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In vitro Evaluation of the Antimycobacterial Activity and Fractionation of *Berberis hispanica* Root Bark

Amina Cherif Haouat[1], Abdellatif Haggoud[1], Suzanna David[2], Saad Ibnsouda[1] and Mohammed Iraqui[1]

[1]Université Sidi Mohammed Ben Abdellah, Faculté des Sciences et Techniques de Fès. B.P: 2202, Fès, Maroc.
[2]Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA,IP), 1649-016, Lisbon, Portugal.

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The antimycobacterial activity of the ethanolic extract from *Berberis hispanica* root bark was evaluated against *Mycobactérium smegmatis* and *M. aurum*. The extract was fractionated and the bands responsible for the activity were identified by bioautography. The results revealed the existence of three of four bands responsible for the antimycobacterial activity of the plant. A phytochemical study evidenced the nature of the main classes of secondary metabolites in the crude extracts and in the active bands of the bioautography. The molecules shown to be responsible for the antimycobacterial activity of the plant included alkaloides, polyphenols and flavonoids.

Key words: Tuberculosis, *Berberis hispanica*, antimycobacterial activity.

Tuberculosis, mainly due to *Mycobacterium tuberculosis*, remains a major public health problem; it generally infects the lungs, but may affect any organ of the human body. Responsible for nearly 2 million deaths annually, tuberculosis is considered as the second main cause of mortality through infection, after AIDS (Acquired Immune Deficiency Syndrome)[1]. In 2011, the estimate was of 8.7 million new tuberculosis cases with the number of deaths reaching 1,4 million. The five countries that registered the highest incidence in 2011 were, respectively, India, China, South Africa, Indonesia and Pakistan[1].

* To whom all correspondence should be addressed.

In Morocco, the number of cases in 2010 reached 27143, of which 70% were reported in the urban areas, particularly those surrounding large cities[2].

Between 1900 and 1980, an important reduction in the number of people infected with tuberculosis was observed in developed countries due to improvements in social economic conditions, the introduction of the BCG (Bacillus Calmette-Guérin) vaccine and the introduction of effective antibiotic therapy. However, as of the end of the 1980s, the resurgence of tuberculosis threatened the world with on one hand multidrug-resistant (MDR) and extensively drug-resistant (XDR) bacilli[3,4], and on the other the pronounced synergy between the human immunodeficiency virus (VIH) infection and tuberculosis[5,6]. In 1993, the World Health Organization (WHO) declared tuberculosis a global emergency[7].

New molecules with an antimycobacterial effect need to be developed especially in the light of emerging multidrug-resistant and extensively drug-resistant *M. tuberculosis* complex strains. Moreover they are needed for the treatment of

opportunistic infections caused by atypical mycobacteria, having also increased in the advent of AIDS. Indeed these atypical, environmental species are usually naturally resistant to the majority of currently available antimybacterial drugs[8-10].

Research for biologically active substances from plants used in traditional medicine has regained interest. Medicinal plants represent a rich reservoir of biologically active substances including compounds that act upon novel molecular targets. New entities with novel mechanisms of action are expected to contribute to the resolution of emerging antibiotic resistance[11-13].

A large number of extracts and pure compounds from plants have been shown to possess remarkable inhibitory activity against *M. tuberculosis* and other mycobacterial species[14-18], including against multidrug-resistant strains[11,19,20].

Within this context, the purpose of the present study was to identify the active substances responsible for the antimycobacterial activity of *Berberis hispanica.*

Berberis hispanica, barberry bush, Boiss. & Reut., is a species belonging to the *Berberidaceae.* It is original of Europe and the British Isles later naturalized in North America. The 1-1.5 m high bush is abundantly ramified with angular branches of a dark reddish color, facetted leaves. The flowers are yellow and the fruit juicy, bacciforme and bluish to blue-black, slightly prunish in color[21]. In Morocco, the plant is abundant in the rocky landscapes of the Middle Atlas, Central and Oriental Middle Atlas, High Atlas and Saharan Atlas ranges and Rif mountains[22,23].

In traditional Moroccan medicine the plant is indicated for the treatment of inflammations, liver ailments, metrorragy and digestive disorders. Infusions of *Berberis hispanica* are used to treat gastro-intestinal atony and hepatic and biliary disorders[22]. The root bark of the plant is used by the Berber tribes of the Middle Atlas in the treatment of eye ailments[22].

