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Wet processing coffee waste as an alternative to produce extracts with antifungal activity: *In vitro* and *in vivo* valorization

Residuos del beneficio húmedo del café como una alternativa para producir extractos con actividad antifúngica: valoración *in vitro* e *in vivo*

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Abstract

Wet coffee waste (WCW) generated by the wet processing of coffee, is mainly made up of coffee pulp and husk, and has been scarcely studied and reutilized. Therefore, the aim of this work was to investigate the *in vitro* and *in vivo* antifungal activity of an WCW ethanolic-extract (EWCW) obtained by ultrasonication (amplitude 70%, sonication time 45 min). EWCW had total phenolic content of 33.8 mg gallic acid equivalents/g and antioxidant activity of 1790.1 μmol Trolox equivalents/g. EWCW showed a potent *in vitro* antifungal activity against *Aspergillus niger*, *Botryris cinerea*, and *Rhizopus stolonifer* equivalent or superior to that of commercial synthetic carbendazim. Strawberries were wounded and inoculated with the three fungi for *in vivo* assays. After 5 d of inoculation, percentage of wounds presenting mold growth was of 16.7% (400 μg/mL of EWCW) and 38.2% (1000 μg/mL of carbendazim) for *A. niger*, and of 36.1% for *B. cinerea* and of 50.12% for *R. stolonifer* (2000 μg/mL of EWCW) compared to the 38.2% and 51.2% (1000 μg/mL of carbendazim), respectively. The efficient antifungal activity of EWCW can be mainly attributed to its majoritarian compounds chlorogenic acid (14.19 mg/g) and caffeic acid (1.18 mg/g), which represented 89.83% of the identified compounds.

Keywords: wet processing coffee waste; ethanolic extract; antioxidant activity; *in vitro* antifungal activity; *in vivo* antifungal activity.

Resumen

Los residuos (WCW) generados por el proceso de beneficio húmedo del café se componen principalmente de pulpa y cáscara de café, y escasamente se han estudiado y reutilizado. Por lo tanto, el objetivo de este trabajo fue investigar la actividad antifúngica *in vitro* e *in vivo* de un WCW extracto etanólico (EWCW) obtenido por ultrasonido (amplitud 70%, tiempo de sonicación 45 min). EWCW tuvo un contenido fenólico total de 33.8 mg equivalentes de ácido gálico/g y una actividad antioxidante de 1790.1 μmol equivalentes de Trolox/g. EWCW mostró una potente actividad antifúngica *in vitro* contra *Aspergillus niger*, *Botryris cinerea* y *Rhizopus stolonifer* equivalente o superior a aquella de carbendazim sintético comercial. Fresas fueron heridas e inoculadas con los tres hongos para ensayos *in vivo*. Después de 5 días de inoculación, el porcentaje de heridas que presentaron crecimiento de moho fue de 16.7% (400 μg/mL de EWCW) y 38.2% (1000 μg/mL de carbendazim) para *A. niger*, 36.1% para *B. cinerea* y 50.12% para *R. stolonifer* (2000 μg/mL de EWCW) en comparación con 38.2% y 51.2% (1000 μg/mL de carbendazim), respectivamente. La actividad antifúngica eficiente de EWCW puede atribuirse principalmente a sus compuestos mayoritarios ácido clorogénico (14.19 mg/g) y ácido cafeico (1.18 mg/g), los cuales representaron el 89.83% de los compuestos identificados.

Palabras clave: residuos del beneficio húmedo del café, extracto etanólico; actividad antioxidante; actividad antifúngica *in vitro*; actividad antifúngica *in vivo*.

