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Antifungal potential of essential oils, aqueous and ethanol extracts of thirteen plants against *Fusarium oxysporum* f. sp *Lycopersici* and *Phytophtora infestans* (Mont.) de Bary as major tomato pathogens in Cameroon

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Abstract

Antifungal activity of essential oils (EO), ethanol extracts (ETE) and cold water extracts (CWE) of thirteen plants was evaluated against *Fusarium oxysporum* and *Phytophtora infestans* causal agents of tomato Fusarium wilt and late blight diseases respectively. The supplemented media and slide germination techniques were carried out to determine the effect of extracts on the mycelial growth and conidia germination of pathogens. The results showed that essential oils exhibited the highest antifungal activity followed by ETE and CWE. *Callistemon citrinus*, *Cymbopogon citratus* and *Ocimum gratissimum* essential oils were the most active inhibiting completely radial growth and conidia germination of *Phytophtora infestans* at 312.5 and 625 µg/ml. Essential oils of *Ocimum gratissimum* and *Cymbopogon citratus* inhibited totally the radial growth and conidia germination of *Fusarium oxysporum* at 625 and 312.5 µg/ml respectively. ETE of *Ageratum conyzoides* and *Callistemon citrinus* were the most active inhibiting radial growth of *Phytophtora infestans*. *Cymbopogon citratus* and *Ocimum gratissimum* were the most active against radial growth and conidia germination of *Fusarium oxysporum* at 6250 µg/ml. The fungi toxic potential of some extracts was comparable to synthetic fungicides used as positive controls. Preliminary phytochemical analysis of ETE and CWE revealed that stronger inhibiting effects were recorded with extracts rich in phenols, flavonoids, tannins, and coumarins. These findings may contribute to develop new green fungicides to protect tomato from Fusarium wilt and late blight diseases.

Keywords: antifungal; Fusarium wilt; late blight; tomato; green fungicides

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Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop in the world after potato with a global production of about 164 million tons (t) of fresh fruit harvested on a 4.7 million hectares (ha) surface in 2013 (FAO, 2015). Tomato is known as a protective food because of its special nutritive value, it provides a major source of minerals and vitamins. This

vegetable has been recently gaining attention in relation to the prevention of some human diseases. This interest is due to the presence of carotenoids and particularly lycopene that appears to be an active compound in the prevention of cancer, cardiovascular risk and in slowing down cellular aging (Abdel-Monaim, 2012). In Cameroon, tomato is cultivated in almost all the agro-ecological zones and its production is about 954384 tons harvested on 69903 hectares (FAO, 2015) however, yields are still very low due to diseases. *Fusarium oxysporum* f. sp *lycopersici* and *Phytophtora infestans* are plant pathogenic fungi causing tomato fusarium wilt and late blight diseases respectively, leading to serious economic loss. *F. oxysporum* f. sp *lycopersici* colonizes the xylem of the host plant and as a result, blockage and breakdown of the xylem leads to wilt disease symptoms such as leaf wilting, yellowing and eventually the death of the plant (Agrios, 1997).

P. infestans attacks foliage and fruits in all the life stages of the plant. Due to the fact that diseases caused by plant pathogenic fungi significantly contribute to the overall loss in crop yield worldwide (Montesinos, 2007), various control measures have been practiced but the control of tomato Fusarium wilt and late blight has been almost exclusively based on application of synthetic fungicides. However, they are not considered as a long-term solution, due to the concerns of expense, exposure risks, the hazards of its residues and threats to human health and environment (Paster and Bullerman, 1988). Moreover, the development of resistance of pathogenic fungi towards synthetic pesticides is a great problem that can affect significantly the efficacy of chemical fungicides (Lumsden and Locke, 1989). The diseases control of crops through the use of plant-based biopesticides and antagonistic microorganisms has become an interesting alternative (Olanya and Larkin, 2006). In Cameroon, local plant extracts have been reported as having inhibitory properties *in vitro* and *in vivo* against various pathogens (Goufo et al., 2010; Djeugap et al., 2011; Galani et al, 2013; Nguefack et al., 2013). Botanicals with antifungal compounds have been identified and can be exploited for the management of plants diseases because they have low mammalian toxicity, target specificity, biodegradability and contain many active ingredients (Kagale et al., 2004).

Plants extracts and essential oils have been tested against *F. oxysporum* and *P. infestans* for the inhibitory effect (Goufo et al., 2010; Galani et al., 2013; Neela et al., 2014; Ramaiah et al., 2015). In most of these *in vitro* studies, the antifungal potential of plants extracts against *F. oxysporum* and *P. infestans* was assessed through their ability to inhibit fungal mycelial growth, their effect on fungal conidia germination is yet to be investigated. Moreover, solvent extracts content in antimicrobial secondary metabolites are to be determined. Therefore, this study investigated the efficacy of essential oils and solvent extracts obtained from thirteen Cameroonian plants on condia germination and mycelial growth of *Fusarium oxysporum* f. sp *lycopersici* and *Phytophtora infestans* under laboratory conditions. Preliminary phytochemical analysis of aqueous and ethanol extracts was performed to determine the group of compounds responsible for antifungal activity recorded against the two pathogens.

Materials and Methods

Plant pathogenic fungi

The strains of *Phytophtora infestans* (Mont.) de Bary and *Fusarium Oxysporum* f. sp *lycopersici* Snyder & Hansen were isolated from tomato fruits, roots and stem obtained from the field and showing symptoms, identified at the Phytopathology Laboratories of the Institute of

Agricultural Research for Development, Nkolbisson (Cameroon) and Institute of Rural Development, Bobo-Dioulasso (Burkina Faso). Identification was based on Agarwal et al. (1989) and Mathur and Kongsdal (2003) manuals. Cultures of a single isolate of *P. infestans* were maintained on V8 agar medium amended with 50 ppm ampicillin, 50 ppm rifamycin and 0.05 g/l β-sitosterol, in 90 mm diameter Petri dishes at 20 ± 2°C in darkness. *F. oxysporum* strain was maintained on Potato Dextrose Agar (PDA) medium. Cultures were allowed to grow for 7 to 10 days to ensure conidia formation, transferred periodically to maintain active growth and stored at 5°C in the dark in tube containing PDA. Cultures aged 21 days were used for antifungal tests.

Preparation of conidia suspension and test samples

The conidia suspension of *P. infestans* and *F. oxysporum* were obtained from 21 days old cultures. Approximately 10-15 ml of sterile distilled water was added to the culture media in the Petri dishes. A sterile glass rod was used to scrape the surface of the culture media to dislodge the conidia. The conidia suspension was adjusted to 5×10^5 conidia/ml with a Malassez cell.