Different parts of the *Berberis hispanica* plant such as the root, bark, leaves and fruit have been used in traditional medicine. Research within the last two decades has shown the different pharmacological and therapeutical effects of the plant[24]. It is largely used in homeopathic medicine against the pain and elimination of renal calculus[25]. Recent studies have shown that *Berberis hispanica* extracts have important antioxidant properties[26,27]. Root bark extracts of *Berberis hispanica* were shown to posses interesting profiles as immunomodulators[28]. El Ouarti and al (2011), also showed that the ethanolic extract of root bark from this plant presents a remarkable antimycobacterial *in-vitro* and *ex-vivo*. With the latter being more accentuated than that observed with amikacine, used as a second line antituberculous agent[29].

These results have led us to pursue investigations for antimycobacterial compounds from this plant. Therefore, the present study consisted in the separation and identification by bioautography followed by phytochemical analysis of compounds responsible for the antimycobacterial activity of *B.hispanica*. To our knowledge this type of study has not been previously pursued.

MATERIALS AND METHODS

Plant material

The plant used in this investigation was *Berberis hispanica* Boiss. et Reut. (= *B. vulgaris* L., *B. aetnensis* auct. = *B. vulgaris* L. subsp. *australis* (Boiss.) Heywood = *B. vulgaris* L. var. *australis* Boiss. = *B. vulgaris* L. subsp. *hispanica* (Boiss. et Reut.) Malag), collected in June 2007 in the region of Imouzzer-des-Marmoucha, Boulmane Province, Marocco (33°29' N - 4°17' W, altitude approximately 1600m). A herbal specimen (exciccata INP76) was registered at the National Institute of Medicinal and Aromatic Plants, University Sidi Mohamed Ben Abdellah, Fès.

The root bark of the plant was air dried in the shade, then ground, in the microbial biotechnology laboratory of the Faculty of Sciences and Techniques of Fès. The powder was stored, in glass hermetically closed flasks, for subsequent preparation of different different extracts.

Mycobacterial strains

The strains used in this study were non virulent mycobacterial strains with faster growth rates than that of the tuberculous bacilli. These include:

M. aurum A+: a non tuberculous

mycobacteria with a generation time of approximately six hours, inhibition of its growth is highly predictive of activity against *M. tuberculosis*[30].

M. smegmatis MC_2155: a non tuberculous, non pathogenic mycobacteria with a generation time of approximately three hours, along with *M. aurum*, its susceptibility to antituberculous agents similar to that of *M. tuberculosis*[31].

These mycobacteria were cultured on Sauton medium at 37°C [32,33].

Preparation of plant extracts

The extraction of active substances was carried out using ethanol as the solvent. Two extracts were prepared from the root bark powder of *B.hispanica*:

Extract 1 : the powder was macerated in the shade for 24 hours in absolute ethanol in a proportion of 8g per 50ml of solvent. After filtration, carried out using a water aspirator vacuum pump, the solvent was dried at 37°C under vacuum using a rotary evaporator. The residue was then diluted in 2 ml of sterile distilled water. The pH of the extract was adjusted to neutral with NaOH 1N. The extract was sterilized by filtration using a 0.45 µm porosity filter.

Extract 2 : 12g of the plant powder were macerated in 50 ml of absolute ethanol for 24 hours, after filtration and evaporation, the extract was recovered in 2ml of ethanol.

Evaluation of the antimycobacterial activity of the ethanolic extract using the disc diffusion method

The antimycobacterial activity of the extracts was measured using the disc diffusion method[34,35]. For this, a sterile 6 mm diameter paper disc was placed at the center of 90 mm plates containing 30 ml of Sauton agar previously inoculated with 100µl of mycobacterial liquid culture at approximately 10^6 CFU/ml. The disc was then impregnated with 20 µl of Extract 1 (corresponding to 80 mg of plant dry matter). A disc impregnated with 20 µl of sterile distilled water was used as control. The plates were incubated at 37ºC for three days. After incubation the inhibition zones around the discs were measured. For each strain the experiment was repeated three times.

The statistical test used was the t student.