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1 Introduction

Coffee is an economically important crop worldwide, and it is the largest product marketed after petroleum (Can *et al*., 2019; Ramírez-Velasco *et al*., 2016). Approximately 55 countries in the world participate in coffee production as part of their primary agricultural production (Chanakya and De Alwis, 2004), making it one of the main food systems. According to data from the International Coffee Organization (2020), the global production of green coffee in 2018 was 10.3 million tons, so the processing of this fruit generates large amounts of waste such as pulp, husk, skin, among others (Esquivel and Jiménez, 2012), that amount to approximately 50% of the total coffee fruit. The wet processing of coffee (WCW) produces approximately 29% of coffee pulp and 12% of husk as by-products respect to the overall coffee cherry (dry weight) (Janissen and Huynh, 2018; Montoya *et al*., 2020). These solid wastes without an apparent economic value are generally discarded impacting negatively the environment. Waste reuse has received much attention recently, as it is seen as a potential source of economic and social benefits, and a way of contributing to the mitigation of the environmental pollution (Ghosh *et al*., 2017). The presence of phytochemicals and antioxidants in coffee and their health benefits have been reported in many studies in recent years (Magalhães *et al*., 2016; Martínez-Ruíz *et al*., 2018), so it is expected that valuable byproducts could be obtained from WCW mainly constituted by coffee cherry pulp (Villa *et al*., 2019; Mussatto *et al*., 2011), which contains considerable amounts of phenolic compounds and caffeine (Heeger *et al*., 2017). Phenolic compounds have a variety of physiological activities, such as antioxidant, antimicrobial, antimutagenic, anti-inflammatory and antiallergic (Martins *et al*., 2011), and are currently used in the fields of biology, medicine, food, among others. Thus, obtaining added-value products from wastes is an ongoing research topic, and to do so in a competent manner is of the utmost importance for obtaining good recovery yields and quality. In this respect, microwave assisted (Yang and Zhai, 2010), ultrasound assisted (Pan *et al*., 2012) and supercritical fluid (Liu *et al*., 2011) extraction techniques have proven to be efficient for phenolic compounds recovery. Several studies have shown that ultrasound assisted extraction is the most efficient technique among specific extraction techniques (Chen *et al*., 2012; Al-Dhabi *et al*., 2017; Vinatoru *et al*., 2017). Likewise, various studies have reported the potential activity of plant extracts against fungal attack in post-harvest fruits with short shelf life (Feliziani and Romanazzi, 2016; de Rodríguez *et al*., 2017; Chen *et al*., 2019). In addition, these extracts have been found to pose a less dangerous threat to human health and the environment than chemical pesticides (Masood *et al*., 2016). Reports have shown that compounds of plant origin are suitable as biopesticides (Suleiman and Yusuf, 2011). Strawberry (*Fragaria* spp.) is a highly perishable fruit even under ideal conditions of storage at 4 ºC, losing its edible quality within a week (Wills and Kim, 1995). Its turgid epidermis and its high rate of respiration make it susceptible to mechanical damage and the invasion by fungal pathogens. Amongst the fungal pathogens, *Botrytis cinerea*, *Aspergillus niger*, *Rhizopus stolonifer*, *Colletotrichum acutatum*, and *Phytophthora cactorum* are of significant importance (Khalid *et al*., 2017). This opens the way to explore, for the first time, the possible antifungal properties of WCW extracts for controlling strawberry fungal postharvest diseases. Based on the above, the objectives of this study were to: (a) determine the sonication conditions (amplitude and time) yielding highest total phenolic content and antioxidant activity by DPPH in the extracts; and (b) evaluate the *in vitro* and *in vivo* antifungal activity of wet processing coffee waste extracts against *Aspergillus niger*, *Botrytis cinerea*, and *Rhizopus stolonifer*.

2 Materials and methods

2.1 Materials

Wet processing coffee (*Coffea arabica* spp.) waste (WCW) generated by a local producer from the community of Zacamitla, municipality of Ixhuatlán del Café, State of Veracruz (GPS coordinates of 19° 05' 06" N and 96° 98" 41' 19" W and an elevation above sea level of 1,343 m) was kindly supplied by the Local Agricultural Association of Coffee Producers and International Center for Training in Coffee and Sustainable Development. All other reagents used were of analytical grade, and distilled water was used in all cases.

2.2 Proximate chemical composition

Moisture, protein (N × 6.25), fat, ash and fiber of WCW were determined using AOAC (2005) methods. The remaining percentage was considered to represent carbohydrates.

2.3 Extraction

WCW was dried at 50 ºC in a hot air oven HCF-62 (Riossa Digital, Mexico City, Mexico) until a final moisture content of 7 g H_2O/per 100 g (~ 48 h), and ground to a fine and homogeneous powder. A 3^2 factorial experiment was used to determine the effects of sonication time (15, 30, 45 min) and amplitude (50, 60 and 70%) on the total phenolic compounds and antioxidant activity by DPPH (see sub-section 2.7) of the extracts. Dry WCW (10 g) was added in a 1/10 w/w solid/liquid ratio with ethanol/water (70:30, v/v). An Ultrasonic Processor (Model VCX 130 PB 500/750 W, with 13 mm diam. stainless steel probe, Sonics and Materials, Inc., Newtown, CT, USA) at a frequency of 20 kHz was used to sonicate the mixtures (100 mL). Sonication was applied to the mixtures in intermittent periods of 5 min, followed by resting periods of 5 min, until total effective applied sonication time completed 15, 30 or 45 min. An ice-water bath was used to dissipate heat produced during sonication (Al-Dhabi *et al*., 2017). The extracts were centrifuged at 4000 × g for 10 min (Centrifuge 5810 R, Eppendorf, AG, Hamburg, Germany) at a temperature of 4 °C. The supernatant obtained after the centrifugation was concentrated in a rotary evaporator at 40 °C and a pressure of 40 mbar, freeze-dried (Lyophilizer Free Zone 2.5, Labconco, Corporation, MO, USA) and stored at - 18 °C until required. Several authors have pointed out that extracts with high antioxidant activity and high phenolic compounds content tend to exhibit high antifungal activity (de Rodríguez *et al*., 2017; Mirón-Mérida *et al*., 2019). So this was the reason why the extract with highest total phenolic content and DPPH antioxidant activity (EWCW) was selected for further *in vitro* and *in vivo* antifungal activity assays against *Botrytis cinerea*, *Aspergillus niger*, and *Rhizopus stolonifer*. Also, flavonoids, total tannins, antioxidant activity by ABTS and FRAP, and phenolic compounds composition by High Performance Liquid Chromatography (HPLC) were determined for EWCW.

