ANTIMYCOBACTERIAL, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF ESSENTIAL OIL OF GALL OF *PISTACIA ATLANTICA* DESF. FROM ALGERIA

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Abstract

Background*:* The aim of this study was to assess the antimycobacterial, antioxidant and the cytotoxic activities of the essential oil from the gall part of *Pistacia atlantica* Desf from Algeria.

Materials and Methods: The antimycobacterial activity was evaluated by the broth microdilution method against three species of mycobacteria: *Mycobacterium smegmatis*, *Mycobacterium aurum* and *Mycobacterium fortuitum*. Antioxidant activity was determined using free-radical scavenging assays. The safety of essential oil was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on C3A and Vero monkey kidney cells.

Results: The minimal inhibitory concentration (MIC) values ranged from 0.16 to 2.5 mg/mL with minimal bactericidal concentration (MBC) values ranged from 0.62 to >2.5 mg/mL. The antioxidant activity showed IC_{50} values ranged between 417.61 - >2000µg/mL and 495.6 - >2000µg/mL for DPPH and ABTS assay respectively. The cytotoxicity assay showed LC_{50} ranged between 26.47 to 93.64 µg/mL against Vero cells and 74.29 to 225.40 µg/mL against C3A. The results of this study show that the essential oils from the gall of *P. atlantica* have low toxicity and moderate activity against fast growing *M. smegmatis* and *M. aurum*.

Conclusion: These results substantiate their potential as used in the treatment of non-tuberculous mycobacterial infections. This finding is predictive of their activity against *M. tuberculosis,* therefore, further study might be considered to investigate the activity against pathogenic *Mycobacterium* strains.

Key words: *Pistacia atlantica*, Essential oil, Antimycobacterial, Antioxidant, Cytotoxicity.

Introduction

Pistacia is a genus of Anacardiaceae family. Five species of the genus grow naturally in the Mediterranean basin and Middle East: *P. lentiscus*, *P. atlantica*, *P. palaestina*, *P. terebinthus* and *P. khinjuk* (Golan-Goldhirsh *et al.*, 2004). *P. atlantica* Desf. is a source of mastic gum. Exudate is used traditionally against coughs, chills and stomach diseases, to strength gums and as a mouth freshener. The gall of *P. atlantica* known in Arabic as "afse" is edible and used in Algeria as insect repellent (Gourine *et al.*, 2011). Moreover, gall is used in the Sahara as expectorant, against asthma and chest diseases (Ozenda, 2004). According to Martinez (2008), the gall has been shown to protect their occupants from natural enemies, such as predators and parasitoids, by various chemical and mechanical means. Less attention, however, has been given to the possibility of defence against microbial pathogens in the humid and nutrient-rich gall environment. Essential oils have been traditionally used for treatment of infections and diseases all over the world for centuries (Rios & Recio, 2005). It's gaining remarkable interest for their potential multipurpose use as antioxidant, antibacterial and antiseptic agent (Zanetti *et al.*, 2010).

Several study emphasized the antimycobacterial potential of essential oil (Billo *et al.*, 2005; Raju *et al.*, 2007; Pinto *et al.*, 2009; Zanetti *et al.*, 2010), but their toxicity have rarely been investigated. The antimicrobial and antioxidant activity of essential oil from different part of *Pistacia atlantica* Desf have been a subject of particular interest to many previous studies (Bachir Raho & Benali, 2010; Gourine *et al.*, 2011). However, the antioxidant activities of essential oil from gall of this plant species as well as the antimycobacterial activity have not been reported to date. This study describes the antimycobacterial and antioxidant activities, as well as the cytotoxicity of essential oil of gall of *Pistacia atlantica* Desf from three regions in Algeria.

Materials and Methods
Plant samples and essential oil

The gall of *Pistacia atlantica* Desf was collected during summer 2011 from three different locations in Algeria: *Aïn-Oussera*, *Laghouat* and *Kheneg*; respectively located at 210, 450, 425 kilometres south of Algiers, the capital of Algeria. The identity of the plant material was confirmed by Professor Salima Benhouhou a botanist from The National Institute of Agrononmy in Algeria. The essential oil was obtained by hydro-distillation, using Clevenger type apparatus, during three hours. The obtained essential oil was treated by anhydrous sodium sulphate filtrated and stored at +4°C until analysis.

