

ANTIMICROBIAL ACTIVITIES OF LEAF EXTRACTS OF *PISTACIA* AND *SCHINUS* SPECIES AGAINST SOME PLANT PATHOGENIC FUNGI AND BACTERIA

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SUMMARY

Leaf extracts of *Pistacia vera*, *Pistacia atlantica*, *Schinus terebinthifolius* and *Schinus molle* were investigated for their antimicrobial effect against *Agrobacterium tumefaciens*, *Pseudomonas savastanoi* pv. *savastanoi*, *Fusarium solani* and *Rhizoctonia solani*. Extracts were prepared from dried and powdered leaves with solvents (hexane, ethanol, methanol and water) with different degrees of polarity. Aqueous and methanolic extracts exhibited a high level of antifungal and antibacterial activity. Leaf extracts from *P. atlantica* showed a high level of antibacterial activity. However, leaf extracts from *Schinus terebinthifolius* and *Schinus molle* showed the best antifungal activities. Total polyphenol contents of extracts was positively correlated with the diameter of the inhibition zones suggesting their potential antimicrobial activity. A preliminary phytochemical screening revealed the presence of tannins, flavanoids and alkaloids. These findings suggest that leaves of *Pistacia* and *Schinus* spp. are potential sources of antimicrobial compounds.

Key words: polyphenols, *Schinus*, *Pistacia*, *Agrobacterium tumefaciens*, *Pseudomonas savastanoi* pv. *savastanoi*, *Fusarium solani*, *Rhizoctonia solani*.

INTRODUCTION

Pesticides have been universally considered for long time as the most efficient solution to control crop diseases. However, synthetic pesticides may enter the food chain and the resistance developed by plant pathogens has rendered some of them ineffective. This has highlighted the need for the use of alternatives compounds that are environmentally friendly and safe to humans.

Plant secondary metabolites, such as essential oils and plant extracts are known to possess insecticidal, antifungal, acaricidal, antibacterial and cytotoxic activities (Tepe *et al.*, 2004). Therefore, they have been intensively screened and applied in pharmacology, pharmaceutical botany, medical and clinical microbiology, plant pathology and food preservation (Daferera *et al.*, 2000). Some plant extracts (Davidson *et al.*, 1989) and essential oils (Kurita *et al.*, 1981) show activity against a wide range of fungi. Among the different screened plant extracts, those from *Allium* and *Capsicum* species showed high levels of antimicrobial activity towards plant pathogens (Curtis *et al.*, 2004; Iorizzi *et al.*, 2002).

Extracts from *Pistacia* species were reported to have anti-atherogenic, hypoglycemic, antioxidant, anti-inflammatory, and anti-insect activities (Giner-Larza *et al.*, 2000; Dedoussis *et al.*, 2004; Hamdan and Afifi, 2004), and the essential oils and leaf extracts of three *Pistacia* species, including *P. vera*, were also studied (Duru *et al.*, 2003; Kordali *et al.*, 2003). Moreover, the resin obtained from *P. terebinthus* is used as urinary and respiratory antiseptic in Turkish popular medicine (Baytop, 1999).

Studies on the effect of *Pistacia* species extracts against plant pathogens are limited. Kordali *et al.* (2003) showed that crude extracts of *Pistacia vera*, *Pistacia terebinthus* and *Pistacia lentiscus* significantly inhibited the growth of *Pythium ultimum* and *Rhizoctonia solani* but had no antifungal activity against *Fusarium sambucinum*. Duru *et al.* (2003) tested the efficacy of essential oils of the three *Pistacia* species and found them to have an inhibitory effect towards *Rhizoctonia solani* but not against *Pythium ultimum* and *F. sambucinum*, whose growth, on the contrary, was improved.

Pistacia and *Schinus* (pepper tree), family Anacardiaceae, have been introduced from South America to most of the tropical and subtropical areas of the world and to the Mediterranean (Taylor, 2005). Pharmacological studies carried out with extracts from *Schinus molle* showed that this plant has hypotensive (Bello *et al.*, 1996), antitumoral (Ruffa *et al.*, 2002), antifungal (Schmourlo *et al.*, 2005), antibacterial (Erazo *et al.*, 2006), anti-inflammatory (Yueqin *et al.*, 2003), analgesic

(Barrachina *et al.*, 1997), and antidepressant (Machado *et al.*, 2007) properties, but there is no evidence of any effect against plant pathogens.

