

Full Length Research Paper

Antimycobacterial activity of *Populus alba* leaf extracts

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Tuberculosis (TB) is the principal cause of death worldwide due to an infectious agent, *Mycobacterium tuberculosis*. The search for new anti-TB drugs, especially from plants, is needed due to the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR) strains and nontuberculous mycobacteria (NTM). The aim of this study was to evaluate the antimycobacterial activity of the white poplar tree (*Populus alba* L.). The incorporation of aqueous and ethanol extracts into the agar medium have shown that extracts of this plant have an antimycobacterial effect against three mycobacteria growth. The disk diffusion method have shown that the ethyl acetate extracts, prepared according to the conventional and the soxhlet extraction methods, had a higher antimycobacterial effect. These results were confirmed by thin layer chromatography (TLC) followed by bioautography. This technique has allowed to localize the active substances from this plant which were responsible for this biological activity. The phytochemical tests carried out showed that these molecules correspond to flavonoids and polyphenols.

Key words: Tuberculosis, *Populus alba*, antimycobacterial activity.

INTRODUCTION

Tuberculosis (TB) is one of the oldest and most widespread diseases in human history. It is caused by *Mycobacterium tuberculosis*, a unique infectious agent, and exceptionally by *M. bovis* and *M. africanum*.

Although its etiological agent was discovered in 1882, TB remains a major public health problem worldwide (Murray, 2004).

In fact, this chronic infection was declared a global emergency in 1993 by the World Health Organization (WHO) (Raviglione et al., 1995).

TB continues as the principal cause of death by infection in the world. The 2009 estimates were of 9.4 million new cases and 1.7 million deaths from TB. The majority of cases were from the African regions, South East Asia and West Pacific (WHO, 2010b).

The discovery of effective antimycobacterial agents, between 1950 and 1970 (ethambutol, isoniazid, pyrazinamide, rifampin and streptomycin), and the

reduction of poverty, had an important impact on the reduction in the number of cases in the western world. However, since the 1980s, on a worldwide scale, the number of cases has steadily increased due to emerging multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (Chan and Iseman, 2008; Gandhi et al., 2006), and also to the growing Human immunodeficiency virus infection / Acquired immunodeficiency syndrome (HIV/AIDS) pandemic (Zumla and Grange, 1998; WHO, 2010a). WHO estimates that between 2000 to 2020, there will be one billion people newly infected by *Mycobacterium tuberculosis*, 200 million developing the disease and 35 million dying from TB if substantial improvement in treatment and disease control is not obtained (WHO, 2000). In this alarming scenario, research for new antimycobacterial substances active against TB and drug-resistance is a major objective.

Furthermore, new antimycobacterial substances are

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also needed for the treatment of infections caused by opportunistic non tuberculous mycobacteria (NTM) which are also an emerging public health concern due to resistance to the majority of the known antituberculosis drugs (Gillespie et al., 2001; Saiman, 2004). Substances extracted from plants are used all over the world in traditional medicine for the treatment of several diseases, including tuberculosis. Indeed, plants are recognized as a source of highly active antimycobacterial metabolites (Gibbons, 2005; Pauli et al., 2005).

The search for new chemical entities, with activities against mycobacteria, from plants and other natural sources has intensified in the last millennium. An important number of extracts and purified compounds isolated from these sources have shown remarkable inhibitory activities against *M. tuberculosis* and other mycobacterial species (Barnes et al., 2003; Copp and Pearce, 2007; El Ouarti et al., 2011, 2012; Jimenez-Arellanes et al., 2007; Negi et al., 2010; Newton et al., 2000; O'Donnell et al., 2006; Sqalli et al., 2009).

These results confirm the interest of pursuing research for antimycobacterial agents from plants.

In this context, the objective of the present investigation is to evaluate the antimycobacterial activity of a plant species *Populus alba* and identify the active compounds of this activity. The white poplar tree (*Populus alba* L.) belongs to the family Salicaceae consisting of some three hundred species (karrenberg et al., 2002). It is a euroasiatic species common in riverine and humid zones of central and meridional Europe, as well as in North Africa. It also grows in central and western Asia, the Himalayas and west of China (Roiron et al., 2004).