Bioautography

In order to identify which molecules in the ethanolic extract of *B hispanica* were responsible for the antimycobacterial activity, an adapted version of a bioautographic method from earlier reports was used[36,37]. Hexane-ethyl acetate 6:4 (v/v) was selected as the solvent system most appropriate for separating the components present in *B. hispanica* Extract 2 by thin layer chromatography (TLC). The bioautography of this extract was obtained as follows: Several spots (approximately 5 mm in diameter), each containing 14µl of the 1:2, v/v, ethanol diluted extract, were spotted onto a silica gel TLC plate (6 cm X 12 cm). The plate was allowed to develop using the selected solvent. After air drying the plate was covered with a thin layer of Sauton agar previously inoculated with a liquid culture of *Mycobacterium smegmatis*, approximately 0,2 absorbance, at 595 nm. For this, 9 ml of the prepared medium was used to cover the plates in 196 cm2 Petri dishes. After 24 hours of incubation at 37°C, the inhibition zone was localized and its diameter measured. The compounds inhibiting mycobacterial growth were characterized by the migration coefficient or R_f ratio of the corresponding TLC bands. The experiment was repeated six times.

Phytochemical study

In order to identify the molecules responsible for the antimycobacterial activity of *B.hispanica*, we determined the presence of total polyphenols, flavonoid, tannin and alkaloid contents in the crude plant extracts as well as in the inhibition zones identified by bioautography. The fraction of the plant extract in these zones was obtained by ethanol elution.

The principal chemical constituents were characterized in the extracts by colorimetric essays. Classic reactions used to detect the various chemical entities were the cyanidin reagent test for the flavonoids[38,39], the ferric chloride reagent for the tannins[40,41], the Folin-Ciocalteu reagent for the polyphenols[42] and the Draggendorf reagent for the alkaloids[43]. The experimental protocols are as follows:

Detection of flavonoids: The test consisted in the addition 5 ml of hydrochloric alcohol (ethanol / distilled water / concentrated hydrochloric acid, V/V/V), some magnesium turnings and 1 ml of isoamyl alcohol to 5 ml of extract. The appearance of a pink-orange, pink purple or red color indicates the presence of flavonoids.

Detection of tannins: 1 ml of a 2% ferric chloride aqueous solution was added to 5 ml of extract. The appearance of a blue-black or brownish-green color indicated the presence of tannins.

Detection of polyphenols: 500 µl of a 10^{-1} dilution of the Folin-Ciocalteu reagent and 400 µl of a Na_2CO_3 solution containing 75 mg/ ml of distilled water, was added to l00 µl of the extract, and incubated for 2hours at room temperature. The appearance of a dark blue color indicated the presence of polyphenols.

Detection of alkaloids: These were detected directly on the TLC plate by spraying with the Draggendorf reagent. The appearance of orange spots indicated the presence of alkaloids.

For all these tests the control consisted of a sample, without the extract, subject to the same conditions. The tests were performed three fold.

RESULTS

Antimycobacterial effect of the ethanolic extract by the disc diffusion method

The study of the antimycobacterial effect of *B. hispanica*, showed that ethanolic Extract 1 was active against *M. smegmatis* and *M. aurum* (Table 1). The effect was evidenced by the presence of growth inhibition zones around the extract impregnated discs.

The ethanolic extract from *B. hispanica* root bark showed an important antimycobacterial activity. This activity increased with the time of incubation (Table 1).

The diameters of the inhibition zones for *M. aurum A+* were significantly larger than those for *M. smegmatis* MC_2. Indeed, statistical results showed that the values for the diameters of inhibition differ significantly ($\alpha = 5\%$).

Bioautography

Results of the TLC fractionation of the ethanolic extract of *B. hispanica*, showed the presence of four separate bands of which three were biologically active (Table 2, Figure 1). This activity was evidenced by the formation of a growth inhibition zone around the band containing the substance/s with antimycobacterial activity. Three inhibition zones were visible around three distinct bands.

Phytochemical tests

Results from the phytochemical tests are shown in Table 3. The presence of flavonoids, tannins, total polyphenols and alkaloids was demonstrated in the *B.hispanica* crude extract. The presence of total polyphenols and flavonoids was shown in the bands BH_1 (R_f : 0,40) and BH_4 (R_f : 0,81). However, in band BH_2 (R_f : 0,65), the presence of polyphenols and alkaloids was shown.

