Original article

Polar *Lippia* extracts as alternatives for the postharvest control of Guazatine®-resistant strains of *Penicillium digitatum* in citrus

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Polar *Lippia* extracts as alternatives for the postharvest control of Guazatine®-resistant strains of *Penicillium digitatum* in citrus.

Abstract — Introduction. *Penicillium digitatum* is a commercially important postharvest pathogen of citrus that is responsible for significant annual global losses. Strains of the fungus, which exhibit strong resistance to widely used synthetic fungicides, are of major concern to the industry. The aim of the study was to investigate the antifungal activities of polar extracts and compounds from *Lippia* species, indigenous to South Africa, against a Guazatine®-resistant strain of *P. digitatum*. **Materials and methods**. *In vitro* tests were done by incorporating the compounds and plant extracts into Malt Extract Agar at concentrations ranging from (0.2 to 1.0) mg·mL^{-1}. An *in vivo* (curative) assay was conducted using the checkerboard technique on 'Valencia' oranges. **Results and discussion**. A strong correlation between the *in vitro* and *in vivo* results was observed. All the compounds and extracts were able to inhibit fungal growth at concentrations above 0.6 mg·mL^{-1}. Verbascoside was the most active compound, while extracts of *Lippia javanica* and *Lippia rehmannii* caused significant inhibition of mycelial growth. The observed activity was largely ascribed to the presence of verbascoside in the plant extracts. **Conclusion**. Polar extracts of *Lippia* species have potential as environmentally friendly alternatives for the control of *P. digitatum* on citrus.

South Africa / *Citrus* / *Penicillium digitatum* / disease control / postharvest control / biological control / *Lippia* / plant extracts

Utilisation d'extraits polaires de *Lippia* pour le contrôle post-récolte d'une souche de *Penicillium digitatum* résistante à la Guazatine® sur oranger.

Résumé — Introduction. *Penicillium digitatum* est un pathogène d'importance commerciale pour le genre *Citrus*, car il est responsable de pertes significatives. L'apparition de nouvelles souches résistantes à la plupart de fongicides couramment utilisés est un problème majeur pour l'industrie. L'objectif de notre étude a été de tester les activités antifongiques d'extraits polaires ainsi que de certains composés présents chez quatre espèces de *Lippia* originaires d'Afrique du Sud, contre une souche de *Penicillium digitatum* résistante à la Guazatine®. **Matériel et méthodes**. Des tests *in vitro* ont été conduits en utilisant la méthode du milieu toxique en incorporant les composés ou extraits à de l'agar enrichi en extrait de malt, à des concentrations allant de (0,2 à 1,0) mg·mL^{-1}. Un test *in vivo* (curatif) avec des oranges du cultivar Valencia a été conduit en utilisant la technique du *checkerboard*. **Résultats et discussion**. Une corrélation significative entre les tests *in vitro* et *in vivo* a été observée. Tous les composés et extraits ont permis de réduire la croissance mycéliale à des concentrations supérieures à 0,6 mg·mL^{-1}. Le composé verbascoside a été le plus actif, alors que les extraits de *Lippia javanica* et *Lippia rehmannii* n'ont montré qu'une réduction partielle de la croissance mycéliale du pathogène. **Conclusion**. Notre étude a établi que les extraits polaires de *Lippia* sp. pourraient être des substances écologiques de substitution, utilisables pour le contrôle de *P. digitatum*.

Afrique du Sud / *Citrus* / *Penicillium digitatum* / contrôle de maladies / lutte après récolte / lutte biologique / *Lippia* / extrait d'origine végétale

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***RESUMEN ESPAÑOL**, p. 82*

1. Introduction

Sweet oranges (*Citrus sinensis* L.) account for more than two-thirds of the global citrus production. Oranges are also the main citrus fruits cultivated in the Republic of South Africa (RSA), although large volumes of kumquats, lemons, limes and soft citrus (mandarin types) are produced and exported[1]. *Penicillium italicum* and *P. digitatum* are the most prevalent citrus pathogens [1] and are responsible for huge financial losses [2]. These fungi infect the fruit through micro-injuries caused during harvesting and processing. It has been proposed that *P. digitatum* conidial growth is stimulated by volatile secondary metabolites produced by wounded oranges [3].

