Salmea scandens (Asteraceae) extracts inhibit *Fusarium oxysporum* and *Alternaria solani* in tomato (*Solanum lycopersicum* L.)

Extractos de *Salmea scandens* (Asteraceae) inhiben el crecimiento de *Fusarium oxysporum* y *Alternaria solani,* patógenos del tomate (*Solanum lycopersicum* L.)

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Abstract

Phytopathogenic fungi from *Fusarium* and *Alternaria* genders affect tomato crops, reducing fruit production and quality. Some plant extracts may constitute an alternative for fungal control. In this regard, this research studied the antifungal effect of *Salmea scandens* extracts against *F. oxysporum* and *A. solani.* The functional groups of the chemical compounds involved in fungal control, were identified. Plant extracts were obtained by three techniques (soxhlet, ultrasound assisted and maceration) using three solvents (water, acetone and ethyl ether). Their biological effectiveness was evaluated against *F. oxysporum* in treated medium, and *A. solani* in tomato fruit. while the functional antifungal groups were identified by the Fourier Transform Infrared Spectroscopy (FTIR) technique. The soxhlet technique resulted the best extraction method for *S. scandens*, using the three solvents. Maceration-acetone extracts at concentrations of 4000 and 5000 ppm showed greater antifungal activity against both phytopathogens. The FTIR analysis confirmed the presence of carboxylic acids, aldehydes, ketones and aromatic compounds in *S. scandens* extracts, constituting probable responsible compounds for antifungal activity. *Salmea scandens* extracts resulted an efficient preventive treatment for *F. oxysporum* and *A. solani*.

Keywords
antifungal activity • FTIR spectroscopy • post-harvest fungi • soxhlet • solvents

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Resumen

Hongos de los géneros *Fusarium* y *Alternaria* afectan significativamente el cultivo de tomate, reduciendo la producción y calidad de los frutos. Los extractos vegetales son una alternativa para el control de hongos fitopatógenos, por lo que, en este trabajo, se evaluó el efecto antifúngico de los extractos de *Salmea scandens* contra *F. oxysporum* y *A. solani* y se identificaron los grupos funcionales de los compuestos químicos. Los extractos se obtuvieron por tres técnicas (soxhlet, ultrasonido asistido y maceración) utilizando tres solventes (agua, acetona y éter etílico). La efectividad biológica de estos extractos, se evaluó contra *F. oxysporum* en medio PDA suplementado con el extracto y *A. solani* en frutos de tomate. Asimismo, los distintos grupos funcionales se identificaron por la técnica de Espectroscopía Infrarroja con Transformación de Fourier (FTIR por sus siglas en inglés). La técnica soxhlet resultó el mejor método de extracción para *S. scandens,* utilizando los tres disolventes. Los extractos de maceración-acetona a concentraciones de 4000 y 5000 ppm, presentaron mayor actividad antifúngica contra ambos fitopatógenos. El análisis de FTIR confirmó la presencia de ácidos carboxílicos, aldehídos, cetonas y compuestos aromáticos en los extractos como los probables responsables de la actividad antifúngica. Se concluye que los extractos de *S. scandens* podrían ser utilizados como tratamiento preventivo en el control de enfermedades provocadas por *F. oxysporum* y *A. solani.*

Palabras clave
actividad antifúngica • espectroscopía FTIR • hongos de poscosecha • soxhlet • disolventes

Introduction

Tomato (*Solanum lycopersicum* L.) is one important vegetable crop worldwide, with high nutritional and industrial value, being Mexico one of the main producing countries (23). This crop is affected by various diseases caused, mainly, by fungal pathogens of the genera *Fusarium* (Link)*, Rhizoctonia* (Kuhn), *Phytophthora (*de Bary), *Pythium* (Pringsheim) and *Alternaria* (Nees) (16, 38). *Fusarium oxysporum* (Schltdl) causes vascular wilt disease (9, 30, 36), decreasing yields up to 60 % (3), while, *Alternaria solani* (Cooke) infects ripe fruit, decreasing its commercial value (18).