The active substances from the genus *Populus* are known in traditional medicine for varied biological activities including antifungal, antioxidant, antitumoral, antiseptic and antiviral (Christov et al., 2006; De Campos et al., 1998). The extract derived from the chloroform extract of *Populus alba* flowers shows an antiproliferative activity against cancerous cell lines (Wamidh and Mahasneh, 2010). Traditionally *Populus alba* is used for its properties as a skin disinfectant (Adam et al., 2009), and in the treatment of herpes and dental cavities (Wamidh and Mahasneh, 2010). The antibacterial and antifungal properties of ethanolic extracts of *Populus alba* leaves against an important number of microorganisms has been clearly evidenced (Al-Hussaini and Mahasneh, 2011). These results have led us to investigate the antimycobacterial effect of this plant that has not been previously explored. The study consists of a chromatographic and phytochemical analysis allowing the separation and identification of chemicals responsible for antimycobacterial activity.

MATERIALS AND METHODS

Plant material

Populus alba (leaves and branches) were collected in March 2009

from the OURDZARH region, Taounate province (Morocco), known for its production of aromatic and medicinal plants.

The plant material was air dried in the shade, at the microbial biotechnology laboratory of the University of Science and Technology of Fès (Faculté des Sciences et Techniques de Fès). The leaves were then ground to a powder for the preparation of the various extracts.

Mycobacteria

The antimycobacterial activity of the *Populus alba* extracts was determined using *Mycobacterium smegmatis* MC₂, *M. aurum* A+ and *M. bovis* BCG (IPP strain), presenting susceptibility profiles to antituberculous agents similar to those of *M. tuberculosis*, which is the reason for the frequent use of these mycobacteria as surrogate models in TB drug discovery (Chung et al., 1995; Mitscher and Baker, 1998; Newton et al., 2002).

The strains were cultured on Sauton medium at 37°C (Nigou and Besra, 2002).

Extract preparation

Extract preparation for agar medium incorporation tests

Aqueous and ethanolic extracts were prepared using 8 g of plant powder in 50 ml of water or solvent

Infusion: The powder was infused in boiling distilled water for one hour, then filtered.

Decoction: The powder was mixed with hot water and boiled for 15 minutes, then filtered.

Ethanol extraction: The powder was macerated in the solvent for 24 h in the shade. After filtration, the solvent was dried at 37°C under vacuum using a rotary evaporator. The residue was then diluted in 5 ml of sterile distilled water.

Filtration of the different extracts was carried out using a water aspirator vacuum pump coupled with a Millipore 0.45 µm porosity filter. The pH of the extract was neutralized when necessary.

Extract preparation for the diffusion test in agar medium

The *Populus alba* powder (8 g) was macerated in the shade for 24 h in different solvents (ethanol, methanol, ethyl acetate, hexane). After filtration, the solvent was evaporated to dryness under vacuum using a rotary evaporator, at 37°C. The residue was, then, dissolved in 2.5 ml of the appropriate solvent.

Screening for antimycobacterial activity

Incorporation of extracts into agar medium

The aqueous and ethanolic extracts were incorporated into Sauton agar medium at a concentration of 160 mg of dried matter/ml, as previously described (Ahmed and Beg, 2001; Cos et al., 2002).

Aliquots of 100 µl of mycobacterial cultures (*M. smegmatis*, *M. aurum* and *M. bovis* BCG), were then streaked on the culture media surface. The cultures contained approximately 10⁶ colony forming units (UFC)/ml. The control corresponded to the culture medium without the plant extract. The plates were incubated at 37°C. They were observed daily for up to six days to evaluate bacterial growth. The study was carried out in triplicate for each strain.

Table 1. Antimycobacterial effect of aqueous and ethanolic extracts of *Populus alba*.

Mycobacteria	Antimycobacterial effect			
	I	D	EE	C
<i>M. smegmatis</i>	–	–	–	+
<i>M. aurum</i>	–	–	–	+
<i>M. bovis BCG</i>	–	–	–	+

I: infusion; D: decoction; EE: ethanol extract; C: control; +: positive mycobacterial growth; -: absence of mycobacterial growth.