In this paper we describe the effect of the leaf extracts of *Schinus molle*, *Schinus terebenthifolius*, *Pistacia vera* and *Pistacia atlantica* against two plant pathogenic bacteria (*Agrobacterium tumefaciens*, *Pseudomonas savastanoi*) and two plant pathogenic fungi (*Fusarium solani*, *Rhizoctonia solani*).

MATERIALS AND METHODS

Plant material. Leaves of *P. vera*, *S. molle* and *S. terebenthifolius* were collected around Sfax (central east Tunisia), whereas samples of *P. atlantica* came from Gafsa (south west Tunisia).

Preparation of plant extracts. Leaves were washed with water, disinfected by immersion in a 2% of sodium hypochlorite solution for 30 min, and thoroughly rinsed with distilled water. Leaves were dried at room temperature in the dark and ground to a fine powder using a laboratory scale mill. Extracts from dried leaves were obtained according to Ljubuncic *et al.* (2005). Briefly, dried leaf powder (10 g) was stirred in 100 ml of solvents with different degrees of polarity (hexane, ethanol, methanol and water) for 15 min at 90°C followed by filtration first through four layers of gauze, then through Whatman #1 filter paper. The filtrate was freeze-dried and stored at 18°C under dry conditions until use. Dried extracts were solubilised in hexane, methanol, ethanol and water to perform the different bioassays.

Antimicrobial activity against phytopathogenic fungi. The fungal species tested were *Rhizoctonia solani* and *Fusarium solani*. They were isolated from rotten olive tree roots by the hyphae point and monosporic techniques (Anguiz, 1989) and identified based on their macroscopic and microscopic features.

The effect of plant extracts on mycelial growth was determined according to Al-Mughrabi (2003). Two ml aliquots of each extract were spread on Petri dishes with PDA medium (200 g potato, 20 g dextrose and 20 g agar) which were left overnight for allowing the extracts to be absorbed. Controls consisted of PDA plates on which 2 ml of the each solvent were spread. Then a PDA plug with mycelium of the tested fungi was placed in the centre of each Petri dish and incubated at 25°C for 9 days. Starting two days after inoculation, colony diameters were measured daily until the plates were overgrown.

Percentage inhibition caused by each plant extract was calculated as follows: % inhibition = (growth in the control – growth in the treatment sample)*100/growth of the control.

To study the effect of plant extracts on conidial germination, spore suspensions were prepared by pipetting 5 ml sterile water containing 0.01% Tween-20 onto the fungal colony and rubbing the surface gently with a spatula. The suspension was filtered through a sterile sintered glass funnel to remove mycelial debris and the spores were collected, washed by centrifuging twice at 5000 g for 15 min, resuspended in sterile distilled water and adjusted to contain 2×10^7 spores/ml using a haemocytometer. Spore suspension (1 ml) was mixed with 3 ml of PDA (0.6% agar) at 45°C and was quickly poured into 9 cm diameter Petri dish containing PDA. Plant extracts (100 µl) were poured immediately on wells dug in the medium with a 5 mm diameter spring-loaded plunger. Water, methanol, ethanol and hexane alone served as negative controls. After 3 days of incubation, the diameter of the inhibition zones was measured.

Antimicrobial activity against phytopathogenic bacteria. Two phytopathogenic bacteria were tested, *Agrobacterium tumefaciens* (strain AR125) and *Pseudomonas savastanoi* pv. *savastanoi* (strain AW7) plus *Bacillus subtilis*, a non plant pathogenic species. *A. tumefaciens* strain AR125 was isolated from tumours from the crown of pear trees grown in the island of Kerkenah (south east Tunisia). *P. savastanoi* pv. *savastanoi* strain AW7 was isolated from knots of olive trees (cv. Chemlali) from Sidi Bouzid (central Tunisia). *B. subtilis* was isolated from olive tree leaves from the region of Sfax. Identification of bacterial strains was by sequencing the 16S rRNA gene (*rrs*) and comparing sequences from GenBank database with BLAST (Altschul *et al.*, 1990). Bacteria were grown in nutrient broth at 25-26°C for 24 h in a shaker and used at a final cell concentration of 10^7 - 10^8 CFU/ml.