2.4 Total phenolic content

The Folin-Ciocalteu method was used to determine total soluble phenolic content (Joaquín-Cruz *et al*., 2015). Briefly, 100 μL of EWCW was mixed with 125 μL of Folin-Ciocalteu reagent and allowed to react for 6 min. The reaction was then neutralized with 1250 μL of saturated Na_2CO_3 solution (19% w/v) and adjusted to a final volume of 3 mL with distilled water. The mixture was vortexed and stored in darkness for 90 min. The absorbance of the samples was measured at 760 nm (Genesys 10S UV-VIS spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Total phenolic content was expressed in dry weight as milligrams gallic acid equivalents/g.

2.5 Total flavonoid content

Total flavonoid content was analyzed using the aluminum chloride assay (Al-Dhabi *et al*., 2017). One mL of sample was diluted with 4 mL of distilled water and 0.3 mL sodium nitrite solution (5% w/v). The mixture was incubated for 5 min after which 0.150 mL aluminum chloride solution (10% w/v) was added. After a further 5 min, 2 mL of 1 M sodium hydroxide solution was added to the mixture. Absorbance was measured at 510 nm. Total flavonoid content was expressed in dry weight as milligrams of quercetin equivalents/g.

2.6 Total tannins

The total tannin content was determined using the potassium iodate test (Choi and Koh, 2017). Five mL of 2.5% KIO_3 solution, preheated for 7 min at 30 °C, was mixed with 1 mL of ten-fold diluted EWCW. The mixture was then placed in a water bath at 30 °C for 2 min, and absorbance was measured at 550 nm. Total tannin content was expressed in dry weight as milligrams of tannic acid equivalents/g.

2.7 Antioxidant activity

The antioxidant activity of the extracts was evaluated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, and the extract having highest total phenolic content and antioxidant activity was selected as EWCW. The antioxidant activity of EWCW was also determined using the 2,2'-azino-bis-3 ethylbenzothiazoline-6-sulphonic acid (ABTS) and ferric reducing antioxidant power (FRAP) methods.

2.7.1 DPPH assay

The DPPH method described by Brand-Williams *et al*. (1995) was followed. A solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol (DPPH solution). Then, 200 μL of WCW extract (100 mg/mL dissolved in a 70:30 v/v, ethanol/water) were added to 2.8 mL of DPPH solution. The absorbance of the samples was measured at 515 nm after incubation in darkness, at room temperature, for 30 min. A Trolox concentration vs absorbance of DPPH standard curve (0.01 a 0.1 mM) was obtained and the results were expressed in dry weight as μmoles of Trolox equivalents/g. The DPPH quenching ability was calculated as inhibition percentage of the DPPH ($I_{DPPH}\%$) absorbance, by the following equation:

$$I_{DPPH}\% = \frac{(A_0 - A_n)}{A_0} \times 100 \quad (1)$$

where A_0 is the absorbance of the blank (DPPH solution) and A_n is the absorbance of the DPPH solution + sample.

2.7.2 ABTS assay

An aqueous 7 mM solution of ABTS was combined (50:50) with a 2.45 mM solution of potassium persulfate, and stored under darkness for 16 h to allow free radical generation. The mixture was diluted with phosphate buffer solution at 75 mM (pH = 7.4) until absorbance at 734 nm was 0.7 (Re *et al*., 1999). EWCW (200 μL) was added to 2.8 mL of the ABTS stock solution in spectrophotometer cells, and absorbance was measured after incubation in darkness, at room temperature, for 10 min (Joaquín-Cruz *et al*., 2015). A standard curve of Trolox (0.1 - 1.5 mM) was obtained and the results were expressed in dry weight as μmoles of Trolox equivalents/g.

The inhibition percentage of ABTS ($I_{ABTS}\%$) was calculated with an equation similar to Eq. (1).

