Webology (ISSN: 1735-188X)
Volume 19, Number 6, 2022

Antibacterial Activity Of Secondary Metabolites Of Medicinal Plants

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ABSTRACT

The objective of this study was to evaluate the antimicrobial activity of essential oils extracted from leaves of Ocimum basilicum and Cymbopogon citratus against Xanthomonas axonopodis pv. manihotis. Plant tissues sampling was carried out in first quarter of 2015 in the municipality of Los Palmitos, Sucre, Colombia. Obtaining essential oils was performed by hydrodistillation assisted microwave. Microdilution technique was used in ELISA plate 96 wells to determine the bactericidal effect of essential oils, concentrations were prepared to 2 500, 1 250, 625, 312, 156, 78, 39, 19 ppm for C. citratus oil and 10 000, 5 000, 2 500, 1250, 625, 312.5, 156.25, 78 ppm O. basilicum oil. The assay was incubated with 180 rpm agitation, to 30 ° C for 24 hours. MTT (3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium) was used as indicator solution and the absorbance was measured in an ELISA reader at 492 nm to reveal the inhibition of bacterial growth. With obtained data the minimum inhibitory concentration (MIC) was determined and the percentage of bacterial inhibition of each oil at different concentrations. MIC values of C. citratus and O. basilicum oils were 312.5 ppm and 2500 ppm respectively. The essential oil obtained from C. citratus showed higher antimicrobial activity against Xam strain.

Keywords: microdilution, clevenger, Cymbopogon citratus, O. basilicum.

INTRODUCTION

One of the diseases that most limits cassava production worldwide is vascular bacteriosis or bacterial blast, caused by the bacterium Xanthomonas axonopodis pv. manihotis (López et al., 2006; Chirinos et al., 2013), an aerobic Gram-negative bacillus, which shares most of the physiological and biochemical characteristics of its genus (Barraza, 2012). Infection begins

with a foliar epiphytic phase and subsequently invades the vascular bundles, triggering a series of symptoms such as angular leaf spots, blistering or burning, wilting, stem exudates, vascular necrosis and downward death of the plant (Ogunjobi, et al., 2008), which can lead to the total loss of the crop (Chirinos et al., 2013).

The control of diseases produced by the genus Xanthomonas is carried out with chemical products such as streptomycin sulphate and cupric compounds, to which these microorganisms have acquired resistance (Farfán et al. 2014), and these compounds also cause serious damage to the environment and human health (Chirinos et al., 2013). Another widely used mechanism to combat this disease has been the use of cassava varieties resistant to bacteriosis, but these have not adapted to all the climatic conditions where they are grown (López et al., 2006). Given the above, there is a need to find other mechanisms to mitigate the damage caused by this disease, making use of biological alternatives to improve the production rate of this root and also contribute to reducing the emission of chemical compounds that are harmful to the environment. In this context, the use of plant bioactives, especially essential oils, is of great interest, as these are known to have medicinal and antimicrobial properties (Pereira et al., 2011).

The plant species Cimbopogon citratus staff, commonly known as lemongrass, is an aromatic plant belonging to the grass family (Barbos et al., 2008), which possesses essential oils, which have been reported to be antifungal, antibacterial and antiparasitic (Tyagi, and Malik. 2010; Taweechaisupapong et al. 2012). The antibacterial activity of C. citratus oil has been little evaluated with phytopathogenic bacteria, however, there are a large number of reported studies where high efficiency of antibacterial activity has been found on human pathogenic bacteria, including Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa (Taweechaisupapong et al. 2012).

On the other hand, the species Ocimum basilucum, commonly known as basil and belonging to the Lamiacea family, produces essential oils with insecticidal, fungicidal, nematicidal and antibacterial properties (Politeo et al., 2007). Several studies have shown that the essential oil of this plant has a strong inhibition against multidrug-resistant bacteria such as Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus faecalis (Opalchenova and Obreshkova, 2003) and Mycobacterium tuberculosis (Tchoumbougnang et al., 2005)), with few studies on phytopathogenic bacteria (Moghaddam et al., 2014).

The aim of this study was to evaluate the antibacterial activity expressed as minimum inhibitory concentration (MIC) and percentage bacterial inhibition (%IB) of the essential oils of C. citratus and O. basilicum against the bacterium Xanthomonas axonopodis pv. manihotis, which causes bacterial blight of cassava, in the department of Sucre, Colombia.

