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Research Paper

Eucalyptus leaf byproduct inhibits the anthracnose-causing fungus *Colletotrichum gloeosporioides*

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ABSTRACT

Colletotrichum gloeosporioides is a major anthracnose-causing agent, causing dark spots in important fruit crops such as banana, papaya and mango. *Eucalyptus* trees and shrubs are native to Australia, but are currently widely distributed. There is evidence that *Eucalyptus* species display activity against *Colletotrichum gloeosporioides*. In this work, the antifungal activity of essential oils and ethanolic extracts of three *Eucalyptus* species cultivated for wood in Colombia (*Eucalyptus camaldulensis* Dehn., *Eucalyptus globulus* Labill. and *Eucalyptus tereticornis* Smith.) was evaluated. Leaf from the three *Eucalyptus* species was extracted by hydrodistillation and ethanol maceration to yield respectively the essential oil and ethanolic extract, which were tested against *C. gloeosporioides* Penz., using an agar macrodilution assay. Ethanolic extracts showed stronger inhibition than essential oils. Although the ethanolic extract of *E. camaldulensis* provided 98% of inhibition at a concentration of 5000 mg/L, the ethanolic extract of *E. globulus* inhibited by 50% at a lower concentration of 500 mg/L. The metabolites from essential oils and ethanol extracts were examined respectively by gas chromatography coupled to mass spectrometry (GC–MS) and ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS). Both *E. camaldulensis* and *E. globulus* ethanolic extracts may be considered for antifungal formulations and as source of interesting anti-*Colletotrichum* compounds.

1. Introduction

Anthracnose is an infection of *Colletotrichum gloeosporioides* and is currently the major postharvest disease of mango and other fruits in many areas of the world (Litz, 2009). The disease occurs as a quiescent infection on immature fruit, however during the postharvest period the fruits are seriously damaged with prominent black spots, being very difficult to control (Kefialew and Ayalew, 2008). To minimize loss during the storage, synthetic fungicides are applied to the fruit. For instance, Benomyl or Prochloraz are recommended to effectively control anthracnose on the harvested mango fruit (Kim et al., 2007). However, the overuse of fungicides in monotherapy can result in pathogen resistance (Shi et al., 2012). In addition, not all the importing countries presently approve the use of postharvest synthetic fungicides (Akem, 2006).

Studies on the traditional use of plants to prevent fungal and bacterial infections have shown the importance of natural products as a viable source of non-phytotoxic and readily biodegradable alternatives to synthetic fungicides (Badrunnisa et al., 2011). Several reports have focused on the application of natural antifungals in the food industry (Appendini and Hotchkiss, 2002; Lanciotti et al., 2004), particularly essential oils (Burt, 2004). *Eucalyptus* is a genus of flowering trees and shrubs, which originated in Australia, and presently includes more than 700 species, mostly native to Australia. *Eucalyptus* species are attractive because of their fast growth, producing large amounts of wood, but also because of their ability to drain swamps and to synthetize large amount of isoprenoids (He et al., 2000). A number of studies have demonstrated the antimicrobial properties of *Eucalyptus* essential oil (EO) against a wide number of microorganisms (Bachir and Benali, 2012; Batish et al., 2008; Cimanga et al., 2002; Cheng et al., 2009; Ramezani et al., 2002a; Safaei-Ghomi and Ahd, 2010), while only a few investigations have been carried out on the alcoholic extracts (Badrunnisa et al., 2011;

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Safaei-Ghomi and Ahd, 2010). Moreover, there is little evidence of the activity of *Eucalyptus* species specifically against *Colletotrichum gloeosporioides* (Barrera-Necha et al., 2008; Combrinck et al., 2011; Padman and Janardhana, 2012), which dramatically affects mango in postharvest commercialization. This study was conducted to examine the *in vitro C. gloeosporioides* inhibitory effect of the leaves of three species of *Eucalyptus*: *E. tereticornis* Smith., *E. camaldulensis* Dehn. and *E. globulus* Labill., which are locally available as waste by-products from the *Eucalyptus* forest-products industry in Colombia.

2. Materials and methods

2.1. Materials and equipment

All solvents were analytical grade and were purchased from Merck. PDA medium and other reagents and antibiotics were purchased from Sigma. The microwave oven used for hydrodistillation was a WM 1107D Whirlpool with a 700 W output, and the rotary evaporator employed was a IKA-RV 10 with cooling unit. GC analysis was carried out on a Trace 1300 gas chromatograph coupled to a ISQ mass detector (Thermo Fisher Scientific, Inc.) using a 100% polydimethylsiloxane capillary column DB-1 (30 m × 0.25 mm × 0.25 μm, Agilent). A Nexera X2 Ultra Performance Liquid Chromatography, UHPLC (Shimadzu Corporation, Japan), equipped with a reversed-phase Acquity UPLC BEH C8 column (1.7 μm, 2.1 mm × 150 mm) (Waters Technologies, Milford, MA) was employed for analysis. Detection was executed in positive and negative ion mode using a maXis 4G Q-TOF mass spectrometer (Bruker Daltonics, Germany) with electrospray ionization (ESI) source. Typical instrument settings were as follows: end plate offset 500 V, capillary voltage 3700 V, nebulizer 4.0 Bar, dry gas 9.0 L/min, dry temp 200 °C, quadrupole ion energy 4 eV, collision energy 8 eV, collision RF 300 Vpp, transfer time 55 μs, pre pulse storage 8 μs and the acquisition spectra rate was 3.00 Hz.