Diffusion in agar medium

The antimycobacterial activity of the extracts was measured using the disc method (Bauer et al., 1966). For this the extracts were prepared according to two different procedures:

Extraction using a conventional method: Sterile paper discs of 14 mm diameter were placed at the center of 90 mm diameter plates containing 30 ml of Sauton agar previously inoculated with 100 µl of a liquid culture of *M. smegmatis*. The discs were then impregnated with 60 and 100 µl of the extracts prepared using different solvents (ethanol, methanol, ethyl acetate and hexane). For each trial, the control corresponded to a disc containing an identical volume of pure solvent. After incubation, at 37°C for 24 to 48 h, the inhibition zones around the discs were measured. Each experiment was repeated three fold.

The culture conditions and bacterial titers used were as previously described.

Extraction using the soxhlet method: The *Populus alba* leaf powder (60 g) was subject to successive extractions in a soxhlet extractor, for 12 h, using the following series of solvents: hexane, dichloromethane, ethyl acetate and methanol. The extracts were then dried under vacuum at 37°C using a rotary evaporator, and stored at 4°C (Derbel et al., 2010). The different extracts were tested for their antimycobacterial effect using the disc method. 20 µl of each extract was placed on 6 mm diameter paper disc placed at the center of plates containing 30 ml of Sauton agar previously inoculated with 100 µl of a liquid culture of *M. smegmatis*. Each experiment was repeated three fold.

The Student t-test was used to assess whether the means obtained for the diameters of the inhibition zones between the different treatments were statistically different.

Evidence of antimycobacterial activity by thin layer chromatography

Thin layer chromatography (TLC) was selected as a rapid and efficient method allowing the separation and purification of different substances from the extract in order to identify those responsible for antimycobacterial activity by bioautography. The details of this method are explained below.

The ethyl acetate extract, obtained by soxhlet extraction, with the highest activity, was analyzed by TLC as follows: The fraction was diluted by ¼ in ethyl acetate and 14 µl of this extract was spotted over a TLC silica gel plate, 6 cm wide and 12 cm high. In order to assess inhibition on the silica gel plates, large 5 mm spots were used to ensure deposition of a sufficient quantity of extract for the observation of the biological effect.

The plate was developed using hexane - ethyl acetate (4:6, v/v).

After migration and once dried, the plate was covered with a thin layer of Sauton agar mixed with a liquid culture of *M. smegmatis* (9 ml of medium 196 cm² petri dishes) (Afolayan et Meyer, 1997; Caccamese et al., 1989). After 24 h of incubation at 37°C, the inhibition zone was measured. Each experiment was repeated four fold. In order to confirm the results from this experiment, the silica gel around the inhibition zone was eluted using ethyl acetate, and 20 µl of the eluate were spotted onto a sterile 6 mm diameter paper disc placed at the center of Sauton agar plates previously inoculated with a liquid culture of *M. smegmatis*. The control corresponded to the eluant obtained from a plate prepared using identical migration conditions, but without extract deposition. Each test was repeated three fold.

Phytochemical study

In order to identify the molecules responsible for mycobacterial growth inhibition, we determined the total polyphenols, flavonoids and tannins contents in the crude plant extracts as well as in the inhibition zones responsible for the antimycobacterial activity by TLC.

These principal chemical constituents were characterized in the extracts by colorimetric essays. To detect the chemical entities the cyanidin reagent was used for the flavonoids (karumi et al., 2004), the ferric chloride reagent for tannins (Kablan et al., 2008) and the Folin-Ciocalteu reagent for the polyphenols (Singleton et al., 1999). The experimental protocols used were as follows:

Detection of flavonoids: The test consisted in the addition several drops of HCl (2N) and 0.5 g of magnesium to a minimum volume of the extract. After three minutes the appearance of a red-orange color indicated 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 Na₂CO₃ solution containing 75 mg/ml of distilled water, was added to 100 µl of the extract, and incubated for 2 h at room temperature. The appearance of a dark blue color indicated the presence of polyphenols.

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

Screening for antimycobacterial activity

The study of the antimycobacterial effect of *Populus alba*, showed that the aqueous and ethanolic extracts of this plant were active against *M. smegmatis*, *M. aurum* and *M. bovis BCG* (Table 1). Complete growth inhibition of the bacteria was observed in culture media containing the different extracts at a concentration of 160 mg/ml.