MATERIALS AND METHODS

Sampling of plant material was carried out in the first quarter of 2015, during which leaves of O. basilicum and C. citratus species were collected in the municipality of Los Palmitos, Sucre, Colombia, located at 9°22'23" north latitude and 75°16'23" west longitude, with an average temperature of 28°C, relative humidity of 65%, average annual rainfall of 1176 mm and altitude of 175 m above sea level.

Obtaining the essential oils.

The essential oils were obtained by the microwave-assisted hydrodistillation method (MWHD), using hydrodistillation equipment with a capacity of 2 L (distillation balloon). Three hundred g of fresh, selected and chopped plant material were weighed and placed in the balloon, which contained 800 mL of distilled water, and the extraction time was 1 hour. A conventional oven was used as a source of microwave radiation, with an irradiation cycle of 60 minutes at 70% power (Torrenegra et al., 2015; Ganjewala, 2009; Chemat et al., 2006). The essential oils obtained were dried with sodium sulphate anhydride and stored in sealed vials at 4°C protected from light until further processing (Rodriguez et al., 2011).

Bacterial inoculum

The bacterial strain was donated by the mycology and phytopathology laboratory of the Universidad de los Andes, Colombia. From a pure culture, five isolated colonies were taken and seeded in a tube with 10 ml of LPG liquid medium (yeast 5 g/L, peptone 5 g/L, glucose 5 g/L), and left in agitation at 150 rpm at 28°C for 24 hours, then the turbidity of the bacterial suspension was adjusted to an absorbance between 0.09 and 0.1 at a wavelength of 600nm, equivalent on the Mc Farland scale to $1.5X10^8$ CFU/mL.

Preparation of the essential oil solutions

Stock solutions were prepared from the essential oils at 5 000 and 20 000 ppm respectively, which were obtained by diluting the oils in 1% dimethyl sulfoxide.

Antimicrobial activity

The antibacterial activity of each essential oil was evaluated separately using the microdilution technique in 96-well Elisa plates (Carvajal et al., 2013). From the stock solutions of the oils, 8 five-fold serial dilutions were made in LPG medium in a 1:1 ratio, leaving a volume of 180µL/well of each dilution, at concentrations of 2 500, 1250, 625, 312, 156, 78, 39 and 19 ppm for the oil of C. citratus oil and 10 000, 5 000, 2 500, 1 250, 625, 312, 156 and 78 ppm for O. basilicum oil, then 20ul of the bacterial inoculum was added to each

well for a final volume of 200uL per well. Each plate included medium sterility controls, growth control, solvent control (1% DMSO) and positive control (Gentamicin 500ppm), plates were capped and sealed with cristaflex and incubated at 150 rpm at 30°C for 24 hours.

After the incubation time, 20 µL of an aqueous solution (0.5 mg/ml) of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to the wells containing the treatments and controls. After incubation for 45 minutes, the contents of the wells were discarded and 200 µL/well of concentrated DMSO was added and the optical density was determined in an Elisa reader, using a wavelength of 492 nm (Ruilova, 2007).

The minimum inhibitory concentration was taken as the lowest concentration at which there was no change in the colouring of the tetrazolium salt (MTT), which changes from yellow to formazan blue in the wells where there is bacterial growth.

Inhibition of bacterial growth was determined by the following formula: %IB = 1- [(AC - CE)/ (CC - CE)]*100, where: %IB is the percentage of bacterial growth inhibition; CC is the absorbance obtained for the growth control; CE is the absorbance obtained for the sterility control; and AC is the absorbance obtained for a given concentration of the oil, CC-CE expresses the actual growth of the bacteria and AC-CE the actual growth of the bacteria at a given concentration of the essential oil (Carvajal, 2013).

Statistical analysis

Results were expressed as mean ± standard deviation. Statistical treatment was performed using the Kruskal-Wallis test (ANOVA by Ranks) followed by Dunn's test for multiple comparisons. Significant differences were considered significant at $p < 0.05$ (rgui).