2.7.3 FRAP assay

The method described by Donlao and Ogawa (2018) was followed for the FRAP assay. For the preparation of FRAP reagent, 300 mM acetate buffer (adjusted to pH 3.6); 10 mM 2,4,6-tris(2-pyridyl)-1,3,5-triazine in 40 mM HCl; and 20 mM $FeCl_3$ were freshly mixed at a ratio of 10:1:1 (v/v), respectively. EWCW (200 μL) was mixed with 1.3 mL of FRAP reagent, and incubated at 37 °C for 30 min in a temperature-controlled water bath. Absorbance was measured at 595 nm using water as a blank. FRAP was expressed in dry weight as μmol FeSO4 equivalents/g dry.

2.8 Quantification of phenolic compounds by high performance liquid chromatography (HPLC)

The composition of the phenolic compounds contained in EWCW (1mg/mL methanol) was determined by HPLC analysis as described by Hečimović *et al*. (2011) using an HPLC equipment (Hewlett-Packard 1100, Palo Alto, CA, USA) provided with a quaternary pump, automatic injector, diode array detector and data treatment station. Separation was achieved on an AQUA® (Phenomenex) reverse phase C18 column (5 μm, 150 mm × 4.6 mm i.d.) at 35 °C. The mobile phase consisted of 3% formic acid (solvent A) and HPLC grade methanol (solvent B) (Merck, Darmstadt, Germany). The flow rate was 0.5 mL/min and the injection volume was 5 μL. The elution was performed with a gradient starting at 2% B to reach 32% B at 20 min, 40% B at 30 min and 95% B at 40 min, and becoming isocratic for 5 min. Detection was at 278 nm. Identification of the phenolic compounds was achieved by comparing their retention time to those of standards (gallic, chlorogenic, protocatechuic, caffeic and ferulic acids, trigonelline and caffeine; Sigma Aldrich, Toluca, State of Mexico, Mexico). The results were expressed as mg/g of EWCW.

2.9 Fungal strains

Aspergillus niger GH1, *Botrytis cinerea* G409, and *Rhizopus stolonifer* FC5 were provided by the Microbiology Laboratory of Universidad Autónoma Metropolitana - Iztapalapa (Mexico City, México), the Mycology Laboratory of the Universidad Nacional Autónoma de México (Mexico City, México) and the Microbiology Laboratory of Universidad Autónoma Chapingo (Texcoco, State of México, México), respectively, and used as target organisms in antifungal assays. The fungal strain cultures were maintained on potato dextrose agar (PDA) slants at 4 °C. The old cultures were transferred to fresh slants every two months in order to avoid a decline in strain viability.

2.10 *In vitro antifungal assays*

2.10.1 *Plate diffusion test*

The purpose of the plate diffusion test is to determine the sensibility or resistance of fungal strains to various antifungal compounds. The fungal strains are grown in PDA agar in the presence of an impregnated filter paper disc with the antifungal compound. The presence or absence of growth around the discs is an indirect measure of the ability of that compound to inhibit that specific fungus strain (Aqueveque *et al*., 2017). The samples were solubilized in sterile distilled water, and paper filter discs (Whatman No.4; 6 mm diameter) were impregnated with 45 μL of OE in different concentration (400, 600, 1500 and 2000 μg/mL). Synthetic fungicide carbendazim (1000 μg/mL) (Derosal 500 SC®, 42.8% carbendazim, Bayer de Mexico, Mexico City, Mexico) was used as positive control and then deposited on Petri dishes (5 cm in diameter) with 20 mL of the fungal inoculum (10^6 spores/mL). Plates were incubated at 37 ± 2 ºC for *Aspergillus niger*, 22 ± 2 ºC for *Botrytis cinerea*, and 25 ± 2 ºC for *Rhizopus stolonifer* and the inhibition halo around the discs was measured (cm) after 72 h (Aqueveque *et al*., 2017).

2.10.2 *Mycelial growth inhibition*

Antifungal activity of the EWCW against mycelial growth of *A. niger*, *B. cinerea*, and *R. stolonifer* was performed according to Aqueveque *et al*. (2017), with modifications. EWCW (45 μL) at different concentrations (400, 600, 1500 and 2000 μg/mL) was deposited and uniformly spread on the agar surface of Petri dishes. Aliquots (45 μL) from carbendazim (1000 μg/mL) were placed on plates as positive control. Mycelial pieces (0.5 cm diameter) scooped from the periphery of 5 day-old cultures of *A. niger*, *B. cinerea*, and *R. stolonifer*, were placed upside down on the center of each plate. Plates of PDA without EWCW or carbendazim were used as negative controls. Plates were incubated at 37 ± 2 ºC (*A. niger*), 22 ± 2 ºC (*B. cinerea*) and 25 ± 2 ºC (*R. stolonifer*), respectively. The diameter of each colony was measured after 72 h. The mycelial growth inhibition was calculated as follows:

$$\text{Mycelial growth inhibition } \% = \frac{D_c - D_o}{D_c} \times 100 \quad (2)$$

where D_c (cm) is the average of colonies diameter in negative control, D_o is the average of colonies diameter in plates with EWCW and with carbendazim.