Plant extracts constitute an important alternative for fungal control. Their high content of secondary metabolites (flavonoids, phenols, terpenes, alkaloids, saponins, polypeptides, among others) inhibit fungal growth and activate plant defense system (40). The Asteraceae family is widely used against fungal pathogens due to its demonstrated anti-phytopathogenic effects. *Flourensia cernua* DC. inhibited *Alternaria alternata* (Keissl), *Penicillum digitatum* (Pers.) and *Colletrotichum gloeosporioides* (Penz) by 91% (21). *Xanthium strumarium* L. inhibited *A. alternata* (5), and *Artemisia annua* L. leaves showed antifungal effects against *F. oxysporum* and *F. solani* (Mart.), presumably due to leaf organic compounds such as camphor, camphene, β-caryophyllene and germacrene D (31). On the other hand, the fungicide effect of garlic plants (*Allium sativum* L.), neem seeds (*Azadirachta indica* A. Juss.), lemon grass (*Cymbopogon proxims* Stapf.), cumin (*Carum carvi* L.) and clove (*Eugenia caryophyllus* Spreng.), against *F. oxysporum* has also been documented (2). The inhibition of *Colletotrichum musae* (Berk and Curtis) Arx growth by *Acacia albida* Delile A. Chev. and *Prosopis juliflora* (Sw.) DC, has been also demonstrated (6), *Aloe vera* L., *A. sativum* L., *Capsicum annuum* L. and *Ginkgo biloba* L. present antifungal activity against *Chalara paradoxa* and *Fusarium guttiforme* (41), while *Annona squamosa* L. extract successfully inhibits growth of *A. alternata*, *Candida albicans* (C.P.Robin), *F. solani*, *Microsporum canis* (Bodin) and *Aspergillus niger* (P.E.L. Van Tieghem) (26).

"Chilemecate", *Salmea scandens* L. is a neotropical plant, with edible leaves widely consumed in Oaxaca, Mexico (48). However, its antimicrobial properties have been slightly studied and the antimicrobial activity of its essential oils has been only documented against *Pseudomonas syringae*, *Clavibacter michiganensis*, *Erwinia carotovora*, *F. oxysporum* and *Phytophthora infestans*, and attributed to its high levels of D-germacrene and elemol (48).

This project aimed to evaluate the antifungal effect of *Salmea scandens* extracts against *Fusarium oxysporum* and *Alternaria solani,* identifying the functional groups of the chemical compounds. The hypothesis stated that the obtained extracts inhibited *in vitro* growth of *F. oxysporum* and *A. solani*.

Materials and methods

Collection and identification of botanical material

Plant tissues were collected in the municipality of Villa Corzo, Chiapas, Mexico, from January to March 2018, coordinates 16° 5' 21" N; 93° 19' 9" W. Plant identification followed known taxonomic keys (17, 30, 32). The identified specimen was registered under number 53106 and deposited in the CHIP Herbarium of the Ministry of the Environment, Housing and Natural History, located in Tuxtla Gutiérrez, Chiapas.

Extraction of plant compounds

A two kg sample of *S. scandens* stems was firstly washed with five liters of tap water, followed by five liters of 2 % NaClO solution for one minute, and three final washes with five liters of sterile distilled water (SDW) for three minutes, respectively. The tissue was dried at 40 °C for 24 h in a hybridization oven (FinePCR Combi-SV12DX, USA), pulverized in an M20 universal mill, IKA, (Staufen, Germany), sieved into a 20 mesh (0.84 mm) and stored in hermetically sealed ziploc plastic bags (Monterrey, N.L, Mexico) at 25 °C and darkness, until use (29).

The extracts were obtained using three solvents, (water, acetone and ethyl ether), and three extraction techniques (soxhlet, ultrasound-assisted and maceration). For the soxhlet extraction, 40 g of pulverized plant material were mixed with 200 ml of solvent, and refluxed (water 100 °C, acetone 56 °C and ethyl ether 34.6 °C). All solvent contained in the extracts was evaporated in a drying oven at 40 °C (20). For ultrasound-assisted extraction (UAE), 40 g of sample were mixed with 200 ml of solvent and incubated in an ultrasound bath at 20 kHz, for 45 min. The extract was filtered (Super Premium #2 filter paper, Melitta brand, USA) and concentrated in a drying oven (FinePCR Combi-SV12DX, USA) at 40 °C (12, 24). For maceration, pulverized plant material was mixed in a 1:4 ratio, with each solvent and incubated at 25 °C, for 24 h and constant shaking (200 rpm). The solvent was filtered and removed in a drying oven at 40 °C (12, 24). The obtained extracts were stored in Pyrex (USA) amber vials at 4 °C until use (34). All the extractions were made in triplicate.

In vitro fungistatic activity of *S. scandens* extracts against *F. oxysporum*

Fungal conidia were obtained from the ceparium of the Laboratory of Plant Pathology, Biological Control and Post-harvest of the Research Center for Food Development (CIAD., A.C., Cd. Cuauhtémoc, Chihuahua, México) and preserved in potato dextrose agar (PDA; BD Difco, USA) medium.