Antimycobacterial activity assay
Mycobacterial culture

Mycobacterium smegmatis (ATCC 1441), *Mycobacterium aurum* (NCTC 10437) and *Mycobacterium fortuitum* (ATCC 6841) were cultured as described by McGaw *et al.* (2008). They were maintained on Löwenstein–Jensen agar slants, supplemented with glycerol. Inocula suspensions were prepared by mixing a few microbial colonies with sterile distilled water. The suspension was diluted with sterile water to render

a concentration of cells equal to standard 1McFarland solution (approximately 4×10^7 cfu/mL), and then diluted with freshly prepared Middlebrook 7H9 broth supplemented with 10% OADC medium to obtain a final inoculum density of approximately 4×10^5 cfu/mL.

Determination of minimum inhibitory and bactericidal concentration (MIC and MBC)

The broth micro-dilution technique using 96-well micro-plates, as described by Eloff (1998) was used to obtain the MIC and MBC values of essential oil samples. A 100µL of each sample of essential oil at an initial concentration of 10 mg/mL were serially diluted, two-fold in 96-well microtitre plates, with equal volumes of Middlebrook 7H9 broth. Then, 100µL of inocula was added to each well to give a final concentration range of 2.5-0.019 mg/mL. The plates were incubated overnight for *M. smegmatis* and 3 days for *M. aurum* and *M. fortuitum* in incubator at 37°C. To indicate bacterial growth, 40µL of 0.2 mg/mL INT (*p*-iodo-nitro-tetrazolium) was added to each well after incubation and the plates incubated further at 37°C for 1 hr. The MIC was defined as the lowest concentration that inhibited the colour change of INT (yellow to purples). The experiment was performed in triplicate.

The MBC was determined by adding 50µl of the suspensions from the wells, which did not show any growth after incubation during MIC assays, to 150µL of fresh broth. These suspensions were re-incubated at 37°C for 24 hr. The MBC was determined as the lowest concentration of extract which inhibited 100% growth of microorganisms.

Antioxidant assay
DPPH·radical-scavenging assay

The antioxidant activities of the samples were measured in term of radical scavenging ability using the stable radical (DPPH) of Brand-Williams *et al.* (1995) with some modifications. Methanol solution (40µL) of the samples at various concentrations (15.62 – 2000 µg/mL), and positive control (trolox and ascorbic acid) at concentration (0.31 to 40µg/mL) were added to 160µL of DPPH in methanol (0.1mM) in a 96 well-microtitre plate. Then, the change in absorbance (517nm) was measured after 30 min with a microtitre plate reader (BioTek®). DPPH free radical scavenging ability (%) was calculated by using the formula:

$$DPPH\text{-}scavenging\ effect\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

The concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting percentage of DPPH· radical scavenged against the concentration.

ABTS·+ radical scavenging assay

The free radical-scavenging activity as a measure of hydrogen donating capacity was determined by using ABTS cation decolourization method of Re *et al.* (1999), with some modifications. ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and kept in the dark at room temperature for 12 hr. Prior to running the assay, the ABTS radical solution was diluted with a 50% methanolic solution to an absorbance between $0.7 – 0.8$ at 734nm. The extracts were serially diluted (40 µL) (15.62 – 2000µg/mL) in 96 well-microtitre plate and 160 µL of ABTS radical solution added to each well. The absorbance were taken exactly after 6 min of reaction (A_{sample}) and blank absorbance (A_{blank}) were prepared using the respective extracts without ABTS radical. ABTS free radical scavenging ability (%) was calculated by using the formula: DPPH-scavenging effect (%)

$$ABTS\text{-}scavenging\ effect\ (\%) = \left[1 - \frac{A_{sample} - A_{blank}}{A_{control}} \right] \times 100$$

The concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting percentage of ABTS· radical scavenged against the concentration.

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP of essential oil samples was determined by direct reduction of potassium ferricyanide ($K_3Fe(CN)_6$) to potassium ferrocyanide ($K_4Fe(CN)_6$) (electron transfer process from the antioxidant). The increase in absorbance from the formation of Pearl's Prussian blue complex following the addition of excess ferric ion was measured as described by Berker *et al.* (2007) with some modification. The reaction medium (210 µL) containing 40µL of the test samples or positive controls (trolox and ascorbic acid; concentration range between: 15.62 – 2000 µg/mL); 100µL of 1.0M hydrochloric acid; 20µL of 1% (w/v) of SDS; 30µL of 1% (w/v) of potassium ferricyanide, was incubated for 20min at 50°C. Then cooled to room temperature. Finally, 20µL of 0.1% (w/v) of ferric chloride was added. The absorbance at 750nm was read and blank absorbance was taken by preparing the reaction medium the same way except the addition of ferric chloride. The TEAC (Trolox Equivalent Antioxidant capacity) was calculated by dividing the slope of each sample (slope obtained from the line of best fit of the absorbance against concentration using the linear regression curve) by that of trolox.