Plant materials

Thirteen Cameroonian plants used in this study were selected based on the knowledge of their ethnobotanical uses and their previously demonstrated antimicrobial activities. They are *Callistemon citrinus*, *Cymbopogon citratus*, *Ocimum gratissimum*, *Eucalyptus tereticornis*, *Oxalis barrelieri*, *Ageratum conyzoides*, *Bidens pilosa*, *Tephrosia vogelii*, *Podocarpus milanjianus*, *Emilia coccinea*, *Euphorbia hirta*, *Commelina benghalensis* and *Erigeron floribondus* collected at Yaoundé, Cameroon in August 2014, identified by the Cameroon National Herbarium in Yaoundé. The collected plant parts were air-dried at room temperature (25-27°C) for 10 to 14 days.

Preparation of solvent extracts

Shade-dried plant material of each species was coarsely powdered in a blender and then 100 g of powder was first defatted by mixing with 600 mL of hexane for 24 hours. After filtration, the residue was spread for complete evaporation of the solvent. Lipid-free powder was then soaked and frequently stirred in 600 mL of cold distilled water or 70% ethanol for 12 hrs followed by filtration through a double folded cheese cloth. The filtrate was conserved while the residue was dissolved into 600 ml of cold distilled water or 70% ethanol for another 12 hours period. The resulting filtrates were passed through Whatman no 1 filter paper. The filtrates were subsequently subjected to centrifugation at 7000 rpm for 10 mins. Ethanol was totally evaporated from the ethanol extract using a rotary evaporator (Buchi). All supernatants were freeze dried using a freeze dryer lyophilizer (Millrock Technology Epic Series) and the yields of cold water and ethanol extracts were calculated as percent of dried plant material weight (% w/w). Powders of cold water extracts and ethanol extracts obtained were preserved in a deep freezer (-20°C) into airtight plastic caps until further use.

Extraction of essential oils: The essential oils were extracted from dried plant material by hydrodistillation for five hours using a Clevenger-type apparatus as recommended by Amvam et al. (1998). Oil collected was dried on anhydrous sodium sulphate (Na_2SO_4) column and preserved at approximately 4°C into airtight brown bottles. Yields of the oils were calculated as percent of dried plant material weight (% w/w). Essential oils from plants with higher yields (≥0.7% w/w) were used for antifungal tests.

Synthetic fungicides

Chemicals used bought in Mfoundi (Yaoundé) market included Banko Plus®, fungicide titrating 550 g/l chlorotalonil and 100 g/l of carbendanzime; Plantizeb® 80WP, fungicide containing 80% mancozeb; and Kocide® 2000 titrating 53,8% copper hydroxide. They are among the most used synthetic fungicides by Cameroonian farmers against late blight and Fusarium wilt management.

Effect of essential oils and solvent extracts on radial growth of the pathogens

Inhibitory effect of extracts and synthetic fungicides on mycelial growth of the pathogens grown on V8 agar and PDA media for *P. infestans* and *F. oxysporum*, respectively, was evaluated using the supplemented media technique as described by Benjilali et al. (1986). Essential oils and synthetic fungicides were added to media at concentrations ranging between 39.0625 and 10000 µg/ml representing 256 fold dilution and initial concentrations, respectively. The solvent extracts have been tested at concentrations ranging from 195.313 to 50000 µg/ml representing 256 fold dilution and initial concentrations, respectively. Sterile double distilled water was used as negative control. Each concentration was repeated five times and Petri dishes were sealed with parafilm paper and incubated in inverted position at 20±2°C in darkness for 14 days. The diameter of mycelial growth of pathogens was recorded after 14 days and results expressed as percentage of mycelial growth inhibition (% I) calculated according to the formula of Pandey et al. (1982): % I = (growth diameter in the control - growth diameter in the treatment sample) x 100 / growth diameter in the control.

Effect of essential oils and solvent extracts on conidia germination of the pathogens: The evaluation of the antifungal activity was carried out using a slide germination test of conidia as described by Ho et al. (2007). The prepared raw plant extracts and synthetic fungicides at 10000 and 50000 µg/ml were used to carry out the two fold serial dilutions in Eppendorf tubes. In each Eppendorf tube, an equivalent volume of conidia suspension of both pathogens at 5×10^5 conidia/ml and plant extract was added. After homogenization, the mixture was deposited into the depression of a cavity slide with a micropipette and covered with a cover slide. The content of each tube was distributed into five slides. Essential oils and synthetic fungicides were added to media at concentrations ranging between 39.0625 and 10000 µg/ml representing 256 fold dilution and first concentrations respectively, while the solvent extracts have been tested at concentrations ranging from 195.313 to 50000 µg/ml representing 256 fold dilution and first concentrations respectively. These slides were then incubated under humid conditions for 24 hrs at room temperature (25-27°C). A photonic microscope was then used to determine the percentage of germination of the conidia by counting germinated and non-germinated conidia with respect of at least 100 conidia per slide. The smallest extract concentration which led to total inhibition of conidia germination enabled the determination of the minimum inhibitory concentration (MIC).

Determination of the nature of inhibition

Fungal discs from plates in which no colony growth occurred 14 days after incubation, were further checked to detect the fungicidal or fungistatic nature of the inhibition. The discs were re-inoculated onto fresh V8 agar or PDA media and fungal growth was observed during 30 days. The inhibition was qualified as fungistatic if renewed mycelial growth was observed or fungicidal if no mycelial growth was observed.

The supplemented conidial suspensions incubated for 24 hrs without any conidia germination were deposited in new slides containing sterile distilled water and fungal conidia germination observed 24 hrs later. The inhibition was qualified fungistatic if renewed conidia germination was observed or fungicidal if no conidium germinated.

Phytochemical analysis

Phytochemical tests for major secondary metabolites of the solvent extracts were performed. Plant extracts were screened for the presence of biologically active compounds such as alkaloids, anthocyanins and cardiac glycosides (Odebiyi and Sofowora, 1978); phenols and flavonoids (Harbone, 1976); triterpenes and sterols, (Schoppe, 1964), saponins (Wall et al., 1952); anthraquinones, hydrolysable and condensed tannins (Trease and Evans, 1989) and coumarins (Kovac-Besović and Durić, 2003). Based on the intensity of coloration or the precipitate formed during the test, secondary metabolites proportion was characterized as strongly present (+++), present (++), weakly present (+) and absent (-) when the test result was negative.