The antibacterial effect of plant extracts was determined according to Tagg and Given (1971), whereby a suspension in sterile distilled water (1 ml) of the target bacterial strain to be tested was mixed with 3 ml of LB (0.6% agar) at 45°C and overlaid to LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl, 20 g agar). After cooling, wells 5 mm in diameter were punched in the agar with a sterile steel borer. Plant extracts and controls (solvents) tested were filled separately in the wells of agar plates. The inoculated plates were incubated for 24 h at their optimum growth temperature, and the diameter of the inhibition zone was measured with a caliper.

Extraction and determination of total polyphenols. Polyphenols were extracted using Kahkonen *et al.* (1999) method modified. Briefly, 0.5 g of ground plant material was extracted with 20 ml of 50% aqueous methanol using a mixer for 1 min. Samples were then centrifugated for 10 min at 5000 rpm and the supernatants were collected. Plant materials were re-extracted twice and the combined supernatants were evaporat-

ed to a volume about 2.5 ml.

The amount of total phenolics in the extracts was determined according to the Folin-Ciocalteu procedure (Singleton *et al.*, 1965). Briefly, extract aliquots of 2.5 ml were mixed with 1 ml of Folin-Ciocalteu's reagent and 2.5 ml of sodium hydroxide (6%). After adjusting the volume to 25 ml, the tubes were allowed to stand for 1 h in the dark. Absorption at 727 nm was measured (Perkin-Elmer15 UV-vis spectrophotometer). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram of dry material.

Preliminary phytochemical tests. Phytochemical tests for some major phytoconstituents of the plant extracts were performed according to Parech and Chanda (2007). Plant extracts were screened for the presence of biologically active compounds such as alkaloids, flavanoids, saponins and steroids and quinones. The test for alkaloids consisted of addition of 200 mg plant material to 10 ml methanol, filtering, addition of 2 ml filtrate to 1% HCl, boiling, filtering again and addition of Mayer's reagent. The presence of flavonoids was determined using 1% aluminum chloride solution in methanol-concentrated HCl, magnesium turnins, and potassium hydroxide solution. The presence of saponins was determined by the so-called frothing test: 0.5 ml filtrate was added to 5 ml distilled water. Frothing persistence indicated the presence of saponins. The presence of steroids was detected by the Liberman-Buchard reaction, i.e. 200 mg of plant material was placed in 10 ml chloroform for 10 min and filtered, 2 ml filtrate was added to 2 ml of anhydrous acetic acid and concentrated H_2SO_4 . A blue-green ring indicated the presence of terpenoides. For quinones, 2 ml of filtrate was added to 2 ml NaOH 1/10. The development of a red colour indicated the presence of quinones.

Data analysis. The data used represent means of all experiments, which were repeated at least three times. Data were analysed using the SPSS statistical Program (version 13) by analysis of variance and comparison of means with the Duncan multiple range test ($p < 0.05$).

RESULTS

Efficacy of plant extracts against plant pathogenic fungi. The inhibitory effect of plant extracts on the mycelial radial growth of *F. solani* is shown in Fig 1. Plant extracts inhibited mycelial growth between 54% and 68%. The maximum inhibition level was observed with methanolic extracts of the leaves of *S. terebenthifolius* (Fig. 1). The highest level of inhibition of mycelial growth was obtained with methanolic extracts. However, there was no inhibitory activity recorded with the hexane extract (data not shown).

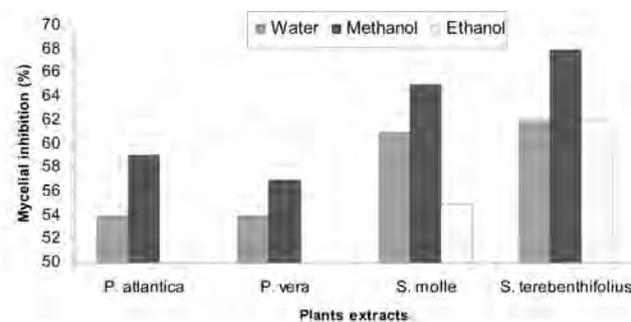


Fig. 1. Percentage of mycelial growth inhibition of *Fusarium solani* obtained with leaf extracts of species of *Pistacia* and *Schinus* prepared in different solvents.