***In vitro* cell cytotoxicity activity (MTT assay)**

Cytotoxicity of essential oil was determined by MTT assay (Mosmann, 1983) using monkey kidney (Vero) cells and human hepatocellular carcinoma (C3A) cells. The C3A cells were obtained from the American Type Culture Collection (ATCC CRL-10741), and the Vero cells line were obtained from the culture collection of the Department of Veterinary Tropical Diseases (University of Pretoria). The preparation of cells culture was followed according to Makhafola *et al.* (2014). The cells were cultured in Minimal Essential Medium (MEM, Highveld Biological) supplemented with 0.1% gentamicin and 5% foetal calf serum (FCS, Highveld Biological) for the Vero cells, and 10% FCS for the C3A cells. The cells of a sub-confluent culture were harvested using trypsin-EDTA (Sigma) and centrifuged at $200 \times g$ for 5 min and re-suspended in growth medium to 5×10^4 cells/mL.

A total of 100µl of the cell suspension is pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. A 200µl aliquot of growth medium was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated at 37°C in a 5% CO_2 incubator overnight and 100µL of different concentrations of essential oil samples prepared in MEM were added. Each dilution of the test sample was tested in quadruplicate. The microtitre plates were incubated at 37°C in a 5% CO_2 incubator for 48 h. A negative control (untreated cells) and positive control (cells treated with different concentrations of doxorubicin chloride, Sigma) as well as an acetone solvent control, were included. After incubation, cells were washed with 200µL phosphate buffered saline (PBS) and fresh MEM (200µL) was added to each well together with 30µL of MTT (Sigma, stock solution of 5 mg/mL in PBS), the plates were incubated for a further 4 hr at 37°C. Then, the medium was carefully removed from the wells without disturbing the MTT concentrate and washed twice with PBS and 50µL of DMSO was added to each well to dissolve the crystallized MTT formazan. The amount of reduced MTT was measured at 570nm using microtitre plate reader (VersaMax, Molecular Devices). The wells in columns 1 and 12, containing medium and MTT but no cells were used to blank the microplate reader. The percentage of cell viability was calculated using the formula below:

$$\% \text{ cell viability} = [OD_{(sample)} - OD_{(blank)}/OD_{(control)} - OD_{(blank)}] \times 100$$

Dose response curves were obtained by plotting the percentage growth of cells versus log concentration of the compound. The LC_{50} (50% lethal concentration) values were calculated from a non-linear regression model of sigmoidal dose-response curve (variable) and computed using SigmaPlot 12.0® software. From the minimum inhibitory concentration (MIC) values and LC_{50} values the selectivity index values of each sample were calculated using the formula: $SI = LC_{50}/MIC$

The selectivity index values indicate relative safety of the plant extract, reflecting the ratio between toxicity and activity. A high selectivity index is an indication of a large safety margin between the concentration of the extract that is able to kill the bacteria and the concentration that is toxic to mammalian cells in this case.

Results

The results of the antimycobacterial activity of essential oil of *Pistacia atlantica* Desf collected from three regions in Algeria against three *Mycobacterium* species are presented in Table 1.

Table 1: MIC and MBC (mg/mL) values of essential oil of *Pistacia atlantica* Desf against three *Mycobacterium* species.

Samples	*M. smegmatis*			*M. aurum*			*M. fortuitum*		
	MIC	MBC	Ratio[a]	MIC	MBC	Ratio[a]	MIC	MBC	Ratio[a]
Ain-oussera	0.62	1.25	2	0.16	0.62	4	2.5	>2.5	1
Kheneg	0.31	1.25	4	0.31	1.25	4	1.25	>2.5	2
Laghouat	0.31	1.25	4	1.25	1.25	1	1.25	>2.5	2
Rifampicin (µg/mL)	50	200	4	3.12	12.5	4	12.5	25	2

[a] *Ratio = MBC/MIC*

The three essential oils tested showed that MIC values ranged from 0.16 to 2.5 mg/mL and MBC values ranged from 0.62 to >2.5 mg/mL. *M. fortuitum* was the most resistant strains towards all the essential oil samples (MIC value varied between 1.25 and 2.5 mg/mL). The MBC values ranged from 0.62 to >2.5mg/mL. The ratio (MBC/MIC) was calculated for all samples of essential oil to show the bactericidal or bacteriostatic effect against mycobacteria.
The antioxidant properties of essential oil of gall of *P. atlantica* were determined by DPPH, ABTS and FRAP. Results are presented in table 2. The IC_{50} values ranged between 417.61 - >2000µg/mL.