Statistical analysis

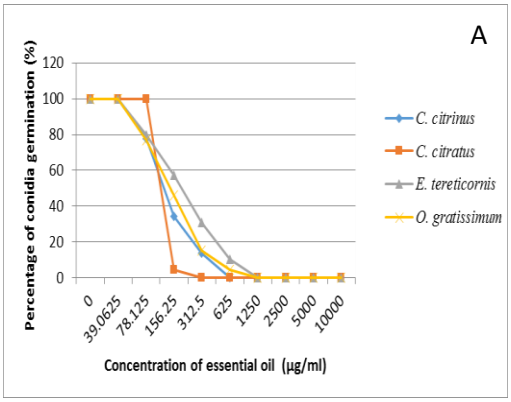
Experiments were set in a Completely Randomized Design with five replications. Data were worked out using Statistical Package for Social Science (SPSS) version 22.1 software by Analysis of Variance (ANOVA) paired to t-test of Student-Newman-Keuls (parametric) and differences among the means were determined for significance at $p<0.05$.

Results

Characteristics of essential oils and solvent extracts

Characteristics of essential oils and solvent extracts vary from one plant species to another and depend on the solvent used and the extraction method. The highest yields were obtained from cold water extracts and the lowest from essential oils. The highest essential oil yield (1.92%) was obtained from *E. tereticornis* and the lowest (0.06%) was recorded from *E. coccinea*. Essential oils obtained from *A. conyzoides, B. pilosa, C. benghalensis, E. floribondus, E. hirta, O. barrelieri, P. milanjianus* and *T. vogelii* were very low compared to the other species and were not enough to perform subsequent antifungal tests. Except *A. conyzoides and B. pilosa* essential oils which were respectively pale green and brown, oils were generally yellow (Table 1).

Fig. 1. Percentage of conidia germination of *F. oxysporum* and *P. infestans* obtained with essential oils of *C. citrinus*, *C. citratus*, *E. tereticornis*, and *O. gratissimum* (**A** - Percentage of conidia germination of *F. oxysporum* obtained with essential oils of *C. citrinus*, *C. citratus*, *E. tereticornis*, *O. gratissimum;* **B** - Percentage of conidia germination of *P. infestans* obtained with essential oils of *C. citrinus*, *C. citratus*, *E. tereticornis*, *O. gratissimum*)



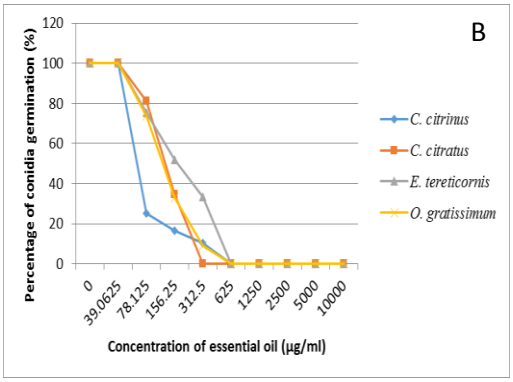
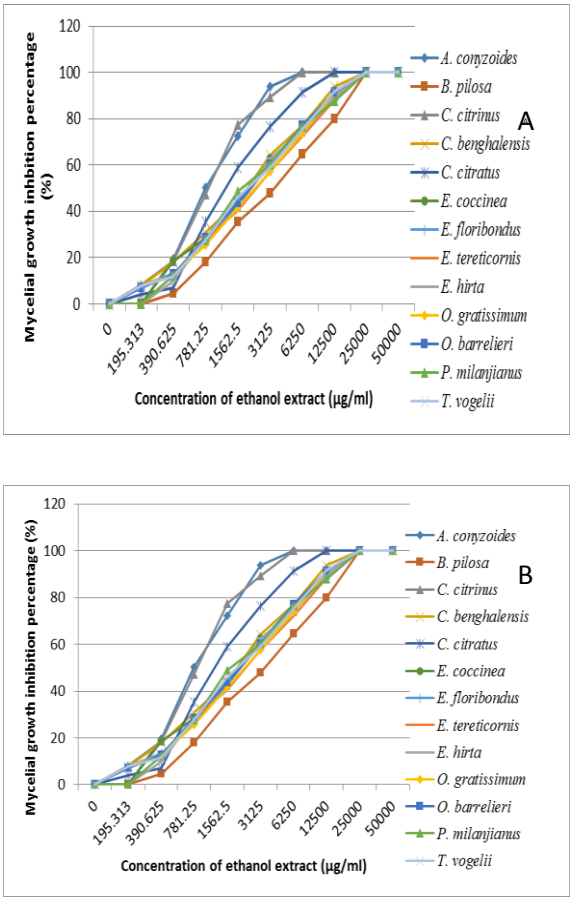


Fig. 2. Percentage of mycelial growth inhibition of *F. oxysporum* and *P. infestans* obtained with ethanol extracts of the thirteen plants extracts (**A** - Percentage of mycelial growth inhibition of *F. oxysporum* obtained with ethanol extracts of the thirteen plants extracts; **B** - Percentage of mycelial growth inhibition of *P. infestans* obtained with ethanol extracts of the thirteen plants extracts)



Efficacy of essential oils against F. oxysporum and P. infestans: The antifungal activities of essential oils (EO) of different plant species were recorded as mycelial growth inhibition and conidia germination percentages of *F. oxysporum* and *P. infestans* (Table 2, Fig. 1). All the tested essential oils exhibited an antifungal activity against both pathogens and the antifungal activity was dose-and plant species-dependant. The essential oils of *C. citratus* and *O. gratissimum* were the most active against *F. oxysporum* with 100% inhibition of mycelial growth of at 625 µg/ml. *C. citrinus* EO was the most active against *P. infestans* with a total mycelial growth at 312.5 µg/ml. EO of *C. citratus* was the most active inhibiting totally the conidia germination of both pathogens at 312.5 µg/ml. The essential oils of *C. citrinus* and *E. tereticornis* were less active against *F. oxysporum* with a total inhibition of mycelial growth recorded at 2500 µg/ml, respectively. EO of *E. tereticornis* was the less active against *P. infestans*. EO obtained from *E. tereticornis* and *O. gratissimum* were the less active against *F. oxysporum*, those obtained from *E. tereticornis*, *O. gratissimum* and *C. citrinus* the less active against conidia germination of *P. infestans*.