2.10.3 *Spore germination*

The effect of EWCW (400 and 2000 μg/mL) and carbendazim (1000 μg/mL) on *A. niger*, *B. cinerea*, and *R. stolonifer* spore germination was assayed. The media were poured into 5-cm-diameter Petri dishes, then 0.05 mL spore suspension (10^6 spores/mL) of the above mentioned fungi placed on the plates center and incubated at 37 ± 2 ºC, 22 ± 2 ºC and 25 ± 2 ºC, respectively for 72 h. After 5 and 20 h of incubation, the number of germinated spores from a total of 100 spores was examined in each of three randomly selected fields under a dissecting microscope (Olympus CX23, Olympus Optical Co., Tokyo, Japan) at 400× magnification. The results were expressed as spore germination (%).

2.11 *In vivo experiments*

Strawberries (*Fragaria x ananassa*) were purchased from local market of the Municipality of Texcoco, State of Mexico, Mexico. Fruits were harvested at commercial maturity stage (only fruits with over 80% surface red color that were uniform in average weight (17 ± 2 g) without visible mechanical damage or fungal infection. Strawberries were disinfected for 10 min using a 1% NaClO solution, washed with sterile water and allowed to dry at room temperature for 10 min. Later, fruits were immersed for 3 min in EWCW aqueous solutions (400 and 2000 μg/mL). Then, the strawberries were inflicted with a 1-mm-deep wound in four points along each fruit equator with a sterile needle, and each wound was inoculated with 30 μL of spore suspension (10^5 spores/mL). Fruits were placed in polypropylene plastic boxes (20.6 ×14.6 × 5.4 mm) and then stored at 25 °C and 96% relative humidity for 5 days. The percentage of inoculated wounds that presented mold growth was calculated (Aguilar-González *et al*., 2015). Carbendazim (1000 μg/mL) was used for comparison purposes. Fruits inoculated with sterile water were used as control.

2.12 *Statistical analysis*

All experiments and measurements were performed in triplicate, using a completely randomized design. The results were submitted to Simple Classification Variance Analysis and when it was adequate to Tukey's mean comparison analysis. Multifactor analysis of variance was performed to determine which factors (amplitude and the sonication time and their interaction) had a statistically significant effect on the antioxidant activity by DPPH and total

phenolic content of the WCW extracts. The data analysis was performed using the statistical package Statgraphics Plus software (Statistical Graphics Corp., Manugistics, Inc., Cambridge, MA, USA). In all cases a significance level of $p \leq 0.05$ was established.

3 Results and discussion

3.1 Proximate chemical composition

Protein, fat, crude fiber, nitrogen-free extract and ash contents in dry basis of WCW were 17.2 ± 0.0, 1.6 ± 0.1, 44.5 ± 0.1, 33.5 ± 0.2 and 3.1 ± 0.5 g/100 g, respectively. These values are within the range of those reported for coffee pulp (11.5% of protein, 2.0% of fat, 60.5% of crude fiber and 8.9% of ash); for coffee husk (8.0% of protein, 0.5% of fat, 24% of crude fiber and 6% of ash); and for silver skin (18.6% of protein, 2.2% of fat, 62.4% of crude fiber and 4.7-7.2% of ash) (Murthy and Naidu, 2012).

3.2 Extract characterization

3.2.1 Effect of sonication

The results of the factorial design showed that as sonication time and amplitude increased, total phenolic content and DPPH antioxidant activity of the extracts increased significantly (Table 1). The interaction between sonication time and amplitude also affected significantly the total phenolic content and the antioxidant activity of the extracts. The total phenolic content ranged from 6.2 ± 0.3 mg gallic acid equivalents/g for the lowest amplitude (50%) and shortest sonication time (15 min) to 33.8 ± 0.4 mg gallic acid equivalents/g for the highest amplitude (70%) and sonication time (45 min) (Fig. 1.a). DPPH antioxidant activity ranged from 370.3 ± 21.1 μmol Trolox equivalents/g at lowest amplitude and shortest sonication time to 1790.1 ± 15.2 μmol Trolox equivalents/g at highest amplitude and longest sonication time (Fig. 1.b). These results can be attributed to the cell wall collapse due to the ultrasonic shock waves and liquid jets during cavitation (Al-Dhabi *et al.*, 2017). Ultrasound, as any other sound wave, is propagated via a series of compression and rarefaction waves induced in the molecules of the medium through which it passes, with the compression and rarefaction cycle depending on the amplitude applied (Vinatoru *et al.*, 2017). The higher the ultrasonic amplitude, greater