Table 2: Antioxidant activities of essential oil of gall of *Pistacia atlantica* Desf by DPPH, ABTS and FRAP assay.

Samples	IC_{50} (µg/mL)		TEAC* (mg/g)
	DPPH	ABTS	FRAP
Ain-oussera	417.61	495.6	0.115
Kheneg	> 2000	1479.0	0.106
Laghouat	> 2000	> 2000	0.111
Trolox	7.68	2.99	–
Ascorbic acid	5.68	1.13	1.534

*TEAC: Trolox Equivalent Antioxidant Capacity (mg of Trolox / g of dried extract)

The results of the cytotoxicity summarized in table 3 and figure 1 showed that all the samples had lower toxicity. According to their collection area, the samples from *Ain-oussera* and *Kheneg* regions showed the lowest toxicity with LC50 values ranged from 93.64±1.07 µg/mL to

225.40±1.19 µg/mL and from 80.58±1.03 – 100.20±1.29 µg/mL on Vero cell and C3A respectively. The selectivity index (SI) varied between 0.016 and 1.734 (table 4). The essential oil of *P. atlantica* collected from *Ain-oussera* had the highest SI (1.734) with *M. aurum*.

Table 3: IC$_{50}$ of essential oil of gall of *Pistacia atlantica* Desf on Vero monkey and C3A cells.

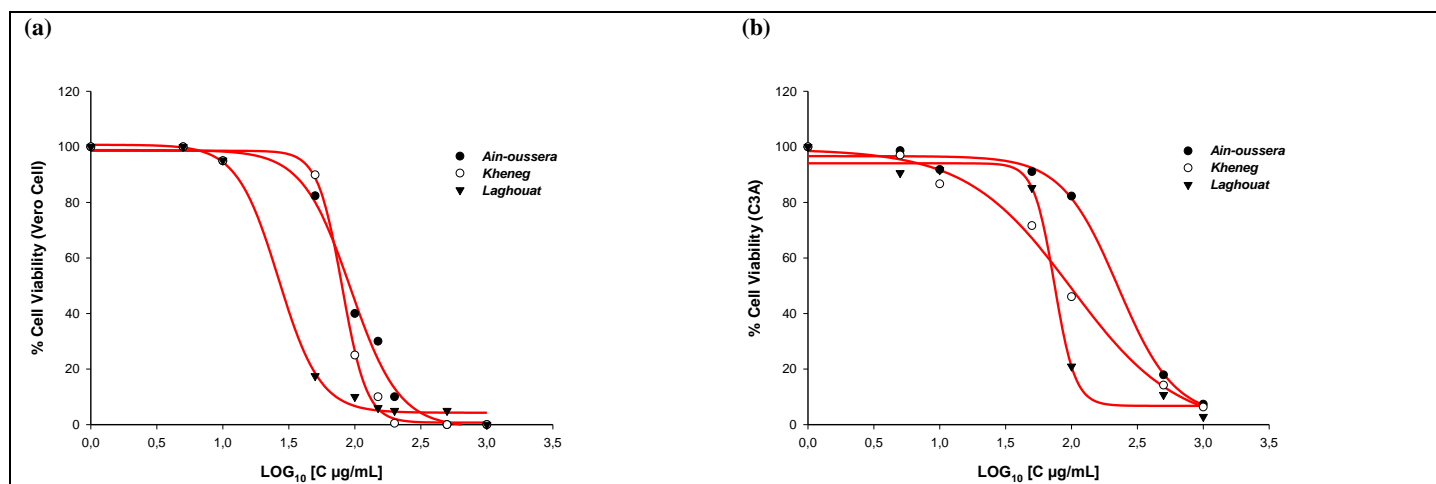| Samples	Vero monkey kidney cells			Human hepatocytes (C3A)		
	LC$_{50}$ (µg/mL)	SD	R^2	LC$_{50}$ (µg/mL)	SD	R^2
Ain-oussera	93.64	1.07	0.993	225.40	1.19	0.995
Kheneg	80.58	1.03	0.997	100.20	1.29	0.993
Laghouat	26.47	1.10	0.998	74.29	1.08	0.992
Doxorubicin (µM)	3.32	1.31		3.104	2.51	

Figure 1:. Effect of essential oils of gall of *P. atlantica* Desf on the growth of Vero monkey (a) and C3A (b) cells.