Gas chromatography coupled to mass spectrometry (GC/MS)

The analysis of essential oils was performed in a gas chromatograph, equipped with a DB-1ms column (30 m x 0.25 mm, 0.25 µm). The initial oven temperature was 50°C and the final oven temperature was 300°C, helium was used as carrier gas. The injector temperature was 175°C, the injection size 0.2 µL and the detector temperature 300°C. The identification of the compounds was established according to their mass spectra, using the database with the highest probability of match for the databases NIST02.L, NIST5a.L and NIST98L. The chemical spectrum information of the metabolites was generated by MSD ChemStation software.

RESULTS AND DISCUSSION

Antimicrobial activity

The concentrations of C. citratus essential oil that inhibited bacterial growth were 2500 ppm, 1250 ppm, 625 ppm and 312.5 ppm (figure 1), the latter being the minimum inhibitory concentration, being the lowest concentration of the oil capable of inhibiting bacterial growth. The blue-violet wells indicate bacterial growth while the yellow wells indicate inhibition. The 1% dimethyl sulfoxide, which was used as a solvent, did not inhibit bacterial growth (Figure 1, row G8-G12).

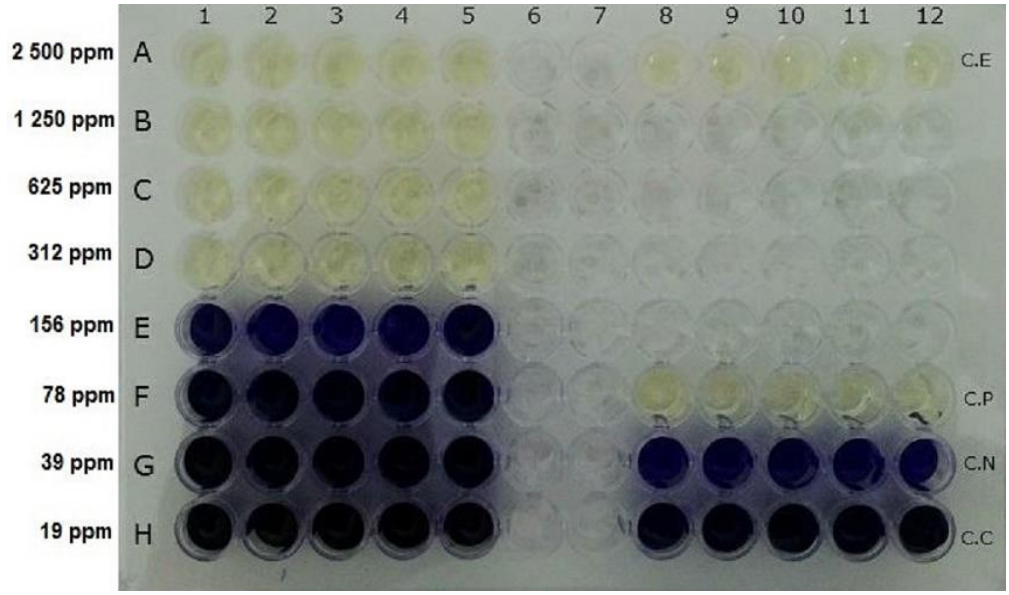

Figure 1: Antimicrobial activity of essential oil of C. citratus **C.E:** sterility control (A8-12 row), **C.C:** growth control (row H8-12), **C.N:** control DMSO cytotoxicity and **C.P:** positive control antibiotic (Gentamicin).

With the absorbance data, the inhibition percentages of the different concentrations of the essential oil of C. citratus were determined (figure 1), showing that at concentrations of 2500, 1250, 625 and 312 ppm, bacterial inhibition was greater than 95%, with the highest bacterial inhibition rate at 2500 ppm.

