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Research Article

Schinus terebinthifolius Raddi (Anacardiaceae) leaf extracts: Antibacterial activity against two *Agrobacterium tumefaciens* strains

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Abstract: Brazilian peppertree (*Schinus terebinthifolius*) leaf extract was applied against two strains of the crown gall disease agent (*Agrobacterium tumefaciens*); strains C58 and AR125. *In vitro*, we used the agar well diffusion method and the extract was tested in different solvents selected according to their polarity indices, at different concentrations and different incubation temperatures. *In vivo*, we tested the extract prepared in different solvents on the stems of young tomato plants (cv. Firenze) which were inoculated with the strain C58.The best results were obtained with the extract prepared in hot sterile distilled water and in methanol (0.3 $g.ml^{-1}$) incubated at 25 °C and 30 °C. The minimum inhibitory concentration (MIC) was evaluated to be 10^{-4} $g.ml^{-1}$. In another aspect, to identify the nature of certain substances from *S. terebinthifolius* leaf extracts, we made a test of fractionation using the Thin Layer Chromatography (TLC) method and phytochemical screening of the crude methanol leaf extract. We noticed the presence of alkaloids and flavonoid compounds which may be responsible for the antibacterial activity. These tests indicated that false pepper leaf extract has an antibiotic effect against *Agrobacterium tumefaciens* both *in vitro* and *in vivo*, which represents a conceptual approach with great promise for future biological control.

Keywords: Brazilian peppertree, Leaf extracts, the crown gall disease, Antibacterial activity

Introduction

Schinus terebinthifolius (Anacardiaceae), commonly known as Brazilian pepper tree or Christmas berry (Cuda *et al.,* 2010) is native to Brazil (Lorenzi 2002), Paraguay and northeastern Argentina. It has become established and naturalized in most tropical and subtropical parts of the world (Ruas *et al.,* 2011). It has been widely grown as an ornamental. Currently, we find this species frequently all around Mediterranean; in southern Africa, Australia and Hawaïi; also present in southern Europe, northern Africa, tropical Asia, New Zealand, western U. S. and the Caribbean (Weber, 2003). Beside its ecological importance, *S. terebinthifolius* has been widely used for therapeutic purposes (Melo *et al.*, 2009; Glienke *et al.,* 2012) particularly in its native area. Nevertheless, it has not defined any antimicrobial effects against three different microorganisms isolated from diarrheic feces inocula using the agar diffusion method (Gonçalves *et al.,* 2011). The phytochemical analysis of this case reveals that the plant contains tannins, alkaloids, flavonoïds, saponins of steroids, terpenes, gums, resins and essential

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oils. The leaves, bark and fruits are rich sources of triterpenes, sesquiterpenes and monoterpenes. The fruits can contain up to 5% of essential oil and leaves can contain up to 2%. A recent chromatographic analysis by GC-MS permitted the identification and quantification of 100% of the integrated components in the essential oil isolated from the fruits of S. *terebinthifolius* (Affonso *et al*., 2012) and from the fresh leaves (Gundidza *et al.,* 2009).Concerning the biological properties of the Brazilian peppertree, it was reported, for a long time, that extracts obtained from the species *S. molle* can be used as an analgesic, anti-inflammatory and anti-tumorous agent (Barrachine *et al.,* 1997; Yueguin *et al.,* 2003; Diaz *et al.,* 2008; Kasimala and Kasimala, 2012). It showed also antibacterial, antiviral, antifungal, insecticidal and repellent properties (Anupam *et al.,* 1986; Dikchit *et al.,*1986; Chopa *et al.,* 2006; Ferrero *et al.,* 2007; Padin *et al.,* 2007; Bayramoglu *et al.,* 2008; Rhouma *et al*., 2009). Researches done on *S. terebinthifolius* were made mainly in medicine. Recently, Gundidza *et al.* (2009) showed that essential oil from fresh leaves of *S. terebinthifolius* has defined potent antibacterial and antifungal activities, and Bendaoud *et al*. (2010), have reported that essential oils from fruits of both false pepper species; *S. molle* and *S. terebinthifolius* were identified and investigated for their antioxidant and anticancer activities. It was indicated that oils induced promising *in vitro* antioxidant activity and cytotoxicity in human breast cancer. Thus, *S. terebinthifolius* essential oil, which is specifically rich of sesquiterpenes, was shown to be the most active. It has been reported also to have insecticidal activity against the African malaria vectors (Kweka *et al*., 2011). Also, Costa *et al.* (2012) reported that hydroalcoholic extracts of this species display an *in vitro* antimicrobial activity, even at low concentrations, against the nosocomial pathogen, *Enterococcus faecalis*, isolated from the rooted-human teeth. These findings suggest that *Schinus* extracts might be a promising source of active compound (s) for innovative therapeutic and/or preventive strategies.