A variety of pre- and postharvest field management practices have been applied in pathogen regulation. These practices involve orchard sanitation before the onset of a growing season, removal of infected material, selection of quality fruit, proper washing and surface disinfection using chemicals alone, or in combination with fungicides [4]. These synthetic fungicides include benzimidazoles, zinc dimethyl-dithiocarbamate, Imazalil®, Guazatine®, ammonia, dichloran, thiophanate-methyl and thiabendazole [5, 6]. Health and environmental concerns have led to legislation in most importing countries, restricting the residues of pesticides on fruit [1, 7]. The more recent occurrence of fungal strains with resistance to many widely used fungicides has serious implications in both the pre- and postharvest environments [3]. In addition, consumers are increasingly aware of the health risks associated with synthetic fungicides, which has resulted in a greater demand for organically cultivated and treated products [8]. Essential oils and other plant extracts have been investigated as environmentally friendly and non-hazardous methods of fungal pathogen prevention and control [1, 6, 9]. Essential oils of thyme, oregano, clove, cinnamon, *Cumium cyminum* and *Aloe vera* gel, as well as extracts of *Thymus capitatus*, *T. serpyllum* and *Eugenia caryophyllata* have been reported to inhibit the growth of postharvest fungi, including *Penicillium expansum*, *P. italicum* and *P. digitatum* [1, 3, 10].

Lippia species (Verbenaceae) are well known as medicinal plants in many parts of the world for the treatment of respiratory and gastrointestinal disorders [11]. Five *Lippia* species (*Lippia scaberrima*, *L. javanica*, *L. rehmannii*, *L. wilmsii* and *L. pretoriensis*) are recognised in the RSA. Infusions of aerial parts of *L. scaberrima* and *L. javanica* are used medicinally as a cure for disorders of microbial origin. The essential oil of *L. scaberrima*, containing high concentrations of limonene, R-(–)-carvone and 1,8-cineole [12], was found to exhibit strong *in vitro* and *in vivo* activities against *Botryosphaeria parva* and *Colletotrichum gloeosporioides* isolated from mango fruit [9]. Recently, in our laboratory, the phenylpropanoids, verbascoside and isoverbascoside were identified in methanolic extracts of four of the indigenous *Lippia* species investigated. These compounds have been shown to have a variety of biological activities, including antimicrobial properties [13–15]. Both verbascoside and isoverbascoside were isolated from brined olive drupes that were found to play a protective role against breast and colon cancers in the Mediterranean region [16]. In addition, an iridoid glucoside, theviridoside, was isolated from methanolic extracts of *L. scaberrima* [17]. This compound is closely related to geniposide, a well-known anti-inflammatory agent present in many Chinese herbal remedies [18].

The aim of our investigation was to evaluate the antifungal activities of polar leaf extracts of four *Lippia* species, as well as the activities of purified secondary metabolites present in these extracts (theviridoside, verbascoside and isoverbascoside), against a Guazatine®-resistant strain of *P. digitatum*.

2. Materials and methods

2.1. Plant materials

Aerial parts of individual specimens of *L. javanica*, *L. scaberrima*, *L. rehmannii*

[1] According to *A century of citrus,* (2007), http://www.cga.co.za/site/files/5438/FPJ%20article%200n%20Century%20of%20Citrus.pdf, accessed 2008/10/07.

and *L. wilmsii* were harvested from the wild, and voucher specimens were deposited in the herbarium (Bosman&Combrinck 15, 16, 21 and 31) of the South African National Biodiversity Institute (Pretoria, RSA). Harvested material was air-dried indoors at ambient temperature (25 °C) for 5 d prior to extraction.

2.2. Pathogen isolation

A Guazatine®-resistant strain of *P. digitatum* was isolated from symptomatic Valencia oranges (*C. sinensis* L.) obtained from Fort Beaufort Packhouse (Eastern Cape, RSA), purified and preserved on Malt Extract Agar (MEA; Oxoid, Johannesburg, RSA) at 24 °C. The resistance was confirmed by *in vitro* exposure to different concentrations of Guazatine®. Spores were harvested after 8 d and suspensions [(10^2 and 10^4) spores·mL^{-1}] were prepared by adding sterile ¼-strength Ringer's solution (Oxoid, Johannesburg, RSA) to colonised petri dishes.

2.3. Plant extracts

Chloroform (analytical grade) (3 × 20 mL portions) was used to remove undesired low polarity compounds such as leaf waxes from the ground leaves (5.00 g) by shaking (Labcon platform shaker, Laboratory Marketing Services CC, Maraisburg, RSA) at 200 rpm for 30 min. After filtering (Whatman No. 4 filter paper), the chloroform extracts were discarded. Aqueous methanol [80% (v/v); 3 × 20 mL] was used to extract polar secondary metabolites from the solid residue remaining after filtration. The methanolic extracts were combined, concentrated using a Büchi rotary evaporator at 40 °C and subsequently diluted with sterile deionised water to 50.0 mL. The concentrations of the extracts were determined using the method described by Eloff [19].