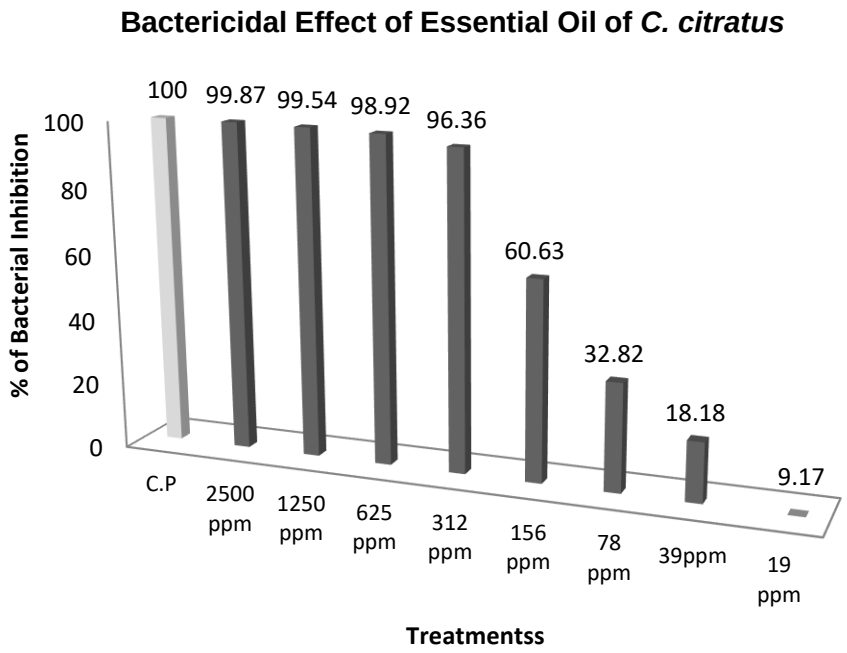

Figure 2. Percentage of bacterial inhibition of C. citratus essential oil **CP:** positive control, **ppm:** parts per million.

The concentrations of O. basilicum essential oil that inhibited bacterial growth were 10 000 ppm, 5 000 ppm, 625 ppm and 312 ppm (figure 1), the minimum inhibitory concentration for this oil being 2500 ppm, figure 2 (row C1-C5).

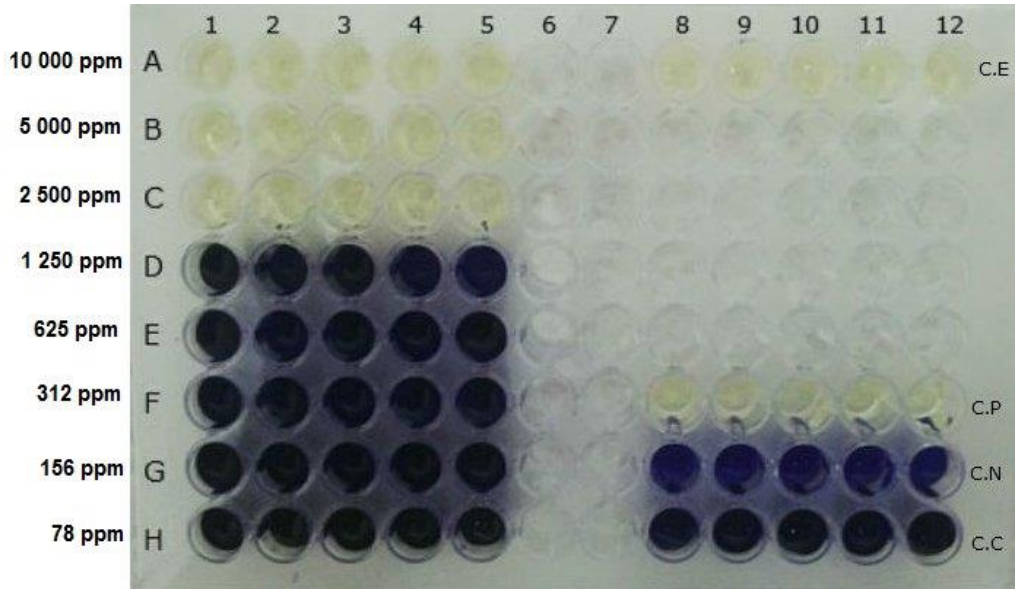

Figure 3: Antimicrobial activity of essential oil of O. basilicum. **C.E:** sterility control (A8-12 row), **C.C:** growth control (row H8-12), **C.N:** control DMSO cytotoxicity and **C.P:** positive control antibiotic (Gentamicin).

On the other hand, the O. basilicum oil treatments that inhibited bacterial growth by more than 95% were 2500 ppm, 1250 ppm, 625 ppm and 312.5 ppm (Figure 4).