The present study was initiated to assess the antibacterial activity of leaf extracts of *S. terebinthifolius* against two strains of the plant tumorigenic bacterium *A. tumefaciens* (Smith and Townsend, 1907 in Bull *et al.* 2010), causative agent of the crown gall disease.

Materials and Methods

Plant material. The *S. terebinthifolius* leaves were harvested from the region of Sfax (in southeast Tunisia), dried at room temperature, then in an oven at 35 °C for 3 to4 days, ground to a fine powder using an electric grinder and then, the resulting material was kept away from moisture in darkness to avoid photodegradation. Extraction was done by maceration in different solvents to prepare the crude extracts which were then kept at 4 °C in the dark for further tests.

Test plants. Tomato plants (cv. Firenze) in cotyledon stage were provided by the Manouba Support Station (MSS) nursery, transplanted into pots containing a sterilized mix of soil and peat (2: 1 by volume)and placed in a glasshouse at INAT (National Agronomic Institute in Tunisia) under optimum growth conditions(photoperiod (16h/24h), temperature (25 °C at day and 15 °C at night), humidity (70%) and irrigated when required).They were subsequently used for inoculation tests with the pathogen and extracts.

Bacterial strains. Two *A. tumefaciens* strains were tested: C58 and AR125 which were provided by the Olive Institute of Sfax (Tunisia) (Table 1). They were cultivated in Mannitol Glutamate Agar (MGA) containing in g/L (5 D-mannitol, 2 L-glutamic acid, 0.5 KH_2PO_4, 0.2 $MgSO_4$, $7H_2O$, agar, 20; pH 7.2).

Table 1 Bacterial strains.

Code	Designation	Origin	Host plant
C58	*Agrobacterium tumefaciens*	USA	Cherry
AR125	*Agrobacterium tumefaciens*	Tunisia	Pear tree

Solvents used for extraction. Five solvents (hexane, toluene, ethanol, methanol and sterile distilled water (SDW)), characterized by different polarity index (Table 2) were used to carry out the Brazilian peppertree leaf crude extracts.

Table 2 Solvents and their polarity index.

Solvent	Polarity index
Hexane	0.1
Toluene	2.4
Ethanol	4.3
Methanol	5.1
Water	10.2

Crude extract preparation. Four concentrations (200, 300, 400 and 500 $mg.ml^{-1}$) were used when preparing crude extracts, in order to study the effect of the extract concentration on the bacterial growth. 10ml of each solvent was added to the different masses of leaf powder: 2g, 3g and 5g to get the concentrations mentioned above. Mixtures were incubated with stirring for a period of two hours. Then filtered through sterile filter paper and filtrates were kept at 8 °C in conical tubes covered by aluminum foil for further tests.

Antibacterial activity *in vitro*. Bacterial suspensions were prepared from 48 hours cultures. Inocula were prepared by adjusting the turbidity of each bacterial culture to 10^8 Colony Forming Units/ml (CFU/ml).Antibacterial activity of *S. terebinthifolius* leaf crude extract was rated by means of agar-well diffusion assay according to the method of Andrews (2005) with some modifications. A mixture of MGA medium (0.6% Agar) and bacterial suspension (3: 1 by volume) was prepared each time and poured into Petri dishes (Ø = 90mm) containing a dry MGA (2% Agar) medium. In each dish, four equidistant wells were drilled out using sterile blue cones (or sterile platinum punch), and for each plate, the extracts (for each concentration already prepared) were added to three of the wells, the fourth was filled by the solvent used during extraction(as negative control).Plates were then incubated at four different temperatures (4 °C, 20 °C, 25 °C and 30 °C) to evaluate the relationship linking temperature and antibacterial activity of extracts. This method was used for extracts prepared with the different solvents. Tests were repeated three times per variable (overall 480 assays).