Efficacy of ethanol extracts against F. oxysporum and P. infestans

All the ethanol extracts inhibited totally mycelial growth and conidia germination of both pathogens at 50000 µg/ml. ETE of *A. conyzoides* and *C. citrinus* were the most active inhibiting totally radial growth of *P.infestans* at 6250 µg/ml followed by ETE of *C. citratus* at 12500 µg/ml. All the other ETE inhibited totally radial growth of *P. infestans* at 25000 µg/ml. ETE of *C. citrinus* and *C. citratus* were the most active against conidia germination with total inhibition at 6250 µg/ml, followed by ETE of *A. conyzoides*, *O. gratissimum* and *O. barrelieri* at 12500 µg/ml. ETE of *O. gratissimum* was the most active inhibiting totally the radial growth of *F. oxysporum* at 6250 µg/ml followed by *C. citratus* and *O. barrelieri* at 12500 µg/ml. ETE of *C. citratus* was the most active on conidia germination with a total inhibition at 6250 µg/ml followed by *C. citrinus* and *O. barrelieri* at 12500 µg/ml (Fig. 2 and 3).

Efficacy of cold water extracts against F. oxysporum and P. infestans: The mycelial growth of both pathogens was lightly inhibited by almost all the cold water extracts at low

concentrations, instead growth stimulation was observed with some extracts at 195.313 and 390.625 µg/ml. The most active extract (100% mycelial growth inhibition) was obtained from *C. citratus* at 12500 µg/ml against *F. oxysporum*. All the other extracts inhibited totally the radial growth of both pathogens at 25000 and 50000 µg/ml. Conidia germination of pathogens was totally inhibited by cold water extract obtained from *C. citratus* at 12500 µg/ml. All the other extracts inhibited the conidia germination of *F. oxysporum* and *P. infestans* at 50000 µg/ml.

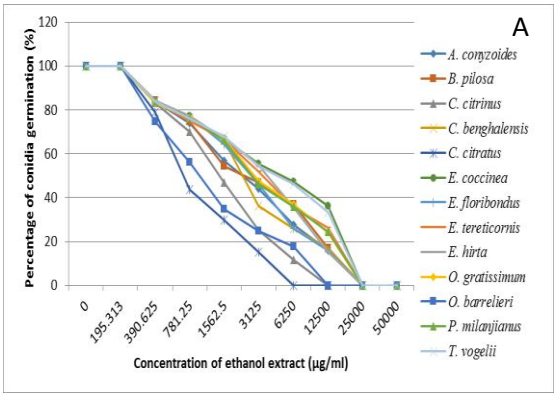
Efficacy of synthetic fungicides against F. oxysporum and P. infestans

Synthetic fungicides Banko Plus® and Plantizeb® 80WP inhibited totally the mycelial growth of *F. oxysporum* and *P. infestans* at 312.5 µg/ml and Kocide® 2000 at 2500 µg/ml. Conidia germination of *F. oxysporum* was inhibited by the Banko Plus® and Plantizeb® 80WP at 312.5 µg/ml, Kocide® 2000 at 625 µg/ml. Banko Plus® and Plantizeb® 80WP inhibited totally the conidia germination of *P. infestans* at 156.25 µg/ml and Kocide® 2000 at 1250 µg/ml. Between the synthetic fungicides, Kocide® 2000 was the less active against both pathogens.

Nature of the antifungal activity

A renewal growth of *F. oxysporum* 30 days after transplanting on fresh media was observed on mycelial discs taken from media supplemented with all the CWE (25000 and 50000 µg/ml), ETE obtained from *O. gratissimum* (12500 µg/ml) and all the other plants (12500 and 25000 µg/ml) and Kocide 2000 (2500 µg/ml). Therefore, the concentrations of 12500, 2500, 25000, 50000 µg/ml were recorded as MIC of *C. citratus* CWE, Kocide 2000 and all the other CWE respectively. ETE were fungistatic against mycelial growth of *F. oxysporum* at 12500 µg/ml for *C. citratus*, *O. gratissimum* and *O. barrelieri* and 25000 µg/ml for the other plants. For conidia germination of *F. oxysporum*, ETE obtained from *C. citrinus* and *O. barrelieri*, CWE of *C. citratus* were fungicidal at 12500 µg/ml, ETE of *C. citratus* was fungicidal at 6250 µg/ml. All the other CWE were fungistatic at 25000 and 50000 µg/ml (Table 3).

Fig. 3. Percentage of conidia germination of *F. oxysporum* and *P. infestans* obtained with ethanol extracts of the thirteen plants extracts (**A** - Percentage of conidia germination of *F. oxysporum* obtained with ethanol extracts of the thirteen plants extracts; **B** - Percentage of conidia germination of *P. infestans* obtained with ethanol extracts of the thirteen plants extracts)



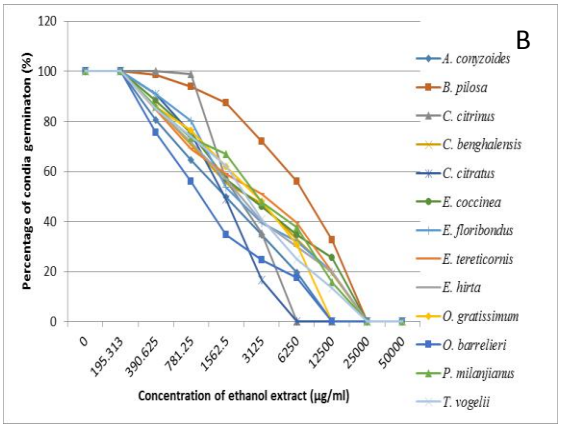


Table 1. Characteristics of plants used and extracts yields

Plant species	Common names	Family	Plant part	EO colours	Yields (%w/w)		
					EO	ETE	CWE
Ageratum conyzoides	Roi des herbes	Asteraceae	Whole plant	Pale green	0.09	3.96	6.23
Bidens pilosa	Hairy Berger	Asteraceae	Whole plant	Brown	0.07	3.55	6.35
Callistemon citrinus	Bottle Brush	Myrtaceae	Leaves	Yellow	1.85	5.62	6.64
Commelina benghalensis	Wandering Jew	Commelinaceae	Whole plant	Yellowish	0.07	4.83	5.21
Cymbopogon citratus	Lemon grass	Poaceae	Leaves	Yellowish	0.81	5.96	6.72
Emilia coccinea	Tassel flower	Asteraceae	Whole plant	Yellow	0.06	2.2	2.73
Erigeron floribondus	Fleabane	Asteraceae	Whole plant	Yellow	0.08	3.22	5.48
Eucalyptus tereticornis	Forest red gum	Myrtaceae	Leaves	Yellow	1.92	3.55	4.35
Euphorbia hirta	Asthma weed	Euphorbiaceae	Whole plant	Yellowish	0.07	1.93	4.32
Ocimum gratissimum	Massep	Lamiaceae	Leaves	Yellowish	0.75	4.47	6.23
Oxalis barrelieri	Oseille-marron	Oxiladeceae	Whole plant	Yellowish	0.07	5.82	6.56
Podocarpus milanjianus	Real yellow-wood	Podocarpaceae	Leaves	Yellowish	0.08	3.65	4.3
Tephrosia vogelii	Tchieuc	Papilionaceae	Leaves	Yellowish	0.09	3.59	5.11

Table 2. Percentage of mycelial growth inhibition of *F. oxysporum* and *P. infestans* obtained with essential oils of *C. citrinus*, *C. citratus*, *E. tereticornis* and *O. gratissimum*.