These results were confirmed by the diffusion method in agar medium. Indeed, the study of the antimycobacterial effect of the different *Populus alba* extracts, using this method, showed growth inhibitory effect for the various extracts tested (ethanol, ethyl acetate, methanol and hexane) (Table 2). The results from the statistical test showed significant differences

Table 2. Antimycobacterial activity of different extracts of *Populus alba* prepared according to the conventional disc method.

Volumes (μ l)	Diameter of inhibition zone (mm)							
	Solvents							
	Ethanol		Methanol		Ethyl acetate		Hexane	
	Test	Control*	Test	Control*	Test	Control *	Test	Control *
60	17.33 \pm 0.44	14.16 \pm 0.22	18.16 \pm 0.22	14.33 \pm 0.22	24.33 \pm 0.22	14.83 \pm 0.22	16.66 \pm 0,44	14.33 \pm 0.22
100	20.5 \pm 0.33	14.66 \pm 0.22	21.66 \pm 0.22	14.66 \pm 0.22	29.16 \pm 0.22	15 \pm 0.00	22.16 \pm 0,22	14.66 \pm 0.22

*: The diameter of the disc was 14 mm.

Table 3. Antimycobacterial effect of *Populus alba* extracts obtained using the soxhlet method

Diameter of inhibition zone (mm)							
Hexane		Dichloromethane		Ethyl acetate		Methanol	
Test	Control*	Test	Control*	Test	Control*	Test	Control*
8.16 \pm 0.22	6 \pm 0.00	21.5 \pm 0.33	9.33 \pm 0.22	35.16 \pm 0.22	7.16 \pm 0.22	22.33 \pm 0.22	8.5 \pm 0.00

*: The diameter of the disc was 6 mm

between the values obtained for the different solvents, indicating that the ethyl acetate extracts had a higher antimycobacterial effect.

The results using the soxhlet method are shown in Table 3. Statistical analysis of these results show that there are significant differences between the values obtained with the different solvents, indicating that ethyl acetate fraction has the more accentuated antimycobacterial effect, corroborating the above mentioned results.

Demonstration of the antimycobacterial activity of thin layer chromatography (TLC) separated components

TLC evidenced the antimycobacterial effect of the ethyl acetate extract, which resulted in the formation of an inhibition zone around the component containing the antimycobacterial substance. The R_f value for this component was 0.56 (Figure 1).

Phytochemical tests

Results for the phytochemical tests are shown in Table 4. The presence of flavonoïds and total polyphenols was observed in the crude extract and in the zone containing the TLC separated components showing antimycobacterial activity (R_f: 0.56).

DISCUSSION

Our investigations showed that *Populus alba* contains one or more, water and ethanol soluble, substances that inhibit mycobacterial growth.

The antimycobacterial activity was observed after heating for 15 min, which indicates that the active principals are not degraded at 100°C, and are therefore not likely to be of protein nature as these would be expected to be degraded after this high temperature treatment.

Results using the disc method showed that the ethyl acetate extract had a greater antimycobacterial effect than that of the other solvents used. It is interesting to note that several other investigations have also pointed to the fact that ethyl acetate extracts from other plants have an effect against *M. aurum* (Eideen and van Staden, 2007, 2008) and *M. tuberculosis* (Lakshmanan et al., 2011).

The antimycobacterial effect *Populus alba* was shown using TLC to localize the active substances from this plant which were responsible for this biological activity.

TLC was also used for the purpose of subsequent purification and identification of the active substances.

The nature of the biologically active substances responsible for the antimycobacterial effect of the components identified by TLC, R_f value of 0.56, was determined using phytochemical tests. These indicated the presence of flavonoïds and total polyphenols in the crude extract and in the TLC components, which leads us to conclude that that the active substances were flavonoïds and other polyphenols.

The point of deposition also showed a slight inhibition that may be explained by the fact that the constituents from the extract do not migrate in their totality. Several studies, based on other plant species, have confirmed such observations and have shown that a fraction of the extract fails to migrate from the point of deposition (Sawaya et al., 2004; Smith et al., 2007)

Several classes of natural products such as polyphenols, terpenoids, fatty acids, aliphatic alcohols and hydrocarbons are found in the genus *Populus*

Table 4. Evidence for the presence of flavonoïds, tanins and total polyphenols in the crude extract and TLC separated components from *Populus alba*.