Antibacterial activity *in vivo*. This experiment was carried out by testing the effect of extracts, which had given the best results in *in vitro* test, against *A. tumefaciens* (strain C58) to control the gall formation after a period of inoculation with the bacterial strain and extracts. These inoculations were performed on tomato plants (cv. Firenze) which were already prepared for this test. Five plants were inoculated by extract with a mean of 2 wound sites per plant which were made on stems. Extracts were injected and left to dry for 30 to 60 min, and then inoculated with equal volumes (10µl) of the bacterial suspension (10^8 CFU). The wounds were covered with water-soaked cotton during the first week. Five plants were used as negative controls (treated with extracts only without bacterial inoculation).The plants were controlled daily, irrigated when needed and checked for the appearance of galls. After a period of 3 to 4 weeks, the percentages of infected plants and numbers and weights of tumorigenic sites were evaluated.

Characterization of antimicrobial metabolites:

Phytochemical tests. Biochemical tests for *S. terebinthifolius* leaf extract extracts were performed according to Allen (1974) and Harborne (1984). Extracts were prepared in Methanol (best choice) and tests were based on the visual observation of color change or formation of a precipitate after the addition of specific reagents. The different chemical constituents tested include sterols and/or triterpenes, flavonoids, tropolones, free quinones and alkaloids.

Thin Layer Chromatography (TLC) Analysis. Extract was prepared in methanol at low concentration (100 $mg.ml^{-1}$) to get the best separation. Silica gel TLC plate was cut into about 9 cm height and 4 cm width. A small amount of compound was spotted 0.5 cm from bottom of TLC plate with the smallest diameter possible, and was allowed to evaporate. TLC solvent was made according to Bloor (2001) with the following composition: water (2.7 ml), acetic acid

(1.1ml), formic acid (1.1ml) and ethyl acetate (10 ml). And then was placed in chamber waiting for 1-5 min to equilibrate the atmosphere inside. After that, TLC plate was placed in chamber and solvent front was allowed to run up the plate. When the solvent front reached approximately 1-0.5 cm from top of plate, this latter was removed from TLC chamber. Finally, after plate drying, the different fractions were revealed to the naked eye or/and under fluorescence. Then, the different fractions were scratched and dissolved in the same solvent (methanol) and then tested against the C58 strain in comparison to the crude extract. This test determined the fraction (s) responsible (s) of the antibacterial activity.

Statistical Analysis. The *in vivo* results were analyzed by a completely randomized design using the SPSS (Version18th) software. The significance of difference was calculated using Duncan test and P values < 0.05 were considered as significant.

Results

Effect of solvent type. The antibacterial activity of *S. terebinthifolius* leaf extracts was examined against two strains of the plant pathogenic species *A. tumefaciens* causing the crown gall disease in Rosaceae crops. Among the tested extracts, MeOH-E Hot DW-E and EtOH-E showed strong antibacterial activities against both strains (Fig. 1). The inhibition diameter of leaf crude extracts prepared in ethanol, methanol and SDW were very effective against both strains AR125 andC58.This efficiency was reflected in large rings of inhibition up to 7mm wide. Unlike those prepared in hexane and toluene that mounted a very weak inhibition of the pathogen, even at the highest concentration (300 mg.ml^{-1}). According to these results, it is assumed that the extract was more soluble in solvents having the highest polarity indexes (ethanol, methanol and water) which allow a good release of active substances. This solubility has been proven by wide zones of inhibition of growth of both strains up to7 mm.

Effect of extract concentration. The effect of concentrations was evaluated for extracts prepared in EtOH, MeOH and SDW. According to Figure 2, the inhibition zone increases as concentration rises. Extracts have defined antibacterial activity even at very low concentrations. The minimum inhibitory concentration (MIC) was equal to 0.1 mg.ml^{-1}. Nevertheless, at this concentration, the extract prepared in cold water (SDW) could not inhibit both bacterial strains. It became inhibitor starting at 1mg.ml^{-1}. The most important inhibition zones were observed at the highest concentrations, especially with extract prepared in hot SD W and in methanol.