2.4. Quantification of compounds in *Lippia* leaves

Analytical standards of verbascoside and isoverbascoside were purchased from ChromaDex Inc. (S. Daimler St., Santa Ana, United States). Theviridoside (4a-hydroxygeniposide) was previously purified in our laboratory [17]. Verbascoside, isoverbascoside and theviridoside contents in leaf samples of the four *Lippia* species were determined in triplicate, using high-performance liquid chromatography (HPLC). The HPLC system consisted of a Varian ProStar pump (model 230) coupled to an ultraviolet/visible detector (model 310), set at 220 nm. A Star Chromatography Workstation was used for data analyses. Manual injection (20 µL) was done. The analytical column was a Varian C18 reversed-phase column (250 mm × 4.6 mm; 5 µm particle diameter) held at room temperature. Gradient elution using acetonitrile (HPLC grade; Ultrafine Limited, Rochelle Chemicals, Johannesburg, RSA) and MilliQ® water was applied at a flow rate of 1 mL·min^{-1}. Initially, the mobile phase consisted of 10% (v/v) aqueous acetonitrile, held for 10 min, increasing to 100% (v/v) acetonitrile over 35 min. Five calibration standards, with concentrations ranging from (10 to 100) mg·L^{-1}, were prepared for each of the pure compounds. Leaf extracts were diluted ten times using methanol (HPLC grade, BDH VWR International Ltd., Poole, England). All standards and extracts were filtered through 0.45-µm nylon Millipore filters (Microsep, Johannesburg, RSA) prior to analysis.

2.5. *In vitro* experiments

Extracts and pure compounds were serially diluted [(0.2, 0.4, 0.6, 0.8 and 1.0) mg·mL^{-1}] using autoclaved 1% (v/v) aqueous methanol. The latter solvent was used as control. An *in vitro* antifungal assay was conducted according to the method proposed by Rojas *et al.* [20], with slight modifications. An aliquot of 50 µL of *P. digitatum* spore suspension (10^4 spores spores·µL^{-1}) was streak-inoculated, using a Hamilton micropipette, onto the surface of petri dishes containing MEA medium supplemented with chloramphenicol (250 mg), to prevent bacterial contamination. The petri dishes were left undisturbed at room temperature (25 °C) in the dark for 5 h to encourage spore germination. Dilutions of each compound and extract were subsequently applied as 10-µL volumes, approximately 3 cm from the

centre of the plate. Four replicates were prepared for each of the five dilutions of either compound or extract. The petri dishes were then incubated at 80–90% humidity and 24 °C for 5 d. Following incubation, the antifungal activities of the compounds and extracts were determined by measuring the diameters of the inhibition zones. Analysis of variance (ANOVA) amongst averages was performed using one-way ANOVA (single factor, and two factors without replication). After applying the least significant difference (LSD) test, differences of $P \leq 0.05$ were considered significant.

2.6. *In vivo* trials

Fresh untreated 'Valencia' oranges were obtained from Crocodile Valley Packhouse (Nelspruit, RSA). Fruit were surface-sterilised by immersion in 70% (v/v) ethanol for 1 min and prepared for inoculation. The curative activities of the test substances were evaluated by wound inoculation using the checkerboard method as described by Korsten [21]. Waterproof ink markers were used to draw (1 × 1) cm squares (three columns and four rows) about 4 mm apart on the rind. A 1-mm diameter needle was used to wound (approximately 3 mm deep in the fruit rind) the oranges at the centre of each square. In the first column, which served as a negative control, a 5-µL aliquot of autoclaved distilled water was applied to each square. A spore suspension (5 µL) of 10^2 spores per mL was applied to the wound of each square in the second column. The same volume of a spore dilution of 10^4 spores per mL was also applied to each square in the third column. Oranges were then incubated at between 80–90% relative humidity and room temperature for 24 h. Following incubation, a 5-µL aliquot of 1% aqueous MeOH was applied to the wound of each square in the first row to represent the curative control. Three dilutions [(0.2, 0.6 and 1.0) $mg \cdot mL^{-1}$] of each test substance in autoclaved 1% aqueous MeOH were then individually applied as 5-µL aliquots across rows 2 to 4 on the injured rind. In this way, the *Penicillium* inoculum pressure increased from left to right, while the concentrations of the test substances increased from top to bottom. Inhibition against fungal growth was monitored after 7 d of incubation at 25 °C and 80–90% relative humidity. The degree of inhibition in each square was evaluated as follows: 'no growth' indicates that no infection was observed, 'no inhibition' corresponds to visible mycelial growth; 'moderate inhibition' implies that some decay was visible, but no mycelial growth could be observed; while 'high inhibition' indicates no decay and that mycelial growth was absent.