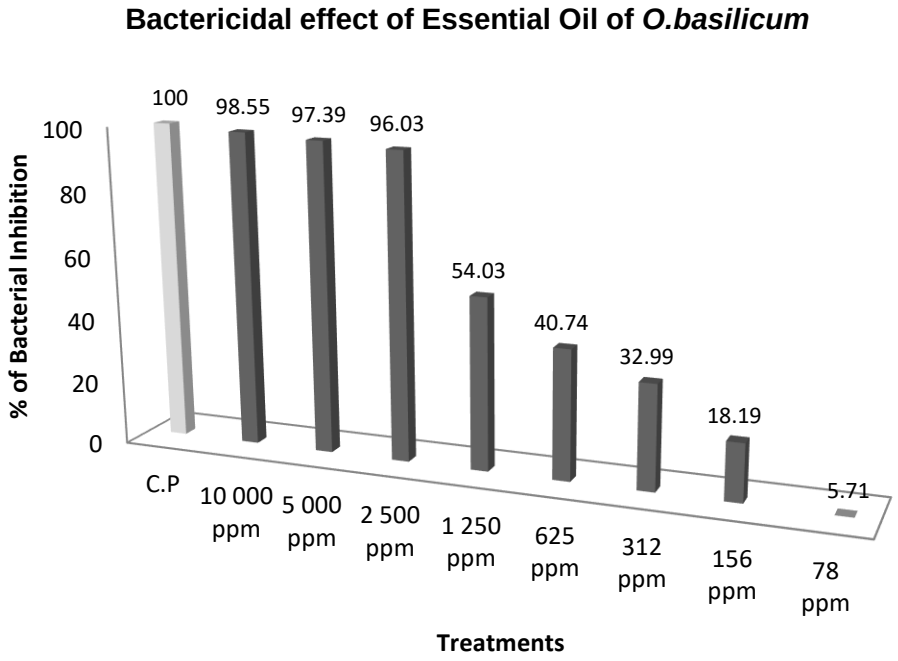

Figure 4. Percentage of bacterial inhibition of O. basilicum essential oil, **C.C:** growth control, **C.N:** Negative Control, **CP:** positive control, **ppm:** parts per million, **D.E:** standard deviation.

Statistical analysis

The non-parametric Kruskal Wallis test showed significant differences (X2= 48.5402, p-value = 0), for the different treatments of C. citratus used with respect to the bacterial inhibition index, with a confidence interval of 95%. Subsequently, using Dunn's test, comparisons were made between the concentrations of the essential oil and the positive control (Gentamicin 300 ppm), and it was determined that the treatments that did not present significant differences ($p > 0.05$) with respect to the positive control were 2500 ppm, 1250 ppm, 625 ppm and 312 ppm. As for the essential oil of O. basilicum, it was determined by Dunn's test that there were no differences ($p > 0.05$) between the treatments at 2500 ppm, 1250ppm, 625 ppm and the positive control (gentamicin). This suggests that at these concentrations, the essential oils of C. citratus and O. basilicum collected in Los Palmitos, Sucre cause a bactericidal effect similar to that caused by Gentamicin at 300 ppm, on Xam.

Gas chromatography coupled to mass spectrometry (GC/MS)

GC/MS analysis of the essential oil of C. citratus allowed the identification of 40 compounds representing 99.99% of the essential oil constituents. The major components identified were the monoterpenes geranial (27.2-33.06%) and neral (25.64-29.76%) which constitute a mixture of isomers known as citral, this compound being the major constituent of the essential oil obtained, representing 62.82%. According to Guerra et al., 2004, the antimicrobial activity of C. citratus oil is mainly attributed to citral, particularly to the geranial (alpha citral) and neral (beta citral) isomers, the high percentage of citral found in C. citratus oil could be the explanation for the high inhibition percentages caused by this oil on the bacterium X. axonopodis pv manihotis.

In the case of basil essential oil, 17 compounds were identified, representing 100% of the constituents of this essential oil. The compounds found in the highest percentage for this oil were Estragole (57.83%) and Linalool (28.36%) followed by fenchone (4.07%).

In the present investigation, the in vitro antibacterial activity of the essential oils of C. citratus and O. basilicum against the bacterium X. axonopodis pv. manihotis was demonstrated. The results presented suggest the use of these essential oils for the biological control of cassava bacteriosis. However, it is recommended that field tests be carried out to verify the bactericidal activity under environmental conditions.

ACKNOWLEDGEMENTS

The authors would like to thank the Microbiological Research Laboratory, part of the Agricultural Bioprospecting Research Group, Faculty of Agricultural Sciences, University of Sucre, Colombia.

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