Effect of incubation temperature. Four different temperatures (4 °C, 20 °C, 25 °C and 30 °C) were used to evaluate the relationship linking temperature and antibacterial activity of extracts. At the lowest temperature (5 °C), the bacterial inhibition zones were limited. The multiplication of both bacterial strains was also not clear. Thus, at low temperature, it was assumed that the extract diffusion is carried out with some difficulty and bacterial strains grow slowly. However, by increasing the incubation temperature, bacteria proliferated rapidly and spread of the extract was also improved. Large and clear inhibition zones, exceeding 7mm, were observed at 25 °C and 30 °C. No significant difference was detected between the two temperatures.

Antibacterial activity *in vivo*. The antimicrobial effects of *S. terebinthifolius* leaf EtOH-E, MeOH-E and SDW-E were confirmed by *in vivo* tests using tomato plants. The results are reported in Table 3. Analysis of variance of the average number and weight of galls from all experiments (Duncan test) showed highly significant differences (P = 0.05) between treatments with extracts and the control mainly with EtOH-E, MeOH-E and Hot SDW-E. These differences were revealed among number, size (Fig. 3) and weight of galls produced in the plants treated with the extracts compared to those in control plants (non-treated by extracts). According to Figure 4, there is no difference among the three extracts EtOH-E, MeOH-E and Hot SDW-E. Nevertheless, all crude extracts were less effective *in vivo* than *in vitro* conditions. They did not completely inhibit bacteria in the plant. It might be related to the fact that natural extracts can be processed in the plant tissue by enzymatic reactions. Figure 5 records the average weight of the galls in the different treatments. A clear

difference was shown among the weight of galls in the control plants (without application of extract) and those produced in the plants treated with the different extracts. According to the histogram, extracts prepared in hot SDW and in methanol have been the most efficient against this pathogen. On the other side, cross-sections were performed into the stems of test plants. We revealed a large difference between the stem diameters at the gall level and those close by it (Figure 5) on tomato stems inoculated with *A. tumefaciens*.

Table 3 Phytochemical test results.

Tests	Results
Sterols	-
Flavonoids	+
Tropolones	-
Quinones	-
Alkaloids	++

(-): Negative test (+): Positive test.
(++): Moderately positive test (+++): Strongly positive test.

Phytochemical test results. The biochemical analysis of the *S. terebinthifolius* leaf MeOH-E showed the presence of alkaloids and flavonoids (Table 3). These compounds present in *S. terebinthifolius* crude extract are known for their wide biological and medicinal activities (Bendaoud *et al.,* 2010; Gundidza *et al.*, 2009).

Fraction Results. The technique of thin layer chromatography (TLC) allowed us to observe spots of three fractions. Results of the agar diffusion method are shown in Figure 6. According to these results, the fraction 3 (F3) was the most active fraction, which induces the highest inhibition of strain C58. It is so comparable to that induced by the crude extract (CE). Presumably, alkaloids and flavonoids are probably responsible for this antibacterial effect. Further analysis will be necessary to identify exactly these compounds using another more sophisticated method such as LC-MS technique.

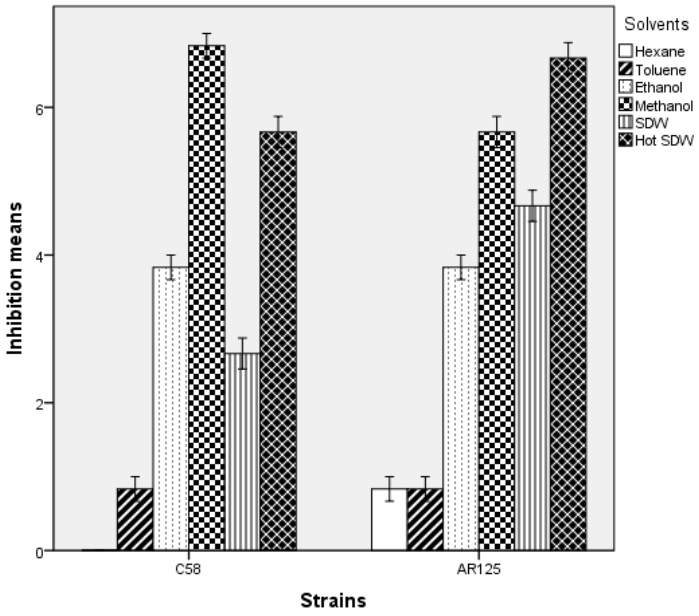

Figure 1 Means (± standard error) of antibacterial activity of leaf crude extracts of *Schinus terebinthifolius*, prepared in different solvents, against both strains C58 andAR125 (after 24h at 25 °C).