3. Results and discussion

3.1. Quantification of compounds in *Lippia* leaves

Theviridoside was only present in *L. scaberrima* leaves at a concentration of 0.45 $mg \cdot g^{-1}$. *Lippia javanica* was found to produce the most verbacoside (1.5 $mg \cdot g^{-1}$), followed by *L. wilmsii* (1.2 $mg \cdot g^{-1}$) and *L. rehmannii* (0.83 $mg \cdot g^{-1}$). *Lippia scaberrima* leaves contained the lowest concentrations of both verbacoside (0.63 $mg \cdot g^{-1}$) and isoverbascoside (0.074 $mg \cdot g^{-1}$). The isoverbascoside contents of the other three species were similar, ranging from (0.13 to 0.18) $mg \cdot g^{-1}$. According to Frum *et al.* [22], verbascoside has been identified in many species of the genera *Oxera* and *Faraday* in variable quantities. It has been isolated from *Lantana camara* (Verbenaceae) [14], which is closely related to the genus *Lippia*.

3.2. *In vitro* antifungal activities of the isolated compounds and extracts against *P. digitatum*

Statistical analysis using one-way ANOVA showed significant differences between diameters of inhibition for each concentration of the test samples. All the compounds and extracts displayed concentration-dependent activities against *P. digitatum* (*figure 1*). The activities of the compounds and extracts were negligible at low concentrations.

Verbascoside was found to be the most active compound at concentrations above 0.6 mg·mL^{-1}. The *L. rehmannii* extract exhibited the highest antifungal activities at the two lowest concentrations. However, at the highest concentration tested (1.0 mg·mL^{-1}), the activity of this extract was found to be comparable to that of *L. javanica* and *L. wilmsii*. Theviridoside and isoverbascoside were the least potent since they exhibited the lowest relative activities, even at high concentrations (1.0 mg·mL^{-1}). In addition, their activities were even lower than those of the *Lippia* extracts. The activities of the leaf extracts corresponded to their verbascoside content, suggesting that this compound could possibly contribute to the antifungal action of the extracts.

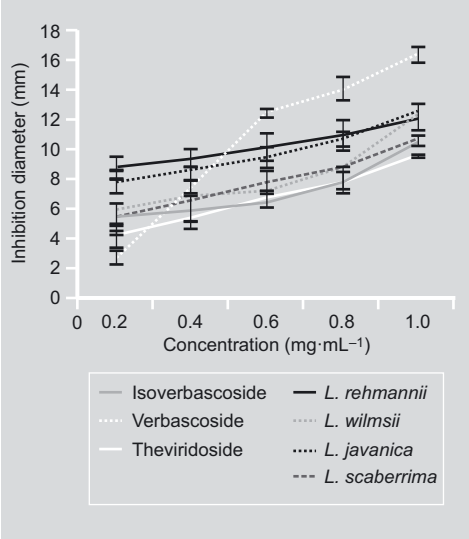

Figure 1.
In vitro inhibition of *Penicillium digitatum* by *Lippia* extracts and purified compounds. Bars represent ± standard deviation from the means (n = 4).

3.3. *In vivo* activities of the isolated compounds and extracts against *P. digitatum* (curative application)

Verbascoside and the *L. javanica* extract showed the strongest activities against *P. digitatum* at concentrations above 0.6 mg·mL^{-1}, with the former appearing to be slightly more active at both inoculum loads (*table I*, *figure 2*). Verbascoside is well known for its antimicrobial properties and has been found to inhibit viruses, bacteria and fungi [23]. With the exception of verbascoside, all the compounds and extracts were ineffective at concentrations below 0.6 mg·mL^{-1}. Of the four extracts tested, once again, *L. scaberrima* exhibited the lowest inhibition of fungal growth, which corresponds to the low verbascoside content of this species.

In our study, the fruits were artificially inoculated with the pathogens at an inoculum concentration as high as 10^4 spores·mL^{-1}. This is not a true reflection of natural conditions, where the spore load would be substantially lower. Verbascoside and the plant extracts would therefore be expected to exhibit stronger inhibition in the packhouse environment against *Penicillium* rot. The *in vivo* and *in vitro* results obtained followed the same pattern.