Essential oil concentration (µg/ml)	Mycelial growth inhibition percentage (%)							
	Fusarium oxysporum f.sp *lycopersici*				*Phytophtora infestans*			
	C. citrinus	*C. Citratus*	*E. Tereticornis*	*O. gratissimum*	*C. citrinus*	*C. Citratus*	*E. tereticornis*	*O. gratissimum*
0	0.00 [a] ± 0.00	0.00 [a] ± 0.00	0.00 [a] ± 0.00	0.00 [a] ± 0.00	0.00 [a] ± 0.00	0.00 [a] ± 0.00	0.00 [a] ± 0.00	0.00 [a] ± 0.00
39.0625	7.69 [b] ± 0.00	23.07 [b] ± 0.00	23.07 [b] ± 0.00	23.07 [b] ± 0.00	7.69 [b] ± 0.00	7.69 [b] ± 0.00	23.07 [b] ± 0.00	23.07 [b] ± 0.00
78.125	15.38 [c] ± 1.53	32.82 [c] ± 0.15	33.33 [c] ± 3.20	35.89 [c] ± 0.88	37.43 [c] ± 2.35	33.33 [c] ± 2.35	40.00 [c] ± 1.53	35.89 [c] ± 0.88
156.25	34.35 [d] ± 2.35	64.61 [d] ± 1.53	48.71 [d] ± 2.35	58.46 [d] ± 1.53	95.38 [d] ± 1.53	75.38 [d] ± 1.53	55.89 [d] ± 0.88	58.46 [d] ± 1.53
312.5	43.58 [e] ± 0.88	82.56 [e] ± 0.88	63.58 [e] ± 2.35	92.82 [e] ± 0.88	100.00 [e] ± 0.00	91.79 [e] ± 1.77	71.79 [e] ± 0.88	92.82 [e] ± 0.88

6250	61.53 [f] ± 1.53	100.00 [f] ± 0.00	81.02 [f] ± 0.05	100.00 [f] ± 0.00	100.00 [e] ± 0.00	100.00 [f] ± 0.00	88.20 [f] ± 0.88	100.00 [f] ± 0.00
12500	78.46 [g] ± 1.53	100.00 [f] ± 0.00	93.33 [g] ± 0.05	100.00 [f] ± 0.00	100.00 [e] ± 0.00	100.00 [f] ± 0.00	100.00 [g] ± 0.00	100.00 [f] ± 0.00
2500	100.00 [h] ± 0.00	100.00 [f] ± 0.00	100.00 [h] ± 0.00	100.00 [f] ± 0.00	100.00 [e] ± 0.00	100.00 [f] ± 0.00	100.00 [g] ± 0.00	100.00 [f] ± 0.00
5000	100.00 [h] ± 0.00	100.00 [f] ± 0.00	100.00 [h] ± 0.00	100.00 [f] ± 0.00	100.00 [e] ± 0.00	100.00 [f] ± 0.00	100.00 [g] ± 0.00	100.00 [f] ± 0.00
10000	100.00 [h] ± 0.00	100.00 [f] ± 0.00	100.00 [h] ± 0.00	100.00 [f] ± 0.00	100.00 [e] ± 0.00	100.00 [f] ± 0.00	100.00 [g] ± 0.00	100.00 [f] ± 0.00

Data are the mean ± SD of five replications. Data in the same column followed by the different letters are significantly different (p<0.05).

Table 3. Nature of inhibition of *F. oxysporum* mycelial growth and conidia germination by plant extracts and synthetic fungicides

Plant species and fungicides	Type of extract	Inhibitory concentration of mycelial growth (µg/ml)	Antifungal property	Inhibitory concentration of conidia germination (µg/ml)	Antifungal property
Ageratum conyzoides	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Bidens pilosa	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Callistemon citrinus	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	12500	Fungicidal
	EO	5000	Fungicidal	625	Fungicidal
Commelina benghalensis	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Cymbopogon citratus	CWE	12500	Fungistatic	12500	Fungicidal
	ETE	12500	Fungistatic	6250	Fungicidal
	EO	625	Fungicidal	312.5	Fungicidal
Emilia coccinea	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Erigeron floribundus	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Eucalyptus tereticornis	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
	EO	2500	Fungicidal	1250	Fungicidal
Euphorbia hirta	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Ocimum gratissimum	CWE	25000	Fungistatic	50000	Fungistatic
	ETE	12500	Fungistatic	25000	Fungistatic
	EO	625	Fungicidal	1250	Fungicidal
Oxalis barrelieri	CWE	25000	Fungistatic	25000	Fungistatic
	ETE	12500	Fungistatic	12500	Fungicidal

Podocarpus milanjianus	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Tephrosia vogelii	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Banko Plus		312.5	Fungicidal	312.5	Fungicidal
Kocide 2000		2500	Fungistatic	625	Fungistatic
Plantizeb		312.5	Fungicidal	312.5	Fungicidal

Table 4. Nature of inhibition of *P. infestans* mycelial growth and conidia germination by plant extracts and synthetic fungicides

Plant species and fungicides	Type of extract	Inhibitory concentration of mycelial growth (µg/ml)	Antifungal property	Inhibitory concentration of conidia germination (µg/ml)	Antifungal property
Ageratum conyzoides	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	6250	Fungistatic	12500	Fungicidal
Bidens pilosa	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Callistemon citrinus	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	6250	Fungistatic	6250	Fungicidal
	EO	312.5	Fungicidal	625	Fungicidal
Commelina benghalensis	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Cymbopogon citratus	CWE	25000	Fungistatic	12500	Fungicidal
	ETE	12500	Fungistatic	6250	Fungicidal
	EO	625	Fungicidal	312.5	Fungicidal
Emilia coccinea	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Erigeron floribundus	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Eucalyptus tereticornis	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
	EO	1250	Fungicidal	625	Fungicidal
Euphorbia hirta	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Ocimum gratissimum	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	12500	Fungicidal
	EO	312.5	Fungicidal	625	Fungicidal
Oxalis barrelieri	CWE	50000	Fungistatic	25000	Fungistatic
	ETE	25000	Fungistatic	12500	Fungicidal
Podocarpus milanjianus	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Tephrosia vogelii	CWE	50000	Fungistatic	50000	Fungistatic
	ETE	25000	Fungistatic	25000	Fungistatic
Banko Plus		312.5	Fungicidal	156.25	Fungicidal
Kocide 2000		2500	Fungistatic	1250	Fungistatic
Plantizeb		312.5	Fungicidal	156.25	Fungicidal