Table 1. Antimycobacterial effect of the ethanolic extract of *Berberis hispanica* root bark by the disc method

Diameter of the inhibition zone against *M. smegmatis* (mm)		Diameter of the inhibition zone against *M. aurum* (mm)	
After 48 h/ 37 °C	After 72 h/ 37 °C	After 48 h/ 37 °C	After 72 h/ 37 °C
13,66 ± 0,94	33,16 ± 0,24	21 ± 2,16	51,66 ± 2,35

*The results correspond to an average of three repetitions.
*The control did not present an inhibition zone.

Table 2. TLC characterization of the *B. hispanica* extracts

Individualized bands	R_f factor
BH_1 [a]	0,40
BH_2 [a]	0,65
BH_3 [b]	0,72
BH_4 [a]	0,81

[a]: active band, [b]: non-active band.

DISCUSSION

Extracts of *B.hispanica* root bark were shown to possess an antimycobacterial effect, indicating the presence of one or more substances, soluble in ethanol and capable of inhibiting the growth of mycobacterial species. These results confirm previous findings showing that in this

plant species ethanol was effective in extracting antimycobacterial compounds[29].

In the literature, ethanolic plant extracts have been shown to exert inhibitory activity against several mycobacterial strains. In his study for the development of plants from the North Central region of Morocco, Sqalli et al (2007) showed that the ethanolic extracts of 14 of the 36 plant species tested showed antimycobacterial activity against *M. smegmatis, M.aurum, M. kansasii, M. bovis* and *M. vaccae*. These included *Juniperus oxycedrus, Pistacia lentiscus, Ruta montana, Populus alba, Cistus albidus, C. monspeliensis, C. salviifolius, Pistacia atlantica, Tamarix africana, Rubia peregrina, Thymus pallidus, Vitex agnus-castus, Verbascum sinuatum* and *Dittrichia viscosa*[44].

Moreover, the ethanolic extracts from *Mallotus philippensis, Vitex negundo, Colebrookea oppositifolia, Rumex hastatus, Mimosa pudica, Kalanchoe integra* and *Flacourtia ramontchii*[45] as well as *K. vesicatoria*[46] were active against *M. smegmatis*. Other studies on the ethanolic extracts of *Acacia nilotica* and *Combretum kraussii* (leaves, bark and roots) have shown a strong inhibitory effect against *Mycobacterium aurum*[47].

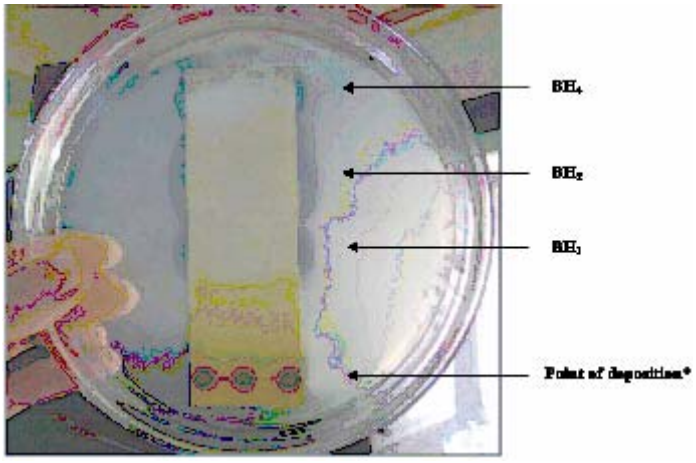
The antimycobacterial activity of *B.hispanica* ethanolic extract increases with the time of incubation. The diameter of inhibition obtained after the first incubation period (48 hours) was due to the inhibition of the mycobacterial cells, whereas the inhibition obtained at the end of the second incubation period (72 hours) was due to the lysis of the cells that have grown during the first incubation. These observations suggest that the extract favored mycobacterial cell lysis.

M. aurum A+ showed greater sensitivity to the extract than *M. smegmatis*. As *M.aurum A+* has a greater generation time than *M. smegmatis* MC_2 this allows more time for the extract to diffuse resulting in larger inhibition zones. This difference in the degree of inhibition between the two species has been reported in several previous studies using the disc diffusion method[48,18,29].