S. scandens extracts were resuspended in dimethyl sulfoxide (DMSO) obtaining a 10 % mass/volume solution (10). Dilutions were prepared at concentrations of 1000, 2000, 3000, 4000 and 5000 ppm using DMS0 as solvent (25), and added to the PDA medium where the final concentrations of DMSO were 0.1, 0.2, 0.3, 0.4 and 0.5 %. Imazalil 44.65 %. CS (Fungaflor 500 EC, Valent, Zapopan, Jalisco, México) at 4 ml/L of water and PDA-DMSO 0.1 - 0.5 %, respectively, were used as controls.

PDA dishes containing the different extract concentrations were inoculated with 10 µl of 1×10^6 conidia ml^{-1} of *F. oxysporum,* resuspended with 0.05 % Tween 60 and incubated at 28 °C. Fungal radial growth was measured every 24 h, until mycelium growth of the control treatment completely covered the PDA dish (34). Inhibition of Radial Growth (IRG), was determined using the formula IRG=[(R1-R2)/R1]×100, where R1 represents control pathogen diameter and R2 is pathogen diameter with *S. scandens* extracts, expressed in millimeters (17). The experiment was conducted in triplicate.

Post-harvest bioassay of *S. scandens* extract against *A. solani*

The *A. solani* strain was obtained from the same laboratory previously mentioned and grown in PDA at 25 °C for 14 d. Conidia were collected with SDW, added with 0.05 % Tween 20, and adjusted to 1×10^6 conidia ml^{-1} (50).

Thirty ripe Saladette tomatoes were used to monitor treatment effects. Fruits were washed with SDW (~5 L) for 2 min and disinfected with 70 % ethanol for 30 s (37). Three longitudinal lesions (~2 mm in diameter and depth) were made on each fruit, later inoculated with 20 µl of *S. scandens* maceration-acetone extract, using the concentration of 5000

ppm, which resulted to be the most effective treatment in the *in vitro* inhibiting of *F. oxysporum*. Thirty percent hymexazol LS (Thachigaren 30 SL, Summit agro, México) and 0.5 % DMSO were used as controls. Subsequently, each lesion was inoculated with 10 µL of *A. solani* spore suspension (1×10^6 spores ml^{-1}). Inoculated fruits were placed in 25×13×10 cm plastic trays (Baquelita brand, México City, México), hermetically sealed, supplemented with moist cotton and incubated under controlled conditions (26 ± 2 °C and 14:10 LD photoperiod) for five days (37). A randomized block design with three replicates per treatment and 10 fruits per replicate, was used. Symptoms incidence caused by *A. solani* was determined as follows: Incidence (%) = (number of damaged fruits/total number of fruits) × 100 (27). Severity was estimated by Alternaria rot injury % = 100 × (Ø of injury on treated fruits/ Ø of untreated fruits), where Ø is diameter (37). Lesion size was determined from five random fruits from each replicate in each treatment according to: Lesion size (mm^2) = π x a/2 × b/2, a: length of the disease spot (mm), b: width of the disease spot (mm) (26). Percentage of fungal inhibition in treatments was determined by: % inhibition = (A-B) /A*100, where A is control pathogen diameter and B is pathogen diameter under treatment (44). The experiment was conducted in triplicate.

Analysis of *S. scandens* extracts by Fourier Transform Infrared Spectroscopy (FTIR)

A spectrophotometer (Spectrum Two, Perkin Elmer, Massachusetts, USA) was used. FTIR spectra were recorded from 400 to-4000 with a resolution of 4 cm^{-1} by 24 scans (14). Finally, a pure extract sample (solid extract obtained with water and oily extract obtained with acetone and ethyl ether solvents) was placed in the cell and covered with the metal cap applying a pressure of 60 Pa. The respective spectra were obtained using attenuated total reflection (ATR). Analyses were conducted in triplicate.

Statistical analysis.

All data obtained were subjected to ANOVA and a Tukey test ($p < 0.05$) using the minitab 18 program (Minitab, State College, PA, USA).

Results

Extraction of plant compounds

The highest mass/volume extraction efficiency yield was 3.94 % with the soxhlet technique, followed by UAE (2.97%) and maceration (2.12%) using water as a solvent (figure 1, page 266).

In vitro* fungistatic activity of *S. scandens* extracts, against *F. oxysporum

Extracts obtained by maceration-acetone technique at 5000, 4000 and 3000 ppm, caused the strongest pathogen inhibition with IRG values of 76.72, 73.32 and 57.77 %, respectively. Soxhlet-acetone extraction obtained, IRG values of 56.86 and 38.6 % at 5000 and 4000 ppm, respectively. The ultrasound-water technique showed low IRG values (11.83 %) obtained at 5000 ppm (figures 2, page 266 and 3, page 267). The extracts inhibited mycelial growth, without affecting conidia production.

Different letters show statistical differences in techniques and solvents (Tukey; $p \leq 0.05$).