Table 5. Preliminary phytochemical analysis of cold water and ethanol extracts

Secondary Metabolites	Solvent extract	*A. cony*	*B. Pilo*	*C. citri*	*C. beng*	*C. citra*	*E. cocc*	*E. flor*	*E. tere*	*E. hirt*	*O. grat*	*O. barr*	*P. mila*	*T. voge*
	ETE	+	++	-	++	-	++	+	++	+	-	++	++	-
Alkaloids	CWE	-	-	-	+	-	+	-	+	+	-	-	++	-
	ETE	+++	+++	+++	+	++	+	++	++	+	++	+++	++	+++
Phenols	CWE	+	+	-	+	-	+	-	+	-	+	++	+	+
	ETE	-	-	+	+	-	++	-	++	-	++	++	++	+
Triterpenes	CWE	+	++	+	+	+	+	+	+	+	+	-	+	-
	ETE	+	+	+	-	-	-	+	+	-	+	+	-	-
Sterols	CWE	-	-	-	-	-	-	-	-	-	-	-	-	-
	ETE	+	++	+++	++	+++	++	+	++	+	+	+++	++	+++
Flavonoids	CWE	+	+	-	++	-	+	+	+	-	+	+	+	+
	ETE	-	-	+	+	+	++	-	++	+	-	+++	++	+
Saponins	CWE	+	+	+	+	+	+	+	++	+	+	+	+	+
	ETE	-	-	-	+	-	++	-	-	+	-	-	+	-
Anthocyanins	CWE	+	+	+	+	+	+	+	+	+	+	+	+	+
	ETE	-	++	++	-	+	-	-	-	+	-	++	+++	+
Anthraquinones	CWE	-	+	+	-	-	-	-	-	+	-	-	+	-
cardiac	ETE	-	-	-	-	+	-	-	+++	-	-	-	-	-
Glycosides	CWE	+	+	-	+	+	-	+	+	+	+	-	-	+
	ETE	++	-	++	+	++	++	-	++	+	++	++	++	-
Coumarins	CWE	-	-	-	-	+	+	-	-	-	+	-	-	-
hydrolysable	ETE	+	-	+++	+	-	+	+	++	+	-	++	-	-
Tannins	CWE	-	-	+	-	-	-	-	+	-	-	-	-	-
Condensed	ETE	++	+++	+	+	++	++	+	-	+	++	++	+	+++
Tannins	CWE	+	++	++	++	-	+	+	-	++	+	-	++	++

Strongly Present: +++; Present: ++; Weakly Present: +; Absent: -

Ethanol extract: ETE Cold water extract: CWE

A renewal growth of *P. infestans* 30 days after transplanting on fresh media was observed on mycelial discs taken from media supplemented with all the cold water (25000 and 50000 µg/ml), ETE of *A. conyzoides*, *C. citrinus* (6250 µg/ml), ETE obtained from *C. citratus* (12500 µg/ml) and all the other plants (25000 µg/ml), and Kocide 2000 (2500 µg/ml). Therefore, the concentrations of 25000 and 50000 µg/ml were recorded as MIC of all the CWE, 6250 µg/ml the MIC of ETE of *A. conyzoides* and *C. citrinus*, 12500 µg/ml the MIC of *C. citratus*, 2500 µg/ml the MIC of Kocide 2000. For conidia germination of *P. infestans*, ETE obtained from *A. conyzoides*, *C. citrinus*, *C. citratus*, *O. gratissimum*, and *O. barrelieri*, were fungicidal at 12500, 6250, 6250, 12500 and 12500 µg/ml, respectively. Except CWE obtained from *C. citratus* which was fungicidal at 12500 µg/ml against conidia germination of *P. infestans*, all the other CWE were fungistatic at 25000 µg/ml for *O. barrelieri* and 50000 µg/ml for the rest of extracts (Table 4). Incubation of *F. oxysporum* mycelial discs taken from the media containing the EO of *C. citrinus*

(5000 µg/ml), *O. gratissimum* (625 µg/ml), *C. citratus* (625 µg/ml), *E. tereticornis* (2500 µg/ml), fungicides Banko Plus® (312.5 µg/ml) and Plantizeb® 80WP (312.5 µg/ml) did not led to any fungal growth: they have exerted a fungicidal activity on *F. oxysporum* and these concentrations were the minimum fungicidal concentrations (MFC) of the extracts and synthetic fungicides (Table 3).

Incubation of *P. infestans* mycelial discs taken from the media containing the EO of *C. citrinus* (312.5 µg/ml), *O. gratissimum* (312.5 µg/ml), *C. citratus* (625 µg/ml), *E. tereticornis* (1250 µg/ml), fungicides Banko Plus® (312.5 µg/ml) and Plantizeb® 80WP (312.5 µg/ml) did not led to any fungal growth: they have exerted a fungicidal activity on *P. infestans* and these concentrations were the minimum fungicidal concentrations (MFC) of the extracts and synthetic fungicides (Table 4).

A renewal germination of conidia of two pathogens in new slides containing sterile distilled water was observed in suspensions supplemented with Kocide 2000 and all the cold water extracts except that of *C. citratus* which was fungicidal together with ETE of *A. conyzoides* (12500 µg/ml), *C. citrinus* (6250 µg/ml), *C. citratus* (6250 µg/ml) and EO of *O. gratissimum* (1250 µg/ml and 625 µg/ml, respectively for *F. oxysporum* and *P. infestans*), *C. citrinus* (625 µg/ml), *C. citratus* (312.5 µg/ml), *E. tereticornis* (1250 and 625 µg/ml respectively for *F. oxysporum* and *P. infestans*) and synthetic fungicides Banko plus (156.25 µg/ml), plantizeb (156.25 µg/ml). For almost all EO, fungicidal concentrations of conidia germination of both pathogens were lesser than fungicidal concentrations of pathogens mycelial radial growth.