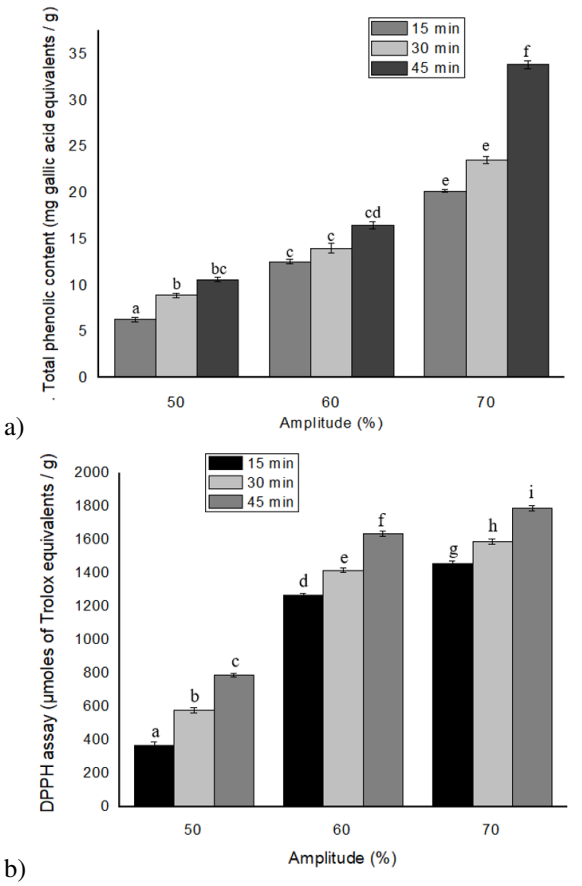

Fig. 1. Effect of the extraction variables amplitude and sonication time on: (a) total phenolic content (mg of gallic acid equivalents/g) and (b) DPPH antioxidant activity (μmol of Trolox equivalents/g). Different letters indicate significant difference between means ($p \leq 0.05$).

is the cavitation engendered, and higher the resulting extraction yield (Al-Dhabi *et al.*, 2017). Parameters such as amplitude, liquid/solid ratio, extraction temperature, sonication time and type of solvent have a direct effect on the ultrasound assisted extraction of total phenolic content. Dahmoune *et al.* (2013) found that total phenolic content optimal conditions for ultrasound-assisted extraction of lemon residues occurred at an amplitude of 77.79% and an extraction time of 15 min. Relatively longer sonication times allow for longer solvent-solid contact, which combined with the cavitation effect, leads to an increased mass transfer rate, resulting in higher extraction efficiency (Al-Dhabi *et al.*, 2017).

Based on our sonication results, an amplitude of 70% and sonication time of 45 min was selected for obtaining a WCW extract (EWCW) having the highest

Table 1. Multifactor variance analysis of the total phenolic content and DPPH antioxidant activity of the extracts obtained from the wet processing coffee waste.

Parameter	Amplitude (%)			Sonication time (min)		
	50	60	70	15	30	45
TPC (mg gallic acid equivalents/g)	8.5 ± 0.7^a	14.3 ± 0.7^b	26.1 ± 0.7^c	13.2 ± 0.7^a	15.4 ± 0.7^b	20.2 ± 0.7^c
DPPH (μmol Trolox equivalents/g)	648.3 ± 8.1^a	1621.6 ± 21.0^b	1717.0 ± 36.9^c	1209.7 ± 12.6^a	1367.1 ± 36.9^b	1411.1 ± 31.4^c

^{a,b,c}Different superscripts in the same row indicate significant difference between means ($p \leq 0.05$).

total phenolic content and DPPH antioxidant activity, which was selected for further evaluation of its *in vitro* and *in vivo* antifungal activity.

3.2.2 Total phenolic content and antioxidant activity

The total phenolic content of EWCW (33.8 ± 0.4 mg gallic acid equivalents/g) was significantly higher than that reported for coffee cherry pulp (9.7 mg gallic acid equivalents/g) (Hegger *et al*., 2017), and for coffee husk (1.0 mg gallic acid equivalents/g) (Moreira *et al*., 2018), both extracted with warm water.

Antioxidant properties of extracts from food matrices are usually evaluated using more than one method, because antioxidants can act by different mechanisms, and a single assay can only provide a partial reductive idea of the antioxidant properties (Leopoldini *et al*., 2011). Moreover, given the chemical complexity of plant extracts, which are often made up by dozens of compounds with different functional groups, polarity, and chemical behavior, it is convenient to employ different assays so that a wider picture of the possible potential applications might be derived (Sacchetti *et al*., 2005).