Letras diferentes muestran las diferencias estadísticas entre técnicas y solventes (Tukey; $p \leq 0,05$).

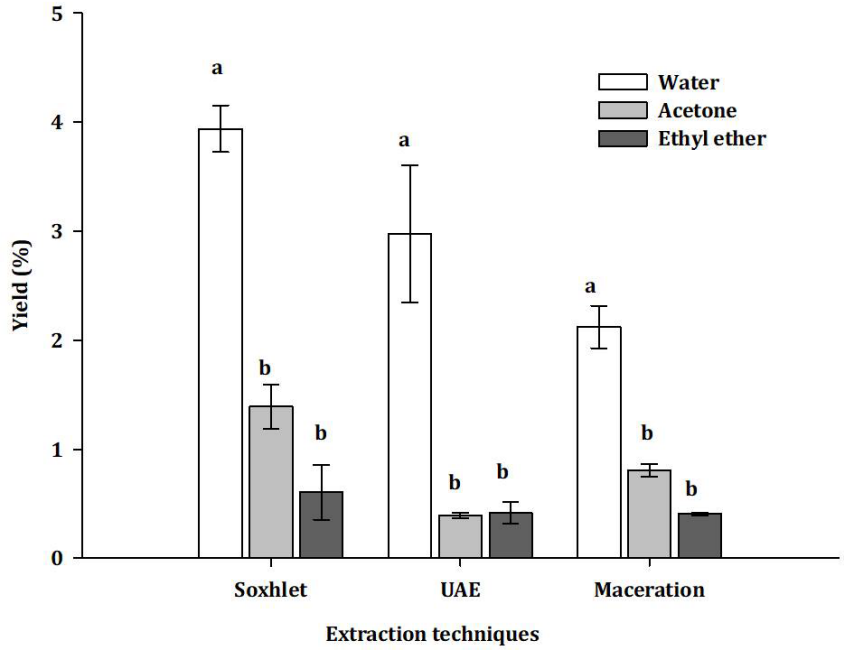


Figure 1. Percentage yield (mass/volume) from *S. scandens* plant extracts obtained by three extraction techniques, soxhlet, UAE and maceration using three solvents (water, acetone and ethyl ether).

Figura 1. Porcentaje de rendimiento (masa/volumen) de los extractos vegetales de *S. scandens* obtenido mediante tres técnicas de extracción soxhlet, ultrasonido asistido (UA) y maceración, usando tres solventes (agua, acetona y éter etílico).

Different letters indicate statistical differences for techniques and solvents (Tukey; $p \leq 0.05$).

Letras diferentes indican las diferencias estadísticas entre técnicas y solventes (Tukey; $p \leq 0,05$).

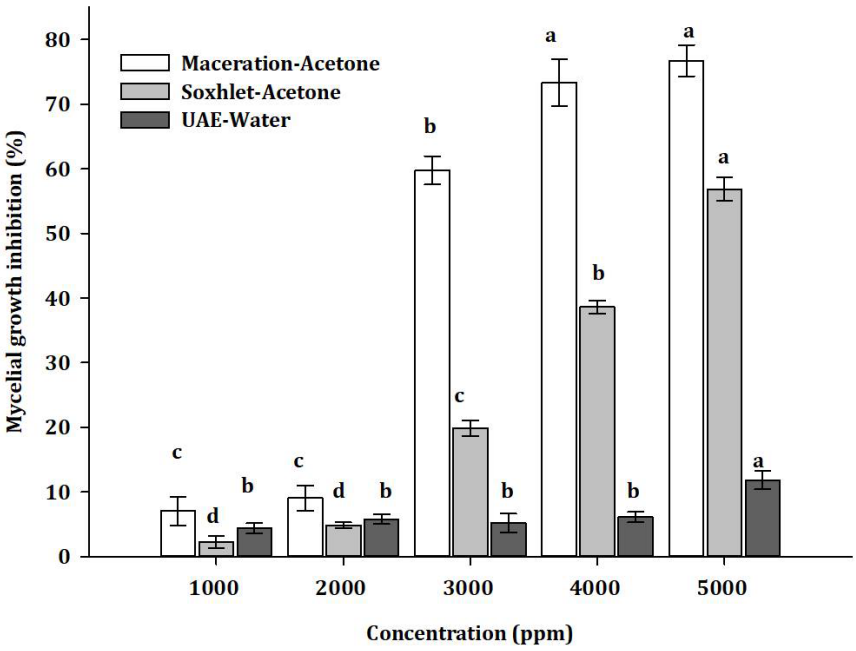


Figure 2. Inhibition of radial growth (IRG) of *F. oxysporum* by different concentrations of *S. scandens* extracts, obtained by different extraction techniques and solvents (maceration-acetone) (soxhlet-acetone) (UAE-water), after 12 days of interaction.