Preliminary phytochemical analysis: Phytochemical screening of solvent extracts showed that their secondary metabolites content varied with botanical species and solvent used for extraction. In general, ethanol extracts contained more secondary metabolites than cold water extracts. Cold water extracts mainly contained anthocyanins, condensed tannins, saponins and triterpens. Phenols, alkaloids, triterpens, flavonoids, hydrolysable tannins, coumarins were shown to be present in almost all ethanol extracts and condensed tannins were almost commonly distributed. Also, sterols were weakly detected in ethanol extracts only. Among ethanol extracts cardiac glycosides were shown only in *C. citratus* and *E. tereticornis*. In cold water extracts, hydrolysable tannins were only detected in *C. citrinus* and *E. tereticornis*, coumarins were identified in *C. citratu, E. coccinea* and *O. gratissimum* (Table 5).

Discussion

In this study, the *in vitro* inhibitory effect of extracts of 13 plants on the mycelial growth and conidia germination of *F. oxysporum* f. sp. *lycopercisi* and *P. infestans* was assessed, and the aqueous and ethanol extracts were screened for their secondary metabolites composition. The extraction yields of essential oils varied with extracts and plant species. Lower EO yields were obtained with *E. coccinea* (0.06%), *B. pilosa, C. benghalensis, E. hirta* and *O. barrelieri* (0.07%), *E. floribundus* and *P. milanjanus* (0.08%), *A. conyzoides* and *T. vogelii* (0.09%). Higher EO yields of 0.75, 0.81, 1.85 and 1.92% were obtained with *O. gratissimum, C. citratus, C. citrinus* and *E. tereticornis*, respectively. The current EO yield (0.81 %) of *C. citratus* differed from the 0.57% yield obtained by Nguefack et al. (2013). Bengyella et al. (2011) obtained 1.46% EO yield from *O. gratissimum*. These disparities confirm the hypothesis that EO extraction yield could be influenced by intrinsic factors such as

botanical species and plant vegetative cycle; and extrinsic factors such as climatic conditions, soil type, place and time of harvest (Bruneton, 1999).

In the present study, essential oils showed the highest antifungal activity against *F. oxysporum* and *P. infestans* as compared to ethanol and cold water extracts of the same plant. Similar observations have been reported by some authors (Mihailović et al., 2011; Bengyella et al., 2011). Mihailović et al. (2011) observed that antimicrobial activity of *Gentiana asclepiadea* essential oil (MIC values: 0.62-2.5 µl/mL) was higher than those of methanolic and n-butanolic extracts (MIC values: 312.5-2500 µg/ml) of the same plant. Bengyella et al. (2011) reported that *O. gratissimum* essential oil at 150 ppm inhibited by 86.17 and 100% the mycelial growth of *Bipolaris oryzae* and *Alternaria padwickii*, respectively. The ethanol extract at 10000 ppm showed 80.92 and 61.54% growth inhibition of *B. oryzae* and *A. padwickii* respectively.

Essential oils of *C. citratus* and *O. gratissimum* were the most active against *F. oxysporum* with 100% inhibition of mycelial growth of at 625 µg/ml. *C. citrinus* EO was the most active against *P. infestans* with a total mycelial growth inhibition at 312.5 µg/ml. Essential oil of *C. citratus* was the most active inhibiting totally the conidia germination of both pathogens at 312.5 µg/ml. Effectiveness of EO on *P. infestans* and *F. oxysporum* has been previously reported (Olanya and Larkin, 2006; Soylu et al., 2006; Al-Reza et al., 2010; Teugwa et al., 2014). Various essential oils demonstrated significant inhibition at 100 and 1000 ppm, over 90% inhibition of *P. infestans* growth was obtained with oregano and Serenade amendments (Olanya and Larkin, 2006). Soylu et al. (2006) have shown that oregano, thyme and fennel oils at 6.4 µg/ml (6.4 ppm) completely inhibited mycelial growth of *P. infestans* whereas growth was totally inhibited by rosemary and lavender essential oils at 12.8 and 25.6 µg/ml (12.8 and 25.6 ppm) concentrations, respectively. Al-Reza et al. (2010) revealed that essential oil of *Cestrum nocturnum* had a remarkable effect on conidia germination of *F. oxysporum* (63.6% inhibition) at 1000 ppm. Teugwa et al. (2014) have shown that essential oils of *Tephrosia vogelii* and *Callistemon citrinus* inhibited radial growth of *F. oxysporum* (100% inhibition) at 1 and 6 µl/ml concentrations, respectively.

According to Amvam et al. (1998), antimicrobial activity of an EO is related to its chemical composition. In addition, the activity of an EO is much related to its proportion in oxygenated terpenes (Hammer et al., 2003; Nguefack et al., 2012). It is therefore evident that *C. citratus* EO which contains 90.4% oxygenated terpenes (Nguefack et al., 2007) was most active. Well-known active ingredients in the chemical composition of EO could justify their high antifungal activities against *P. infestans* and *F. oxysporum*. Thus, the strong activity of *C. citratus* might be due to its proportion in neral and geranial, which represent the major constituents (84.21% of total oil composition). Similarly, activity of *O. gratissimum* essential oil could be linked to thymol, γ-terpinene and p-cymene (73.20%) (Nguefack et al., 2007) whereas 1, 8-cineole, α-pinene and α–terpineol (94.90%) could be responsible for *C. citrinus* activity (Jazet et al., 2009).

The activity of these terpenes results from their high solubility in aqueous media and in microbial membranes (Hammer et al., 2003). Moreover, antifungal activity of EO could not only be due to the action of the major components, all the compounds may act synergistically (Nguefack et al., 2012). Essential oil active compounds inhibit *P. infestans* and *F. oxysporum* by provoking

considerable morphological alterations in fungi hyphae such as cytoplasmic coagulation, vacuolations, hyphal shrivelling and protoplast leakage (Soylu et al., 2006). The solvent extracts revealed diverse antifungal activities, depending on the type of extract or plant species. In general, ethanolic extracts were more active than cold water extracts. These results are in agreement with previous studies showing the antifungal activity of *O. gratissimum* extracts against *B. oryzae* (Bengyella et al., 2011). ETE of *A. conyzoides* and *C. citrinus* were the most active solvent extracts inhibiting totally radial growth of *P. infestans* at 6250 µg/ml whereas ETE of *C. citrinus* and *C. citratus* were the most active against its conidia germination with total inhibition at 6250 µg/ml. ETE of *C. citratus*, and *O. gratissimum* were the most active inhibiting totally radial growth of *F. oxysporum* at 6250 µg/ml. *C. citratus* was the most active on the conidia germination with total inhibition at 6250 µg/ml. These results are different to those obtained by Singha et al. (2011) with different plant extracts against *P. infestans* and *F. oxysporum*. Several other studies reported that plant solvent extracts play an important role in controlling the late blight and tomato wilt pathogens. Methylene chloride and methanol (1:1 V/V) extracts of *Cupressus benthamii* and *Vetiveria zizanioides* at 3% (30000 ppm) shown 23% and 35% inhibition of sporangial germination of *P. infestans* respectively (Goufo et al., 2010).