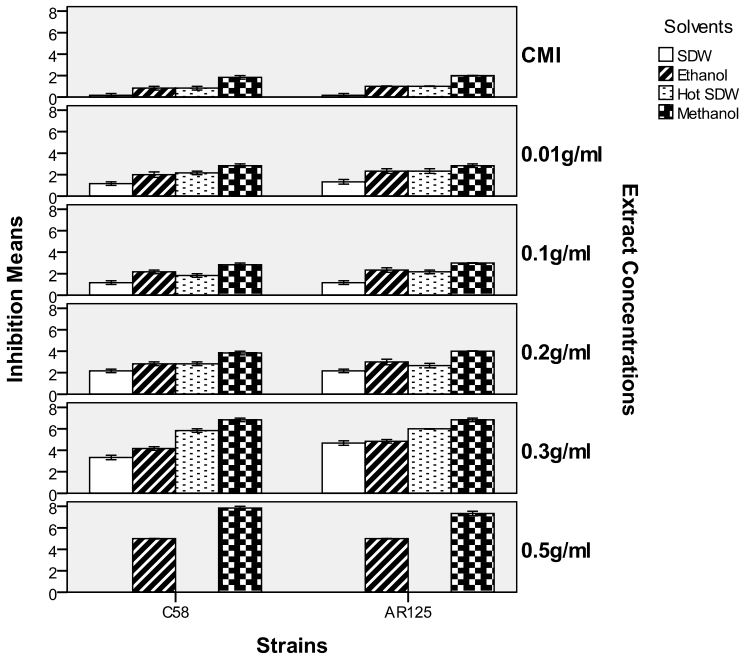

Figure 2 Means (± standard error) of inhibitory potential of Brazilian pepper tree leaf extracts against C58 and AR125 at different concentrations.

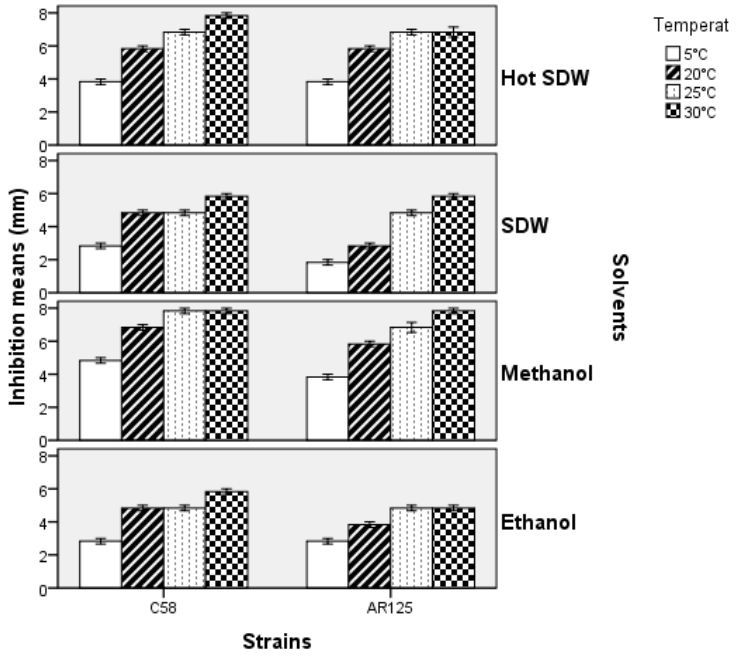

Figure 3 Means (± standard error) of effectiveness of Brazilian pepper tree leaf extracts prepared in different solvents (0.3 g/ml) against both strains *Agrobacterium tumefaciens* (C58 and AR125) at different incubation temperatures.