Figura 2. Inhibición del crecimiento radial (ICR) de *F. oxysporum* por diferentes concentraciones de extractos de S. scandens, obtenido mediante diferentes técnicas extracción y solventes (Maceración-acetona) (Soxhlet-acetona) (UAE-Agua) después de 12 días de interacción.

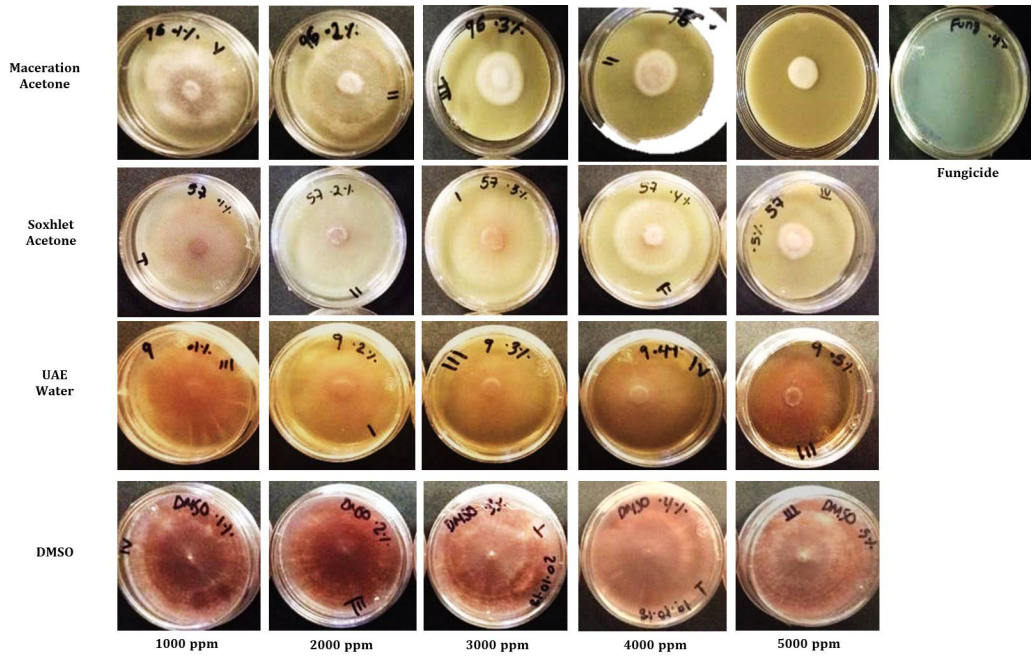


Figure 3. Schematic representation of *in vitro* inhibition of radial growth (IRG) of *F. oxysporum* in PDA medium with increasing concentrations (1000, 2000, 3000, 4000 and 5000 ppm) of *S. scandens* extracts, obtained by maceration-acetone, soxhlet-acetone and UAE-water. DMSO was used as absolute control after 12 days of interaction.

Figura 3. Representación esquemática de la inhibición del crecimiento radial (ICR) *in vitro* de *F. oxysporum* en medio de PDA adicionado con diferentes concentraciones (1000, 2000, 3000, 4000 y 5000 ppm) de extractos de *S. scandens* obtenido mediante Maceración-acetona, Soxhlet-acetona y UAE-agua. El DMSO se utilizó como control absoluto después de 12 días de interacción.

Biological effectiveness of *S. scandens* extract against *Alternaria solani* in post-harvest

In this experiment, the maceration-acetone extract at 5000 ppm was evaluated against *A. solani,* showing comparatively higher inhibition of mycelial growth (>92%) than the commercial fungicide Hymexazol, after five days of interaction (table 1; figure 4A-F, page 268). The extract inhibited mycelial growth, without affecting conidia production.

Identification of functional groups in *S. scandens* extracts by Fourier Transform Infrared Spectroscopy (FTIR*)*

The FTIR analysis showed strong peaks in extracts obtained by the soxhlet and maceration techniques using the acetone solvent. At 2927 cm^{-1} and 2849 cm^{-1}, peaks belonged to the C–H group, whereas peaks at 1734 cm^{-1} and 1656 cm^{-1} belonged to(C=O)and limited amine group (–NH), respectively. Spectrums at 1459cm^{-1}, 1273 cm^{-1} and 1161 cm^{-1} (CO), are characteristic of the vibrational modes of carboxylic groups, while at 1387 cm^{-1} and 1124 cm^{-1}, C=O, hydrolysable tannins have been documented. The extract obtained by water-assisted ultrasound technique showed weak peaks at 2892 cm^{-1}, 1545 cm^{-1}, 1249 cm^{-1} and 1084 cm^{-1} indicating the presence of amides, alkynes, alkanes, carboxylic acids, alkenes and aromatic compounds (figure 5, page 269).