Yanar et al. (2011) demonstrated that out of 26 plant extracts, *Xanthium strumarium, Lauris nobilis, Salvia officinalis* and *Styrax officinalis* were most active on daily radial growth of *P. infestans* and completely inhibited mycelial growth of the pathogen at 4% (40000 ppm) concentration. Abd-El-Khair and Wafaa (2007) observed that cold water extracts of basil leaves (*Ocimum bacilicum*), chilli fruits (*Capsicum frutescens*), eucalyptus leaves (*Eucalyptus globulus*), garlic bulbs (*Allium sativum*), lemon grass leaves (*Cymbopogon citratus*), marjoram leaves (*Majorana hortensis*), onion seeds (*Allium cepa*) and peppermint leaves (*Mentha piperita*) reduced the conidia germination of *P. infestans* from 30 to 56%, 41 to 72% and 58 to 81% at concentrations of 2.5%, 5.0 and 10.0% respectively. The highly inhibition of mycelial growth of *P. infestans* was obtained with lemon grass leaves followed by garlic bulbs, onion seeds, basil leaves, eucalyptus leaves, peppermint leaves, marjoram leaves, chili fruits and lantana leaves and fruits, respectively. Also, hexane, chloroform, ethyl acetate and methanol extracts of *Cestrum nocturnum* showed radial growth inhibition percentages of 50.2, 52.3, 52.3 and 67.0% respectively against *F. oxysporum* at 1500 µg/disc (Al-Reza et al., 2010).

A tentative correlation between antifungal activity and phytochemical composition of solvent extracts suggests that ethanol extracts with significant antifungal activity mainly contain phenols, flavonoids, condensed tannins, coumarins, and alkaloids. This suggests a high solubility of these secondary metabolites in ethanol, or the absence of their inhibitors in ethanol extracts (Lapornik et al., 2005). Phenols at high concentration have been reported to have very high antimicrobial activity. Also, high activity of coumarins such as phytoalexins produced by plants in response to fungal attack has been reported by many authors (Lapornik et al., 2005). This could explain the fact that extracts containing coumarins (*A. conyzoides*, *C. citrinus*, *C. citratus* and *O. gratissimum* ethanol extracts; and *C. citratus* cold water extract) were very active against *P. infestans* and *F. oxysporum*. Stimulation of fungal radial growth by almost all cold water extracts at

195.313 and 390.625 µg/ml for *F. oxysporum* and *P. infestans* respectively was observed in this work. Galani et al. (2013) mentioned stimulation of pathogens growth by plant extracts at low concentrations. This could be explained by low phenols content of the extracts as revealed by phytochemical analysis. In facts, low concentrations of phenols (3-5 µg/ml) are required by fungi during normal metabolism but higher concentrations (20 µg/ml) are inhibitory to fungal growth (Mohapotra et al., 2000). Furthermore, the lesser activity of cold water extracts could be due to their lesser concentrations in active secondary metabolites as shown from phytochemical screening, because of the low water solubility of these metabolites. It could also be due to the water extraction of polysaccharides, amino acids and proteins that could reduce the quantity and the activity of active metabolites.

The effectiveness of EO obtained from *C. citratus* (CMF=625 µg/ml), *O. gratissimum* (MFC=625 µg/ml) on radial growth of *F. oxysporum* was greater than synthetic fungicide Kocide 2000 (MIC= 2500 µg/ml). Moreover, EO of *C. citrinus* and *O. gratissimum* have the same antifungal activity as synthetic fungicides Banko Plus® and Plantizeb® 80WP (CMF=312.5 µg/ml) on *P. infestans*. EO obtained from *C. citratus* was as much active as synthetic fungicides Banko Plus® and Plantizeb® 80WP (CMF=312.5 µg/ml) on conidia germination inhibition of both pathogens. The difference in sensitivity observed can be explained by difference in mechanism of action of extracts and/or the constitution of the two pathogens. The antimicrobial activity is strongly influenced by the physical, morphological and chemical characteristics of the components of the microbe (Hammer et al., 2003). To a better understanding of the mode of action of these extracts, there is a need of more studies on the chemical and structural characteristics of these pathogens. Considering the different plant species, it appeared that the composition of secondary metabolites varies from one specie to another. Javed et al. (2011) also reported a diversified phytochemical composition of essential oil, aqueous, methanol and chloroform extract of *Eucalyptus citriodora* leaves. Methanol extracts were most rich in secondary metabolites and considerable proportions of sterols and phenols were predominantly found in extracts. The presence of active compounds in a sample is influenced by the extraction method, age of the plant, harvest time and the extraction solvent (Lapornik et al., 2005)

Conclusion

In conclusion, the present study explored the possibilities of controlling *F. oxysporum* f. sp. *lycopersici* and *P. infestans* by using plants extracts. The fungitoxic effects of essential oils of *C. citrinus*, *C. citratus*, *O. gratissimum*, and *E. tereticornis* as well as their ethanol extracts indicated the potential use of these selected species as a source of green fungicidal material. The inhibitory activity of essential oils of *C. citrinus*, *C. citratus*, *O. gratissimum* and *E. tereticornis* was comparable to that of synthetic fungicides Kocide® 2000, Banko Plus® and Plantizeb® used as positive controls. The EO exhibited the strongest antifungal activity against *F. oxysporum* and *P. infestans* followed by ethanol extracts which were more active than cold water extracts. For almost all essential oils, fungicidal concentrations of conidia germination of both pathogens were lesser than fungicidal concentrations of radial mycelial growth. This study has also revealed the presence of bioactive groups (phenolic compounds, sterols, flavonoids, tannins, and coumarins) in plant extracts with highest inhibiting effects. The findings of the present

investigation are important first steps towards the possibilities of using natural plant products of (list of most active plants) as green pesticides to control tomato diseases caused by *F. oxysporum* f.sp. *lycopersici* and *P. infestans*. Nevertheless, essential oils composition, bioactive molecules of aqueous and ethanol extracts of all active plants need to be characterized as well as the investigation of their mode of action against both pathogens.

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