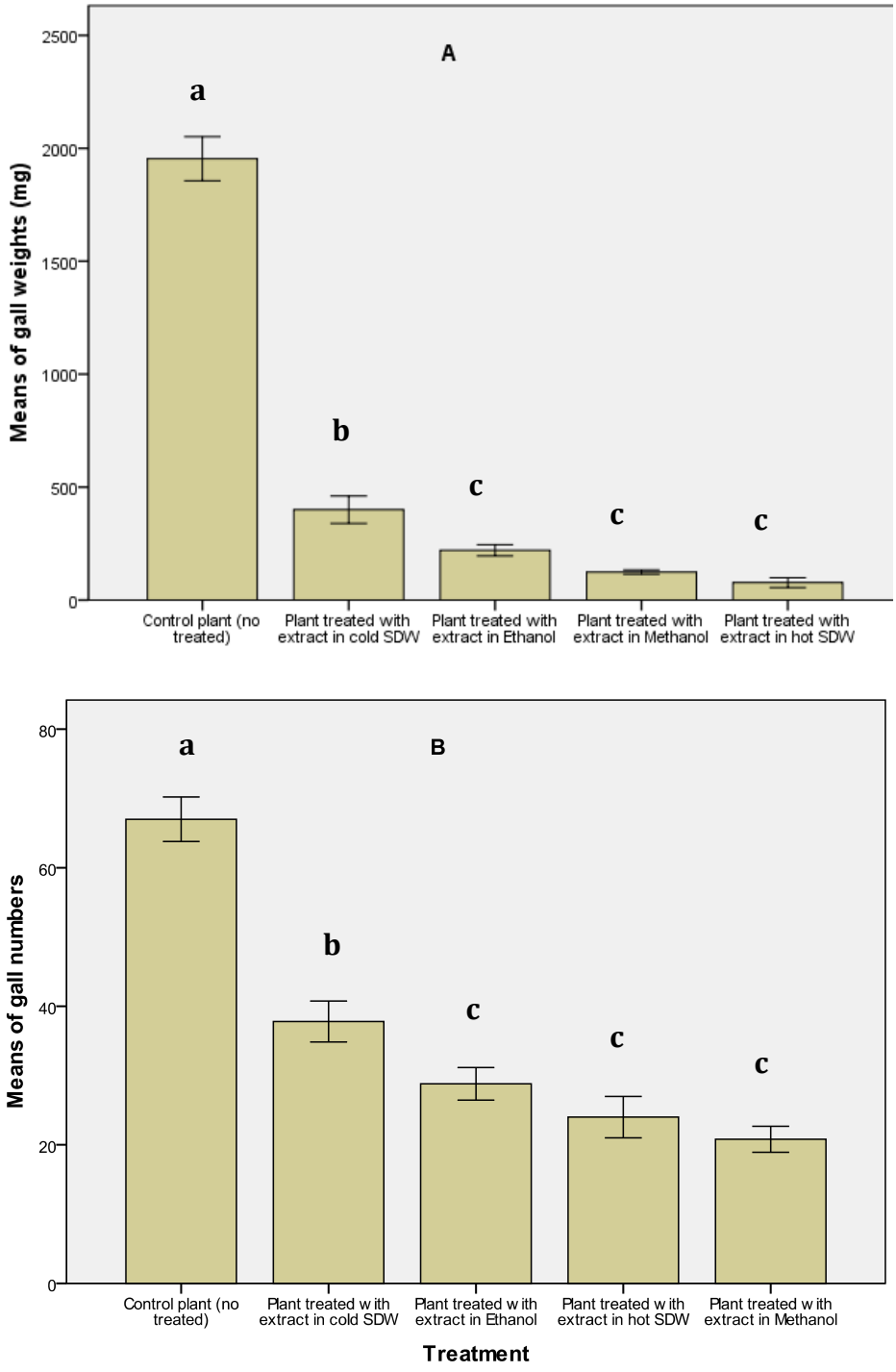

Figure 4 Means (± standard error) of weights (A) and numbers (B) of tumorigenicites.