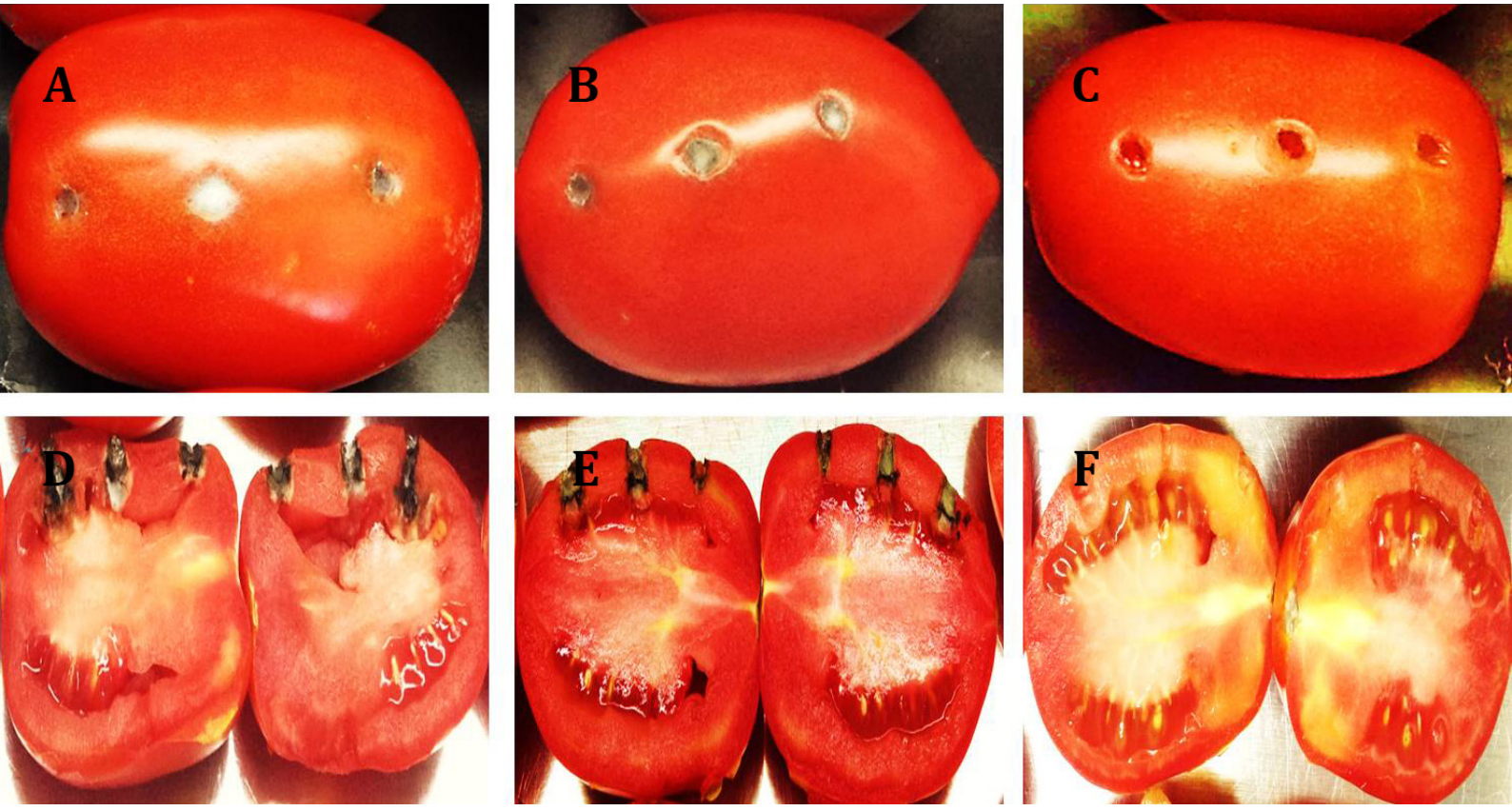
Table 1. Biological effectiveness of *S. scandens* maceration-acetone extract at 5000 ppm against *A. solani* in tomato fruit after five days of interaction.

Tabla 1. Efectividad biológica del extracto de maceración-acetona de *S. scandens* 5000 ppm contra *A. solani* en frutos de tomate después de cinco días de interacción.