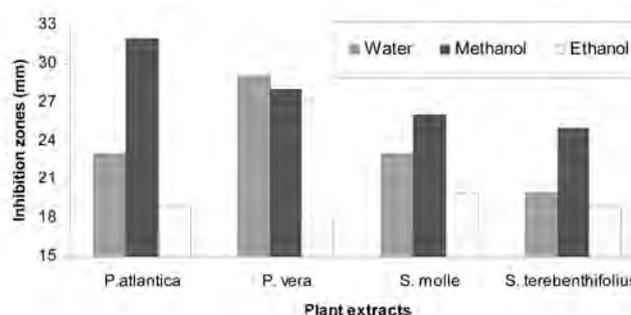


Fig. 2. Diameter of inhibition zones (mm) recorded with leaf extracts prepared in different solvents used to study effect on conidial germination of *Fusarium solani*.

Plant extracts of the different species of *Pistacia* and *Schinus* showed an inhibitory effect on conidial germination of *F. solani*. Methanolic extracts were more efficient than the other tested solvents (Fig. 2). Leaf extracts of *P. atlantica* exhibited a high level of antifungal activity against conidial germination of *F. solani* (Fig. 2 and 3). The analysis of variance showed highly significant differences between extracts and solvents, although the interaction was not significant.

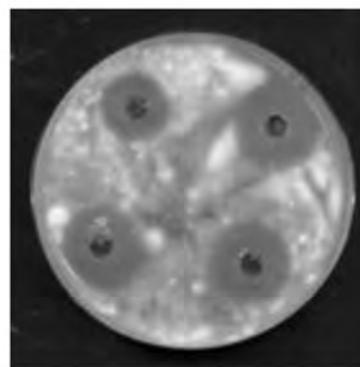


Fig. 3. Inhibition zones observed with the methanolic extracts of leaves of *Pistacia atlantica* used to study effect on conidial germination of *Fusarium solani*.

The inhibitory effect of plant extracts on the mycelial radial growth of *R. solani* showed that methanolic extracts were highly inhibitory (data not shown). The maximum level of inhibition was recorded with leaf extracts of *S. terebenthifolius* reaching almost 80%.

Efficiency of the plant extracts on the phytopathogenic bacteria. Leaf extracts of *Schinus* and *Pistacia* showed a high level of antimicrobial effect against the bacterial strains and the best results were obtained with *P. atlantica* and *S. molle* against *A. tumefaciens* and *B. subtilis* (Table 1, Fig. 4). There was no antibacterial effect with leaf extracts prepared in hexane (data not shown).

As to the sensitivity of bacterial strains to leaf extracts, it was observed that *A. tumefaciens* and *B. subtilis* were very susceptible since the diameter of inhibition zones reached 28 and 29 mm, respectively (Table 1). *P. savastanoi* pv. *savastanoi* was more resistant to the leaf extracts than the other bacteria for the diameter of the inhibition zone did not exceed 20 mm. There was no significant difference between the diameters of inhibition zones induced by leaf extracts in water, methanol and ethanol.

Polyphenols contents and their antimicrobial effect. *P. atlantica* leaves contained the highest level of total polyphenol reaching about 25 mg GAE/g of dry weight.