The molecular basis for the antioxidant properties of polyphenols arises from the direct reaction with free radicals, and from the chelation of free metals (which ultimately are involved in reactions generating free radicals) (Leopoldini *et al*., 2011). In this work the antioxidant activity of EWCW was assessed by DPPH, ABTS and FRAP. Antioxidant activity (μmol Trolox equivalents/g) for EWCW was of 1790.1 ± 15.2 for DPPH and of 2000.6 ± 20.9 for ABTS, while it was of 1295.0 ± 10.8 μmol $FeSO_4$/g for FRAP. The DPPH and ABTS % inhibition of EWCW were 93.6 ± 0.5% and 97.4 ± 0.4%, respectively. Andrade *et al*. (2012) reported an ABTS antioxidant activity of 381 μmol Trolox equivalents/g for coffee husk extracted with ethyl acetate, while Magoni *et al*. (2018) reported a DPPH antioxidant activity of 141.7 μmol Trolox equivalents/g for coffee pulp extracted with a water-ethanol (30:70% w/w) mixture as solvent. Composition of the WCW (coffee pulp and husk) and the extraction conditions could be responsible for the differences between our results and those of the aforementioned authors. The recovery of polyphenols and compounds with antioxidant properties from plant materials is influenced by the solubility of the phenolic compounds in the solvent used for the extraction process (Jimenez *et al*., 2011). Al-Dhabi *et al*. (2017) reported that the liquid/solid ratio, extraction temperature, ultrasound power and the sonication time significantly affect the total phenolic content yield and antioxidant activity of the plant extracts.

Torres-Valenzuela *et al*. (2020) reported I_{DPPH}% and I_{ABTS}% activity of 45 and 91% in coffee pulp extracts with supramolecular solvents. The process variables used for extraction reactions, such as the reaction time, temperature, liquid/solid ratio, and solvent nature usually have great influence both on the kinetics of phenolic compounds release from the solid matrix as well as on the antioxidant activity of the produced extracts (Ballesteros *et al*., 2017). Phenolic (hydroxycinnamic acids, feruloylquinic acids, and other chlorogenic acids) and nonphenolic compounds might contribute to the antioxidant activity (Bravo *et al*., 2012).

3.2.3 Total flavonoids and total tannins contents

Flavonoids and tannins are secondary metabolites in plants that have antioxidant capacity and diverse bio-functionalities. Such properties make possible the application of these compounds in different areas (Ballesteros *et al*., 2017; Sharma *et al*., 2019). In

particular, plant extracts having high contents of these compounds have been tagged as excellent antifungal agents (de Rodríguez *et al*., 2017). Total flavonoids and total tannins contents of EWCW were 9.1 ± 0.5 mg quercetin equivalents/g and 11.6 ± 0.1 mg tannic acid equivalents/g, respectively. Total flavonoids content was lower than 11.4 mg of quercetin equivalents/g reported for coffee cherry pulp (Do *et al*., 2019), while total tannins content found was higher to that reported by Murthy and Naidu (2012) for pulp coffee (3.0 mg of acid tannic equivalents/g).

3.2.4 Determination of phenolic and nitrogenous compounds

The phenolic and nitrogenous compounds of EWCW were identified by the relative retention times (Table 2). Quantification of these compounds was performed with the calibration curve of the respective standards. The major phenolic compounds found (mg/g) in EWCW were: chlorogenic acid (14.19), caffeic acid (1.18), gallic acid (0.78), protocatechuic acid (0.73), ferulic acid (0.23) and nitrogenous compounds including caffeine (1.2) and trigonelline (0.16). Chlorogenic acid and caffeic acid were the dominant polyphenols, representing 89.83% of the identified phenolic compounds.

Antioxidant activity of coffee depends on the phenolic compounds characteristics, and in particular, on chlorogenic acid content, which possesses strong *in vitro* and *in vivo* antioxidant capacity, and high *in vitro* bioavailability (Jeszka-Skowron *et al*., 2016). Chlorogenic acid has a wide number of biological activities, including hypoglycemic, hepatoprotective, antiviral, antibacterial, anti-carcinogenic, and antiinflammatory activities, which are mostly related to its potent antioxidant activity (Ballesteros *et al*., 2017; Ruiz-Palomino *et al*., 2019). Chlorogenic acid can also exert antifungal activity. It has been reported that it is able to inhibit the growth of pathogenic fungi such as *Fusarium solani* f. sp. *eumartii*, *Sclerotinia sclerotiorum*, *B. cinerea* and *Cercospora sojina* among others (Martínez *et al*., 2017). The chlorogenic acid content in this work was significantly higher than the 1.0-4.0 mg/g found for *Coffea arabica* pulp (Heeger *et al*., 2017). The variations in phenolic compounds and differences in their contents in coffee cherry pulp have been attributed mainly to differences within the execution of the wet processing or other factors like growing conditions (altitude, climate, soil and agricultural practices) or harvest time (Heeger *et al*., 2017).