Treatments	Injury size (mm^2)	Injury diameter (mm^2)	Severity of injury (%)	Incidence (%)	Inhibition (%)
DMSO + *A. solani* Control (+)	19.74±3.75 a	1.69±0.10 B	100 a	100	0.0 a
Hymexazol + *A. solani* Control (-)	15.39±0.43 a	4.38±0.07 A	94.94±3.79 a	100	5.06±3.71 a
S. scandens extract Maceration-Acetone 5000 ppm	0.91±0.29 b	0.33±0.15 C	7.36±3.71 b	13.33	92.64±3.71 b

Different letters between rows show significant difference (ANOVA, Tukey test, $p<0.05$).

Letras diferentes entre filas muestran diferencia significativa (ANOVA, prueba de Tukey, $p<0,05$).



(A, D). fruits inoculated with DMSO-*A. solani*, (B, E), fruits inoculated with *A. solani*-Himexazol; (C, F), fruits inoculated with *A. solani*-extract of *S. scandens* at 5000 ppm.

(A, D). frutos inoculados con DMSO-*A. solani*, (B, E). frutos inoculados con *A. solani* y el fungicida comercial (Himexazol); (C, F). frutos inoculados con *A. solani* y extracto de *S. scandens* a 5000 ppm.

Figure 4. Schematic representation of the effects of *S. scandens* extract on growth inhibition of *A. solani* in tomato.

Figura 4. Representación esquemática de los efectos del extracto de *S. scandens* sobre la inhibición del crecimiento de *A. solani* en jitomate.

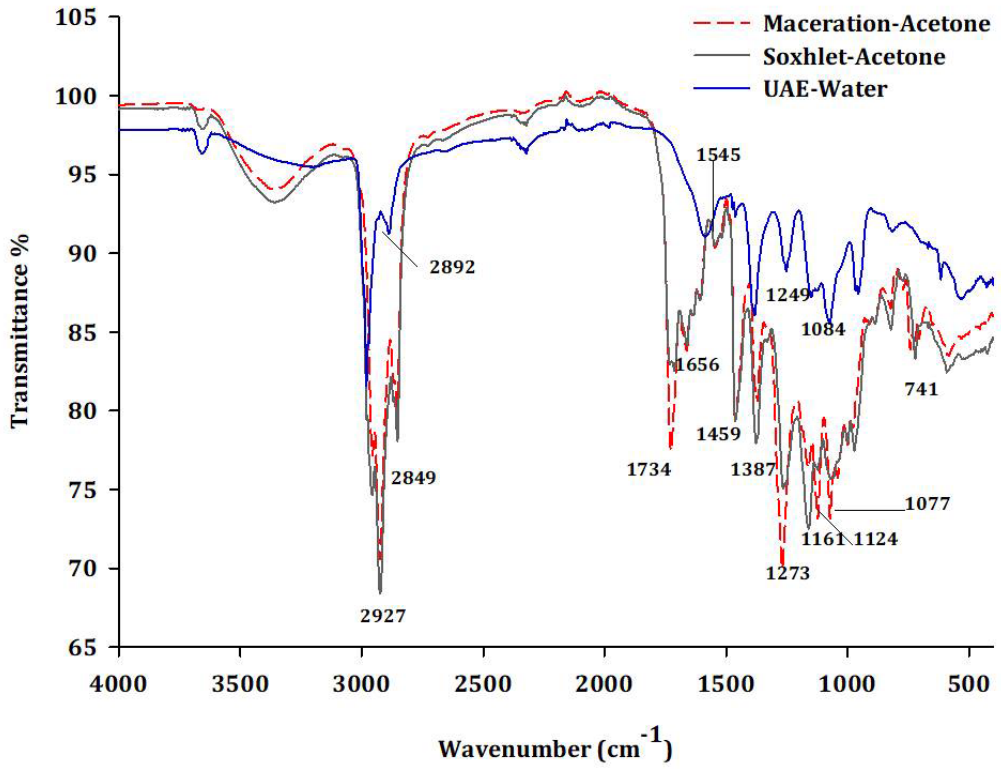


Figure 5. FTIR spectrum of *S. scandens* extracts obtained by the maceration-acetone, soxhlet-acetone and UAE-water techniques.

Figura 5. Espectro FTIR de los extractos de *S. scandens* obtenido mediante las técnicas de Maceración-acetona, Soxhlet-acetona y UAE-Agua.