Table 4: Selectivity index values of essential oils of gall of *P. atlantica* Desf on *M. smegmatis*, *M. aurum* and *M. fortuitum* and Vero monkey and C3A cells.

Cell lines	Collection area		
	Ain-oussera	Kheneg	Laghouat
Vero monkey kidney cells			
M. smegmatis	0.150	0.258	0.085
M. aurum	0.720	0.193	0.025
M. fortutium	0.022	0.064	0.016
Human hepatocytes (C3A)			
M. smegmatis	0.361	0.321	0.238
M. aurum	1.734	0.240	0.071
M. fortutium	0.054	0.080	0.045

Discussion

The overall antimycobacterial activity of the three essential oils varied from moderate to weak. Similar results were revealed in an earlier study on the essential oils from plants collected in Colombia (Bueno *et al.*, 2011). Nonetheless, the antimycobacterial activity of plant-derived compounds against fast growing *Mycobacterium* species is well documented by several previous studies (Mossa *et al.*, 2004; Seidel & Taylor, 2004). Chung *et al.* (1995) showed that the activity against the rapidly growing *M. aurum* is highly predictive of the activity against *M. tuberculosis*, as the two species have similar drug sensitivity profiles. In this study, essential oil from *Ain-oussera* had the highest activity (MIC value of 0.16 mg/mL) against *M. aurum,* giving some interest for further screening against pathogenic *Mycobacterium* species. According to Oussou *et al.* (2008), essential oil activity can be considered as bactericidal if the ration MBC/MIC < 4 and bacteriostatic if the ration MBC/MIC > 4. Taking into account this cut-off, the activity of the three essential oils tested could be considered as bactericidal against *M. smegmatis* and *M. aurum.* The moderate antimycobacterial activity of the essential oil obtained in this study could be attributed to terpenoids derivatives. The

antimycobacterial potential of terpenoids derivatives have been previously demonstrated (Copp, 2003; Paduch *et al.*, 2007). Gourine *et al.* (2011) reported monoterpenes such as α-pinene and carene as major constituents of essential oils gall of *P. atlantica.*

The three various *invitro* assays used in this study are widely used to evaluate the free radical scavenging ability of plants. The use of at least two different assays in evaluating antioxidant activity of plant extracts has been recommended by Moon & Shibamoto (2009). Compared to trolox and ascorbic acid used as standard antioxidant, the essentials oils tested possess weak antioxidant activity. These results are different of those previously obtained on the antioxidant activity of the essential oils from other parts of *P. atlantica* (Bachir Raho & Benali, 2010; Gourine *et al.*, 2010). However, the dissimilarity observed in this study could be explained by the differences observed in their chemical composition (Mecherara-Idjeri *et al.*, 2008).

For a new anti-infectious drug candidate to be useful in clinical application, the preparation must be selectively toxic towards the targeted microorganism without major effects on host cells or interference with normal physiological pathways. Consequently, the three essential oils studied were tested for their toxicity against Vero monkey and C3A normal cell lines. In the categorization of crude extract safety, LC_{50} value of 20 µg/mL and below is considered as cytotoxic in according to US National Cancer Institute (NCI) plant screening program (Kuete *et al.*, 2011). In this study, LC_{50} values greater than 20 µg/mL were recorded for the three essential oil tested, therefore their toxicity could considered as relatively low. Interestingly, the SI obtained with the essential oil of *P. atlantica* collected from *Ain-oussera* was promising. A selectivity index value greater than 1 for a sample increases the likelihood that its toxic and antibacterial components are different (Cho-Ngwa *et al.*, 2010). Thus, elimination of these toxic components by manipulation of this essential oil may yield more suitable antimycobacterial product.

Conclusion

The results of this study showed that the essential oils from the gall of *P. atlantica* have low toxicity and moderate activity against fast growing *M. smegmatis* and *M. aurum*. These results substantiate their potential as used in the treatment of non tuberculous mycobacterial infections. This finding is predictive of their activity against *M. tuberculosis,* therefore, further study might be considered to investigate the activity against pathogenic *Mycobacterium* strains.

Acknowledgements

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