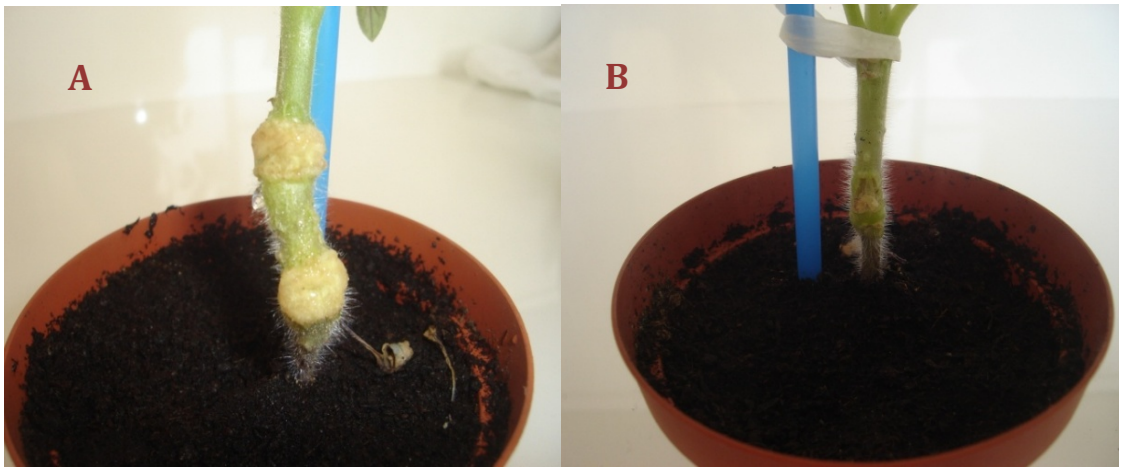

Figure 5 Symptoms of *Agrobacterium tumefaciens* on tomato stems. A: plant without application of the leaf extract, B: plant treated with the leaf extract of Brazilian pepper tree prepared in hot sterile distilled water.

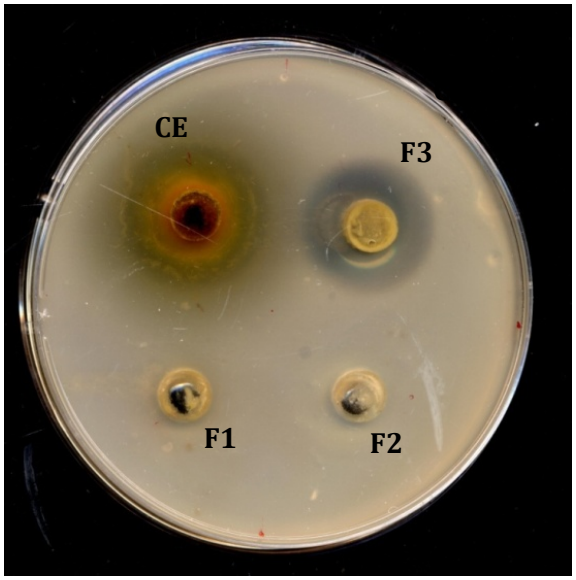

Figure 6 Leaf crude extract (CE) and fraction (F1, F2 and F3) activities against *Agrobacterium tumefaciens* strain C58.

Discussion

Resistance to antimicrobial chemicals today, remains a major problem in plant health care. The Brazilian peppertree species, especially *S. molle*,have since long ago been described as producers of metabolites with antimicrobial activity. Lots of researches have shown that this species controls many pathogenic microorganisms in particular plant pathogens (Anupam et *al.*, 1986; Dikchit *et al.*, 1986; Chopa et *al.*, 2006; Ferrero *et al.*, 2007; Padin et *al.*, 2007; Bayramoglu *et al.*, 2008; Rhouma *et al*., 2009). It is also used for the repellence of certain insects. It contains potential natural products in its crude extracts as well as in its essential oils. However, researches made on the species *S. terebinthifolius* are few. Recently, GC-MS analysis has allowed identification of chemical composition of essential oil from fresh leaves (Gundidza *et al.*, 2009) and quantification of the integrated components from the fruits of *S. terebinthifolius* (Affonso *et al.*, 2012).

Our study by means the agar diffusion method showed that the crude leaf extracts of the Brazilian peppertree *S.terebinthifolius*, when prepared by solvents of high polarity index (Ethanol, Methanol and SDW), have potential antibacterial properties., it is deduced that the extractability of compounds (kind and/or rate) depends on the solubility of the leaf powder in the solvent. According to our work, solvents with high polarity index were more efficient in extracting these compounds.