Discussion

The higher extract yield obtained with the soxhlet technique, might have been given by water low volatility, compared to acetone and ethyl ether (4). In this regard, different yield percentages of *Mentha spicata* L. extracts had been also found by the soxhlet technique using different solvents; methanol (267.33 mg/g), ethanol-water (70:30) (257.66 mg/g) and petroleum ether (30.47 mg/g) (7). Extraction efficiency can also be increased by rising water content, facilitating protein and carbohydrate solubility, (13). Extract yield is affected by several factors like sample preparation, extraction methods and time, boiling temperature, and the specific nature of the bioactive compound (22). In this sense, water and acetone solvents are frequently used for hydrophilic compounds extraction from plants (46). Therefore, polar compounds result easier to extract with polar solvents, leading to the assumption that the most abundant compounds of *S. scandens* are hydrophilic ones (7).

Although maceration and ultrasonic-assisted extraction techniques showed lower yield than soxhlet, they demonstrated interesting attributes such as low operating temperature by using less solvent, no compounds degradation and faster and more selective extraction of target compounds (7). According to our best knowledge, this is the first comparative study of different extraction techniques using different organic and aqueous solvents for the obtention of bioactive compounds from *S. scandens*.

The 76.72 % inhibition of *F. oxysporum* growth could have been due to solvent natural polarity and extraction capacity (29). Acetone extracts of *Cinnamomum burmanni* (Nees & T. Nees) Blume, *Nerium oleander* L. and *Parrotia persica* (DC.) C. A. Mey. inhibited *F. oxysporum* f.sp. *lycopersici* (Sacc.) radial growth by 48.43 %, given their content of antimicrobial metabolites like flavonoids, alkaloids, terpenes, tannins and saponins (42, 43). Likewise, *S. scandens* essential oil is known to inhibit *F. oxysporum* and *P. infestans* growth (48), attributed to high levels of germacrene D (47.1 %) and elemol (15.3 %), and to N-isobutyl-(2E, 4E, 8Z, 10E/Z)-dodecatetraenamide isomers (39.7 %) contained in the stem bark (48).

The use of plant extracts for post-harvest disease control in fruits and vegetables constitutes a sustainable alternative in relation to synthetic fungicides (49). In the present study, the inhibitory effect of acetone-soluble extract of *S. scandens* against *A. solani*, in tomato plants and fruits, is documented for the first time, providing an alternative for protection and control during transport and storage (1). Antifungal plant derived compounds had already shown to be advantageous for *Alternaria* control in cherry tomatoes (47).

Regarding the identification of functional groups, similar results were also obtained with the extract from *Chamaemelum nobile* (L.) identifying several functional groups (C–H, –NH, phenolic groups of tannins, C-O, amines, carboxylic acids, aldehydes and ketones) (15). On the other hand, absorption intensity variation between1743 cm^{-1} (C=O) and 1160 cm^{-1} (CO) are characteristic of the vibrational modes of carboxylic groups (45). In this experiment, close bands of 1734 and 1161 cm^{-1} were obtained, while in the 1722–1702, 1368 and 1157 cm^{-1} peaks, the C=O stretching of esters from hydrolysable tannins were found, especially those derived from gallic acid, since condensed tannins show intense signals of C=C-C stretching between the 1555–1503, 1361-1340 and 1284-1283 cm^{-1} regions (39). In this sense, *S. scandens* extracts showed absorption bands at 1545 cm^{-1}, predicting the presence of hydrolyzed tannins. Stretching of C-C aldehyde (1459 cm^{-1}), CN nitrile (1273 cm^{-1}) and C-C aromatic hydrocarbons (741 cm^{-1}) had previously been found, determining that the stretching vibration of C=O at 1554 cm^{-1} in aldehydes and ketones, indicates the presence of phenolic acid and/or terpenoid (51). Other functional compounds of *Albizia lebbeck* (L) Benth had also been identified, confirming the presence of amides, alkines, alkanes, carboxylic acids, alkenes, aromatic compounds and aliphatic amines, showing main peaks at 2918.44 and 2849.92, 1643.73, 1454.46, 1054.13 and 510.34 cm^{-1}, respectively (8). Finally, other easily identified stretches are the aromatic bonds C=C-C in the region of 1611–1444 cm^{-1} and C-O between 1368–1157 cm^{-1} and 1031–1023 cm^{-1} (11).

Therefore, the FTIR analysis on *S. scandens* indicated the presence of carboxylic groups, hydrolysable tannins, amides, alkenes, alkylic acids, alkenes, aromatic compounds, aliphatic amines and alkyl halides as possible antifungal molecules. Aromatic compounds, monoterpenes, sesquiterpenes, alkylamides and aliphatic and oxygenated hydrocarbons in *S. scandens,* were identified (48). However, few studies about the characterization of the biological activity of the compounds of this plant species, are available. Additional work characterizing the antimicrobial activity of the compounds already reported should be conducted.

Conclusions

Salmea scandens extracts demonstrated strong antifungal effects against *Fusarium oxysporum* and *Alternaria solani.*

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