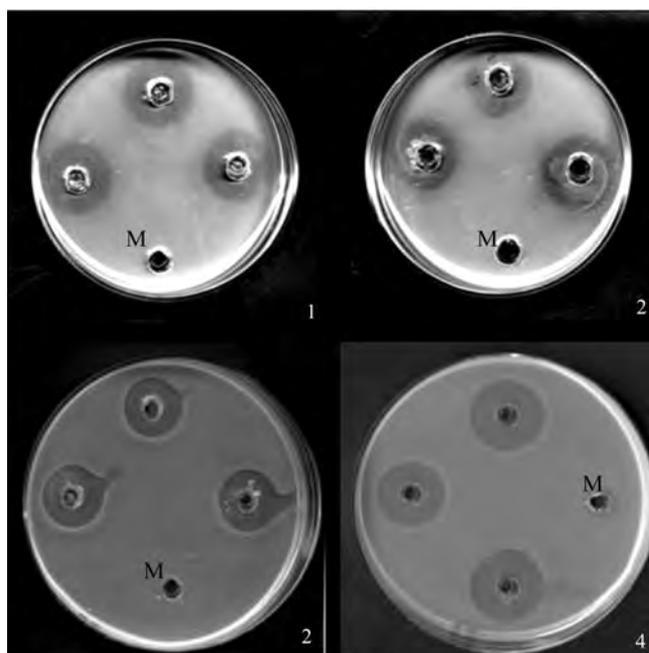


Fig. 4. Inhibition zones observed with methanolic extracts of leaf extracts of *Pistacia atlantica* and *Schinus molle* against *Agrobacterium tumefaciens* strain AR125 and *Bacillus subtilis*. 1. *Pistacia atlantica* against *Agrobacterium tumefaciens*, 2. *Schinus molle* against *Agrobacterium tumefaciens*, 3. *Pistacia atlantica* against *Bacillus subtilis*, 4. *Schinus molle* against *Bacillus subtilis* M, negative control (methanol).

Table 1. Diameter of inhibition zones (mm) observed with leaf extracts prepared in different solvents against plant pathogenic bacteria.

Plant extracts	Bacterial species	Water	Methanol	Ethanol
<i>P. atlantica</i>	<i>Agrobacterium tumefaciens</i>	23 aA	28 aA	22 aA
<i>P. vera</i>		9 cA	20 aB	17 bB
<i>S. molle</i>		13 bA	22 aB	20 aB
<i>S. terebenthifolius</i>		20 aA	15 bB	11 cB
<i>P. atlantica</i>	<i>Pseudomonas savastanoi</i> pv. <i>savastanoi</i>	7 cA	10 cA	12 cA
<i>P. vera</i>		14 bA	20 bA	12 cB
<i>S. molle</i>		9 cA	10 cA	9 cA
<i>S. terebenthifolius</i>		9 cA	10 aA	9 cA
<i>P. atlantica</i>	<i>Bacillus subtilis</i>	28 aA	28 aA	29 aA
<i>P. vera</i>		21 aA	23 aA	20 aA
<i>S. molle</i>		15 bA	11 cA	13 bcA
<i>S. terebenthifolius</i>		17 bA	14 cA	17 bA

Values represent means of three replicates. Values in the same columns followed by different low case letters are significantly different at $p < 0.05$. Values in the same rows followed by different high case letters are significantly different at $p < 0.05$.

The polyphenol content of the other species did not exceed 15 mg of GAE/g. The polyphenol content was significantly correlated with the diameter of the inhibition zones recorded with *A. tumefaciens* strain C58 (data not shown).

Phytochemicals analysis. Preliminary phytochemical analysis revealed the presence of alkaloids and flavanoids. Other secondary metabolites like steroides, saponins and quinones were absent.

DISCUSSION

Aqueous and methanol extracts showed the highest antibacterial and antifungal effect. This confirms previous reports that methanol is a better solvent for more consistent extraction of antimicrobial substances from medicinal plants as compared to other solvents, such as water, ethanol, and hexane (Ahmad *et al.*, 1998; Etof *et al.*, 1998; Lin *et al.*, 1999). Total absence of inhibitory activities by hexane extracts suggests that the antimicrobial compounds of the plant studied are not soluble in non-polar solvents.

Our data show that there was no uniform response within or between the bacterial and fungal strains investigated, in terms of susceptibility to antimicrobial compounds in the methanol extract of *Schinus* and *Pistacia*. These differences in susceptibility shown by different microorganisms against antimicrobial substances in plant extracts may be explained by the differences in cell wall composition.