2.2. Plants and fungal strain

Leaves of the three species of *Eucalyptus* were collected in the non-windy season from March to May 2014, in forestry farms from the Departments of Magdalena and Nariño, Colombia. The species were authenticated by the Herbario Nacional Colombiano (COL) from the Instituto de Ciencias Naturales – Universidad Nacional de Colombia. The leaves of *Eucalyptus tereticornis* Smith. and *Eucalyptus camaldulensis* Dehn. were collected by Ivan Vanegas in the farms Terranova, Pijiño del Carmen, Magdalena, and Nueva Esperanza, San Zenón, Magdalena, respectively. The leaves of *Eucalyptus globulus* Labill. were collected by Libio Guerra in the Vereda Briceño, Pasto, Nariño. The vouchers given from the National Colombian Herbarium (COL) for the collected species *E. tereticornis* S., *E. camaldulensis* D. and *E. globulus* L. were COL578345, COL578346 and COL578339 respectively. The fungus strain *Colletotrichum gloeosporioides* Penz. (C-935, Corpoica) was isolated from postharvest fruits of mango (*Mangifera indica* L.) by Corporación Colombiana de Investigación Agropecuaria (Corpoica). The culture was maintained on potato dextrose agar (PDA) medium and stored at 4 °C.

2.3. Extraction

The EOs of the three *Eucalyptus* species was obtained by microwave-assisted hydrodistillation (MWHD). Fresh leaves were cut into small pieces and weighted. The material (around 150 g) was introduced into 4 L round-bottom flasks with 500 mL of distilled water, under a plant material/water ratio of 0.3 g/mL. A Clevenger-distillation head with Dean-Stark trap was employed to collect the EO. The hydrodistillation was performed for 80 min with warming periods of 15 min and 5 min breaks in between. The ethanolic extract (EE) of the *Eucalyptus* species was extracted by maceration at room temperature. Briefly the weighted dried and milled leaves (around 220 g) were extracted with 500 mL of 96% ethanol, under a plant material/ethanol ratio of 0.4 g/mL, by soaking the plant material with the ethanol for a week. The mixture was filtered through filter paper and the solvent was removed under reduced pressure using a rotary evaporator. The EOs and EEs obtained were collected in test tubes and glass vials respectively and were closed with hermetic lid and stored at 4 °C.

2.4. Antifungal activity

The antifungal activity was evaluated by adapting of the spot culture growth inhibition assay e.g. (Guzman et al., 2013) to Petri dishes and fungal inoculation by passage from fully grown solid media culture with sterile hole punch. EOs and EEs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 g/L. A small amount of each extract in DMSO was added into sterile 85 mm disposable Petri dishes, and followed by the addition of 13 mL of warm and sterile PDA medium so that the final concentrations of the extracts were 50, 100, 250, 500, 1000, 2000, 3000, 4000, 5000 mg/L. The fungus was passaged by cutting a plug of grown agar and placing it into the middle of freshly prepared PDA medium in a Petri dish and cultured for 10 days at 26 °C. With a sterile hole punch the grown fungus was cut from the surface of the agar and the plug placed in the middle of the extract-containing Petri dishes. After transfer of the mycelium, the testing cultures were incubated at 26 °C for 10 days. Each experiment consisted of a negative control (DMSO) and nine different concentrations of the extracts. The commercial fungicide Prochloraz was used as a positive control at 0.3, 1, 3, 10, 30 mg/L concentrations (Gullino et al., 1985). The diameter of mycelial growth was measured with a caliper when the mycelium for the negative control reached the edges of Petri dish. The growth inhibition percentage (GIP) was obtained by comparing the growth of the extracts with the negative control. The lowest concentration with 90% growth inhibition was determined as minimum inhibitory concentration (MIC_{90}) as a measure of antifungal activity (Griffin et al., 2000). Each experiment was performed by triplicate. Descriptive statistical analysis was performed using SPSS (version 17.0) for Windows (SPSS, Inc., Chicago, IL). The data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test.

2.5. Chemical identification

The essential oils were dissolved at 5% (*v/v*) in dichloromethane and were analyzed by capillary gas chromatography − mass spectrometry (GC–MS). The GC–MS equipment was operated in split mode (1:33, injected volume 1 μL), and the temperature program started from 40 °C, increased to 100 °C at 10 °C/min, hold for 8 min and then to 280 °C at 12 °C/min. The temperature of the injector and detector were 200 °C and 280 °C respectively. The carrier gas was helium at 1.0 mL/min. The temperature of the ion transfer line was maintained at 300 °C. Mass spectra were obtained by electron ionization (EI, 70 eV) in a quadrupole mass detector with a mass range 40–800 *m/z*, in the full scan mode with 0.35 s intervals and a solvent delay of 10 min. The linear retention indices (RI) were calculated using with linear hydrocarbons (C5–C15). The components of the EOs were identified by comparison of their mass spectra with those of a computer library search (NIST05) and confirmed by comparison of their RI reported in the literature (Babushok et al., 2011; Botnick et al., 2012; Mariotti et al., 1997; Schreyen et al., 1979). The relative abundance of each identified component was determined from the area under the curve. The composition analysis of EEs for the three species was performed by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). A methanolic solution of the extracts was prepared at a 1 mg/mL concentration using methanol, filtered through a 0.22 μm membrane filter and then injected into the UHPLC–MS equipment. One μL of each leaf extract was injected for separation. The following chromatographic gradient program was applied: 0–1 min isocratic 90% A (deionized water, 0.1% [v/v] formic acid) and 10% B