On the other hand, TLC method is only qualitative but simple and a relatively cheap technique to test the inhibitory activity of a large quantity of organic extracts. Results

revealed that compounds present in the crude extracts of peppertree leaves as well as in the fraction3, have been responsible for the antimicrobial activity of the plant. More sophisticated methods are needed to use for further studies giving more details on these compounds. Using the HPLC method, Queires *et al.* (2006) have reported that polyphenol fractions purified from the Brazilian peppertree leaf extract inhibited the DU145 (human prostatic carcinoma cell line) cell proliferation more than 30-fold compared to the crude extract.

Biochemical tests made on methanol leaf extract have reported the presence of alkaloids and flavonoids which have revealed an antibacterial effect with the agar diffusion method and the *in vivo* test. Thus, the crude extract and active fractions of the methanol leaf extract create the hypothesis that peppertree antimicrobial activity could be caused by these compounds along with other bioactive secondary metabolites that might be produced by the plant.

In conclusion, our results suggest that crude extracts of *S. terebinthifolius* leaves could be a potential source of effective antibacterial compounds against *A. tumefaciens* and other plant pathogenic bacteria and fungi. Therefore, these findings revealed that the pepper tree species, widely used in traditional medicines and as ornamental adapted to Mediterranean climate, could be used also in agriculture as source of natural compounds to control plant pathogenic organisms. Furthermore, the development and formulation of natural extracts will be helpful to decrease the negative effects of synthetic pesticides at a time when all stakeholders in the agricultural sector recognize the urgency to minimize hazards caused by the misuse of chemicals. The use of plant extracts is potential and wiser approach with great promise for the future as well as means of control that acts directly on the pathogen or as biological elicitor that induces defense mechanisms of the plant. The latter hypothesis could be verified by further detailed studies.

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عصاره برگ *Schinus terebinthifolius Raddi*: فعالیت ضدباکتریایی علیه استرین‌های *Agrobacterium tumefaciens*

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چکیده: عصاره برگ درخت فلفل برزیلی (*Schinus terebinthifolius*) علیه دو استرین C58 و AR125 عامل گال طوقه (*Agrobacterium tumefaciens*) به‌کار گرفته شد. برای این‌منظور از آزمون درون شیشه‌ای با روش نشت در آگار استفاده شد. هم‌چنین از عصاره‌هایی که با حلال‌های مختلف که براساس شاخص قطبیت‌شان انتخاب و استخراج شده بودند در غلظت‌های مختلف و دماهای نگهداری متفاوت استفاده شد. در آزمایش روی گیاه از عصاره‌های تهیه شده با حلال‌های مختلف روی ساقه‌های جوان گوجه فرنگی (رقم فیرنزه) مایه‌زنی شده با استرین C58 استفاده شد. بهترین نتیجه از عصاره تهیه شده با آب مقطر سترون و متانول (۰/۳ گرم در لیتر) در دماهای ۲۵ و ۳۰ درجه سلسیوس به‌دست آمد. حداقل غلظت بازدارنده (MIC) برابر $۱۰^{-۴}$ گرم در میلی‌لیتر به‌دست آمد. در بخش دیگر برای شناسایی ماهیت برخی مواد موجود در عصار برگی *S. terebinthifolius* از روش کروماتوگرافی روی لایه نازک (TLC) و مطالعات فیتوشیمیایی عصاره‌های برگی ناخالص در متانول استفاده شد. براین اساس ترکیبات آلکالوییدی و فلاونوییدی در این عصاره‌ها یافت شد که ممکن است عامل فعالیت ضدباکتریایی آنها باشد. این آزمایش‌ها نشان داد که عصار برگ فلفل برزیلی در شرایط درون شیشه‌ای و روی گیاه دارای خواص آنتی‌بیوتیکی علیه *Agrobacterium tumefaciens* می‌باشد که می‌تواند یافته‌ای نوید بخش برای به‌کارگیری آن در کنترل بیولوژیک در آینده باشد.

واژگان کلیدی: درخت فلفل برزیلی، عصاره‌های برگی، بیماری گال طوقه، فعالیت ضدباکتریایی