J. Environ Public Health. 2012;727630:1-7.
24. Nyberg K, Johansson U, Johansson A, Camner P. Phagolysosomal pH in Alveolar Macrophages. Environ Health Persp. 1992;97:149-152.

25. Porcaro I, Jouvert S, Mallié M, Boudard F, Vidal M, Giaimis J. Rôle du phagolysosome des macrophages sur la croissance et la filamentation de *Candida albicans*. J Mycol Med. 2002;12(2):58-64.
26. Eswaran S, Roy MA. Medical management of acid-peptic disorders of the stomach. Surg Clin N Am. 2005;85:895–906.
27. Kristoffersen SM, Ravnum S, Tourasse NJ, Økstad OA, Kolstø AB, Davies W. Low concentrations of bile salts induce stress responses and reduce motility in *Bacillus cereus* ATCC 14570. J Bacteriol. 2007;189(14):5302–5313.
28. Kokosharov T. Acid tolerance, bile salts and serum resistance of *Salmonella* Gallinarum from hens. Vet Arhiv. 2003;73:277-283.
29. Shi L, Sohaskey CD, North RJ, Gennaro ML. Transcriptional characterization of the antioxidant response of *Mycobacterium tuberculosis* in vivo and during adaptation to hypoxia in vitro. Tuberculosis. 2008;88:1–6.
30. Cordone A, Audrain B, Calabrese I, Euphrasie D, Reyrat JM. Characterization of a *Mycobacterium smegmatis* uvrA mutant impaired in dormancy induced by hypoxia and low carbon concentration. BMC Microbiol. 2011;11:231.
31. Hu Y, Kendall S, Stoker NG, Coates RMA. The *Mycobacterium tuberculosis* sigJ gene controls sensitivity of the bacterium to hydrogen peroxide. FEMS Microbiol Lett. 2004;237:415–423.
32. Lucas-Elío P, Gómez D, Solano F, Sanchez-Amat A. The Antimicrobial activity of Marinocine, synthesized by *Marinomonas mediterranea* is due to hydrogen peroxide generated by its lysine oxidase activity. J Bacteriol. 2006;188(7):2493-2501.
33. Russo R, Curtis EW, Yanong RP. Preliminary investigations of hydrogen peroxide treatment of selected ornamental fishes and efficacy against external bacteria and parasites in green swordtails. J Aquat Anim Health. 2007;19(2):121-7.
34. Shahriari S, Mohammadi Z, Mokhtari MM, Yousefi R. Effect of hydrogen peroxide on the antibacterial substantivity of Chlorhexidine. Int J Dent. 2010;946384:1-4.
35. Climo MW, Patron RL, Archer GL. Combinations of Vancomycin and B-lactams are synergistic against *Staphylococci* with reduced susceptibilities to Vancomycin. Antimicrob Agent Ch. 1999;43(7):1747–1753.
36. Mayer I, Nagy E. Investigation of the synergic effects of aminoglycoside-fluoroquinolone and third generation cephalosporin combinations against clinical isolates of *Pseudomonas spp*. Antimicrob Agent Ch. 1999;43:651-657.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=489&id=8&aid=4479