4. Conclusions

The activities of the polar extracts against a Guazatine®-resistant strain of *P. digitatum* are possibly linked to the presence of high concentrations of verbascoside, since this compound was observed to be the strongest fungal inhibitor. This is the first report on the potential application of polar *Lippia* extracts for the control of postharvest pathogens, but the results must be confirmed by a semi-commercial trial. The use of these polar extracts could provide safer and more acceptable alternatives to synthetic fungicides. Further investigations should focus on the identification of chemotypes or even other genera that produce high concentrations of verbascoside. Furthermore, extraction methods leading to enrichment of the compound in the extract should be investigated.

Acknowledgements

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Table I.
Effect of *Lippia* sp. extracts and purified compounds on the *in vitro Penicillium digitatum* mycelial growth observed 7 days after inoculation in a Malt Extract Agar medium. For no spores of *P. digitatum*·mL^{-1} (control), no growth of the fungus was observed with any plant extracts.

Compound or plant extract tested	Extract concentration (mg·mL–1)	*P. digitatum* (10^2 spores·mL^{-1})	*P. digitatum* (10^4 spores·mL^{-1})
Theviridoside	0.2	No inhibition	No inhibition
	0.6	No inhibition	No inhibition
	1.0	No inhibition	Moderate inhibition
Verbascoside	0.2	No inhibition	Moderate inhibition
	0.6	High inhibition	High inhibition
	1.0	High inhibition	High inhibition
Isoverbascoside	0.2	No inhibition	No inhibition
	0.6	No inhibition	No inhibition
	1.0	Moderate inhibition	High inhibition
L. javanica	0.2	No inhibition	No inhibition
	0.6	Moderate inhibition	Moderate inhibition
	1.0	High inhibition	High inhibition
L. scaberrima	0.2	No inhibition	No inhibition
	0.6	No inhibition	No inhibition
	1.0	Moderate inhibition	Moderate inhibition
L. rehmannii	0.2	No inhibition	No inhibition
	0.6	Moderate inhibition	Moderate inhibition
	1.0	Moderate inhibition	Moderate inhibition
L. wilmsii	0.2	No inhibition	No inhibition
	0.6	Moderate inhibition	Moderate inhibition
	1.0	Moderate inhibition	Moderate inhibition

Figure 2.
Checkerboard method of wound-inoculated 'Valencia' orange treated with increasing concentrations (top to bottom) of *Lippia javanica* extract and increased inoculum load (left to right).

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Uso de extractos polares de *Lippia* para el control pos-cosecha de una cepa de *Penicillium digitatum* resistente a la Guazatine® en naranjo.

Resumen — Introducción. *Penicillium digitatum* es un patógeno de importancia comercial para el género *Citrus*, debido a que es responsable de pérdidas significativas. La aparición de nuevas cepas resistentes a la mayoría de fungicidas habitualmente empleados es un problema mayor para la industria. El objetivo de nuestro estudio fue de testear las actividades antifungicidas de extractos polares, así como el de ciertos compuestos presentes en cuatro especies de *Lippia* originarios de Sudáfrica, contra una cepa de *Penicillium digitatum* resistente a la Guazatine®. **Material y métodos**. Se realizaron unos tests *in vitro* mediante el empleo del método del medio tóxico, incorporando los compuestos o extractos en agar enriquecido en extracto de malta, en concentraciones que oscilan de (0,2 a 1,0) $mg{\cdot}mL^{-1}$. Se realizó un test *in vivo* (curativo) con naranjas del cultivar Valencia mediante el empleo de la técnica del *checkerboard*. **Resultado y discusión**. Se observó una correlación significativa entre los tests *in vitro* e *in vivo*. La totalidad de los compuestos y extractos permitieron reducir el crecimiento micelial en concentraciones superiores a 0,6 $mg{\cdot}mL^{-1}$. El compuesto verbascoside fue el más activo, mientras que los extractos de *Lippia javanica* y *Lippia rehmannii* solamente mostraron una reducción parcial del crecimiento micelial del patógeno. **Conclusión**. Nuestro estudio estableció que los extractos polares de *Lippia* sp. podrían ser sustancias ecológicas de sustitución, utilizables para el control de *P. digitatum*.

Sudáfrica / *Citrus* / *Penicillium digitatum* / control de enfermedades / control de plagas (postcosecha)/ control biológico / *Lippia* / extractos vegetales