The two *Schinus* species proved effective against some of the plant pathogens tested in the present study. This finding is in agreement with previously reported results of Dikshit *et al.* (1986) who found that oils of *S. molle* exhibited antifungal activity towards storage fungi. To the best of our knowledge, this is the first study showing that methanolic extract of *S. terebenthifolius* leaves contains antimicrobial substances against phytopathogenic bacteria and fungi. Erazo *et al.* (2006) found that essential oils of *S. polygamus* fruits have antimicrobial activity against bacteria pathogenic to human. These authors showed that the composition of essential oil was dependent on the geographic site and the most abundant compounds were α -pinene and α -phellandrene and limonene.

Leaf extracts of *P. vera* were effective against the tested plant pathogenic fungi and bacteria. These results are in accord with those by Kordali *et al.* (2003) who reported that crude leaf extracts from *P. vera*, *P. terebinthus* and *P. lentiscus* were effective against *Pythium ultimum* and *R. solani*. As far as *Fusarium* is concerned, our results show that the extracts of *P. vera* are effective against *F. solani*, whereas the results of Kordali *et al.* (2003) and Duru *et al.* (2003) showed no activity of

crude extracts and essential oils of *P. vera* against *F. sambucinum*. According to Ozcelik *et al.* (2005), the lipophytic extracts of *P. vera* were effective against bacteria, fungi and viruses.

Leaf extracts of *P. atlantica* proved to be very efficient against plant pathogenic fungi and bacteria. *P. atlantica* has been used as a rootstock for *P. vera* in Tunisia and other pistachio-growing countries for many centuries. It is well adapted to drought conditions and grows in degraded forests in many parts of Tunisia. However, no more than 1,500 trees occur in the country so that there is a serious danger of extinction for this species (Padulosi and Hadj-Hassan, 1998). *P. atlantica* is valued because it is the source of mastic gum, an exudate that strengthens gums, deodorizes breath and combats coughs, chills and stomach diseases (Bellakhder, 1997). Our results are consistent with those recently obtained by Benhammou *et al.* (2008) who also determined an antifungal activity against *Fusarium* sp. Several *in vitro* studies have shown that leaf extracts of *P. terebinthus* and its resin are also effective against human pathogenic bacteria (Magiatis *et al.*, 1999).

Polyphenols were found to be more abundant in *P. atlantica* than in the other species studied and exhibited a high level of antimicrobial activity against plant pathogens. Many authors have suggested that polyphenols inhibit the growth of microorganisms by forming complexes with either microbial enzymes or proteins. One of the known inhibition mechanisms consists of iron depletion (Mila *et al.*, 1996). Among polyphenols, flavonoids are able to chelate some metals and consequently inhibit Fenton and Haber-Weiss reactions, which are important sources of active oxygen radicals (Manach *et al.*, 1996; Shahidi *et al.*, 1992). Pistachio leaves contain several types of water-soluble polyphenols which have a high antioxidant and antimicrobial potential (Barotto *et al.*, 2003; Ljubuncic *et al.*, 2005). In the case of *P. lentiscus*, the abundant polyphenols are acid gallic, certain derivatives from galloyl, myricetin-like glycosides and quercetin (Romani *et al.*, 2002). Based on these results, the antibacterial and antifungal activities seem attributable mainly to flavanoids.

Preliminary phytochemical screening revealed the presence of alkaloids, flavanoids and tannins, confirming the findings of Mansouri *et al.* (2005) who showed that *P. vera* contains saponins, tannins and flavanoids. These compounds have been associated with antimicrobial effects in various studies using plant extracts (Nwaogu *et al.*, 2007; Newze *et al.*, 2004; Abo *et al.*, 1999).

In conclusion, our findings suggest that *Pistacia* and *Schinus* species are a potential source of compounds effective against *A. tumefaciens*, *P. savastanoi* pv. *savastanoi*, *F. solani* and *R. solani*. They confirm the results of previous studies, but this is the first report of the antimicrobial activities of *S. molle*, *S. terebenthifolius* and *P. atlantica* against the above-mentioned plant patho-

genic bacteria and fungi. Further research is, however, necessary to determine the nature of the active principles present in the extracts of these plant species. The identification of the molecules involved in the antimicrobial effect could lead to the discovery of new biopesticides for use in the control of plant diseases.

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