Table 3. Detection of flavonoids, total polyphenols and tannins in the crude extracts and biologically actives fractions of *B. hispanica*

	Flavonoids	Total polyphenols	Tannins	Alkaloids
CE	+	+	+	+
BH_1	+	+	-	-
BH_2	-	+	-	+
BH_4	+	+	-	-

+ : presence ; - : absence ; CE : Crude extract; BH_1, BH_2, BH_4 : Active bands.



* At the point of deposition, the spots of the same extract were deposited.

Fig. 1. Bioautography of the *B.hispanica* extract.

The *B.hispanica* extract inhibited the growth of *M. smegmatis* and *M. aurum* that present a susceptibility profile to antituberculous agents similar to that of *M. tuberculosis*[31]. Their growth inhibition is highly predictive of the activity against *M. tuberculosis*[30]. This suggests that the *B .hispanica* extract could be equally active against tuberculous bacilli. Indeed, these two non-pathogenic mycobacteria have been considered as surrogates for the evaluation of active substances against *M. tuberculosis* growth[17,48].

The antimycobacterial activity of *B.hispanica* was demonstrated by bioautography. By this method the TLC separation of the different constituents from the ethanolic extract of the plant was first obtained, followed by the evaluation and localization on the TLC plate of the active substances. The identification of three active bands with different R_f coefficient suggested the existence of several biologically active components with antimycobacterial activity in the *B.hispanicai* extract. These compounds were found to belong to different chemical classes.

The chemical analysis that was carried out on the crude extract and products from the TLC bands responsible for the antimycobacterial activity showed that the active substances responsible for the antimycobacerial activity observed in bands BH_1 (R_f : 0,40) and BH_4 (R_f : 0,81), were polyphenols and flavonoids. However, the active substances of band BH_2 (R_f : 0,65), were alkaloids and polyphenols other than flavonoids. The results obtained also showed that tannins were not responsible for the observed biological activity.

Polyphenols are products of the secondary metabolism of plants and cover a large range of chemical substances characterized by the presence of a phenol group. Flavanoids and tannins are polyphenols. However, alkaloids are not polyphenols.

Polyphenols are very well known for their excellent biological activities[49-51]. Moreover, several studies have shown that these molecules posses an antimycobacterial effect. In 2004, Okunade et al., showed that several polyphenols extracted from medicinal plants, tradicionally used in the treatment of respiratory diseases, could inhibit the *in vitro* growth of *M. tuberculosis*[52-54,48]. Another study showed that polyphenols from green tea inhibited the *in vivo* growth of *M. tuberculosis*[55].

Several studies, carried out on the biological activity of plant flavanoids, have revealed that these molecules posses an antibacterial effect[56-58], as well as an antimycobacterial effect[59,60,57,53].

Recent studies have shown that alkaloids represent a potential source in the development of new antituberculous agents[61-63]. Berberine from *Hydrastis canadensis* was reported as responsible for the observed activity against resistant strains of *M. tuberculosis*[64] and that originating from *Xanthorhiza simplicissima* showed an antimycobacterial effect against *M. intracellularae*[65]. Berberine from *Thalictrum rugosum* and from *Berberis fremontii* was also capable of inhibiting *M. smegmatis* growth[66]. Berberine, the principal alkaloid and the most biologically active substance from *B.hispanica*[67,24], could be the alkaloid responsible for the antimycobacterial effect observed in band BH_2. However, berberine was not the only molecule responsible for the antimycobacterial activity of this plant, as alkaloids were absent from the other two active bands.

CONCLUSIONS

This investigation focused the study of the antimycobacterial activity of the ethanolic extract of *Berberis hispanica* root bark as well as that of its biologically active fractions. The ethanolic extract from this plant revealed important antimycobacterial activity. The active bands (BH_1, BH_2, BH_4) identified by bioautography, also showed antimycobacterial activity. The phytochemical study showed that this activity could be attributed to several biologically active substances including polyphenols, alkaloids and flavonoids. In perspective, this investigation should be completed with an *ex vivo* study on the effect of the active molecules, responsible for the observed bacterial inhibition, on mycobacteria infecting macrophages, and with the identification of their chemical structure.

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