Substance	Flavonoids	Tanins	Total Polyphenols
CE	+	-	+
TLC separated components (Rf: 0.56)	+	-	+

CE: crude extract; Rf: retention factor; +: presence; -: absence.

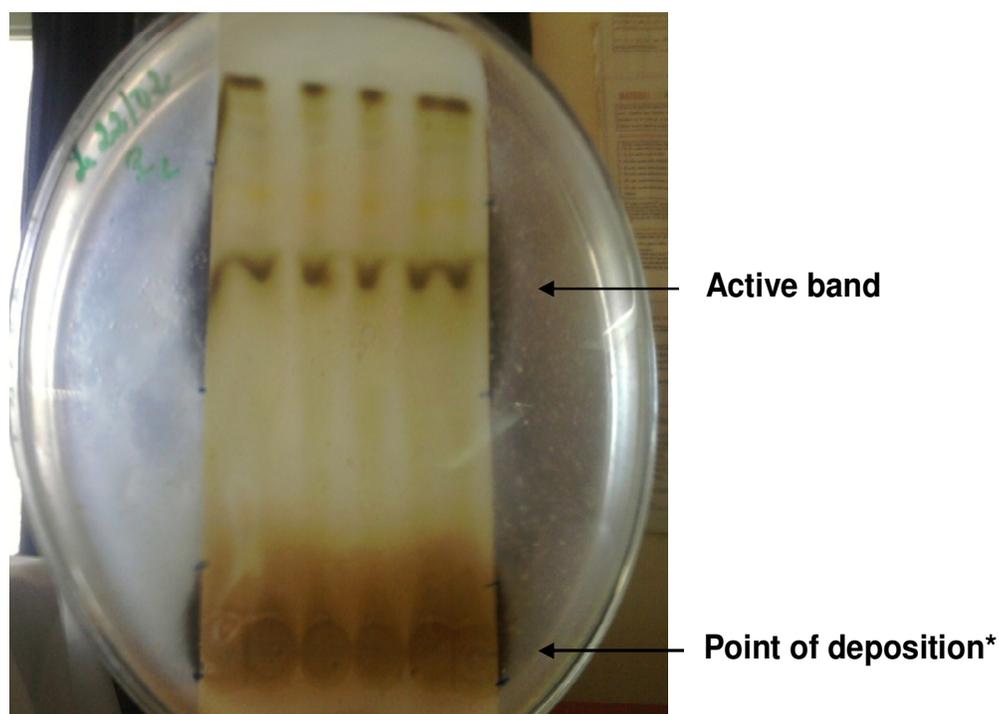


Figure 1. Bioautography of the *Populus alba* extract. * At the point of deposition, the spots of the same extract were deposited. This result was also confirmed using the disc method. In this case the diameter of the inhibition zone was 11 mm. No effect was observed for the control.

(English et al., 1991). The presence of some phenolic compounds can be used as a 'fingerprint' to identify poplar species (Wollenwebe, 1975). Previous studies on *Populus* species have shown the presence of flavonoids, salicin derivatives, organic acids, and coumarins (Kim et al., 2006; Pearl and Darling, 1971).

Indeed, polyphenols are well known for their excellent biological activities including the inhibition of dental cavities (Sakanaka et al., 1989), inhibition of allergies (Yeo et al., 1995), reduction of blood pressure (An, 1998), prevention of gout (An et al., 1996) and the inhibition of oxydation.

Moreover, several studies show that these molecules have an antimycobacterial effect. Indeed, in 2004, Okunade and collaborators., reported that several polyphenolic extracts from medicinal plants, traditionally used in the treatment of respiratory illnesses, inhibit *M. tuberculosis* growth *in vitro* (Newton et al., 2002; Okunade et al., 2004; Seephonkai et al., 2002). Another

study showed that polyphenols from green tea inhibit *M. tuberculosis* growth *in vivo* (Anand et al., 2006).

Several studies concerning the biological activity of flavonoïds from other plants, have demonstrated the antibacterial properties of these molecules (Hernandez et al., 2000; Kuete et al., 2010; Schinor et al., 2007), including in mycobacteria (Koysomboon et al., 2006; Kuete et al., 2008, 2010; Okunade et al., 2004). In perspective, this study should be completed with the chemical identification of the active molecules and by the study of their effect on mycobacteria inside macrophages (*ex vivo* tests).

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