Table 2. Phenolic and nitrogenous compounds contents in the selected extract obtained from the wet processing coffee waste (EWCW) by using HPLC.

Compound	Retention time (min)	Content (mg/g)
Chlorogenic acid	2.6	14.19
Caffeic acid	7.7	1.18
Gallic acid	1.8	0.78
Protocatechuic acid	2.4	0.73
Ferulic acid	6.7	0.23
Trigonelline	1.4	1.20
Caffeine	3.0	0.16
Total		18.47

Caffeic acid is a secondary metabolite synthetized by plants, and its natural and synthetic derivatives show potent antioxidant activity, even at low concentrations. Moreover, it has been proved in many biological investigations that caffeic acid and its analogues also display anti-inflammatory, antibacterial, antiviral, antitumor (Sidoryk *et al*., 2018) and antifungal activities (Sardi *et al*., 2016). Gallic acid isolated from methanol extracts of the fruits of *Diospyros virginiana* L. exhibited good antifungal activity against *Aspergillus fumigatus*, *Aspergillus versicolor*, *Aspergillus ochraceus*, *Aspergillus niger*, *Trichoderma viride*, *Penicillium funiculosum*, *Penicillium ochrochloron*, and *Penicillium* var. *cyclopium* (Rashed *et al*., 2014). The caffeine values recorded in this work were lower than caffeine contents (3.5-7.0 mg/g) found in coffee cherry pulp by Heeger *et al*. (2017). Caffeine has been reported to inhibit the growth of the mutualistic fungus of *Atta sexdens rubropilosa* (Miyashira *et al*., 2012). The main components in coffee pulp were reported as being ferulic acid, protocatechuic acid, chlorogenic acid, 3-p-coumaroylquinic acid and 3-feruloylquinic acid by Londoño-Hernandez *et al*. (2020), and chlorogenic acid, ferulic acid, protocatechuic acid, and nitrogenous compounds including trigonelline and caffeine (Duangjai *et al*., 2016). In our study we found that EWCW contained caffeic and gallic acids which have not been reported by other authors for *Coffea arabica*.

3.3 In vitro experiments

Table 3 shows the inhibition halo around the discs (in cm) measured after 72 h. The activities were classified according to the diameter of the inhibition halos as:

Table 3. Inhibition of fungal growth by EWCW in disk diffusion

Fungal strain	Treatment (μg/mL)	Diameter of the inhibition halo (cm)
A. niger	$EWCW_{400}$	3.1 ± 0.1^{a}
	$EWCW_{600}$	5.0 ± 0.1^{b}
	$EWCW_{1500}$	5.0 ± 0.0^{b}
	$EWCW_{2000}$	5.0 ± 0.0^{b}
	$Carbendazim_{1000}$	3.2 ± 0.1^{a}
B. cinerea	$EWCW_{400}$	2.1 ± 0.3^{a}
	$EWCW_{600}$	2.8 ± 0.2^{b}
	$EWCW_{1500}$	3.7 ± 0.0^{c}
	$EWCW_{2000}$	4.9 ± 0.1^{d}
	$Carbendazim_{1000}$	4.0 ± 0.3^{c}
R. stolonifer	$EWCW_{400}$	0.7 ± 0.1^{a}
	$EWCW_{600}$	0.8 ± 0.0^{a}
	$EWCW_{1500}$	2.3 ± 0.1^{b}
	$EWCW_{2000}$	3.8 ± 0.3^{d}
	$Carbendazim_{1000}$	3.3 ± 0.1^{c}

Forty five μl of EWCW at the specified concentrations were added to the disks. Subindexes in the treatment column refer to concentration used. ^{a,b,c,d}Different superscripts within the same column indicate that the means differ significantly ($p \leq 0.05$).

Active =1.5 cm or more, moderate =1.0-1.5 cm, and weak =less than 1.0 cm or diffuse (Aqueveque *et al*., 2017). The carbendazim (1000 μg/mL) control showed an active antifungal activity against the three fungi, with halo inhibition diameters ranging from 3.2-4.0 cm. The antifungal activity of EWCW against *A. niger* was significantly higher at any concentration than that of carbendazim, except the 400 μg/mL treatment, which was non-significantly different. The antifungal activity of EWCW at 2000 μg/mL was significantly more effective against *B. cinerea* and *R. stolonifer* than the carbendazim control.

EWCW exhibited a superior mycelial growth inhibition against *A. niger* in comparison to the carbendazim control, except the treatment at 400 μg/mL, which was non-significantly different (Table 4). Mycelial growth inhibition ranged from 89.1-99.7%. On the other hand, 2000 μg/mL EWCW treatment was significantly more efficient against *B. cinerea* and comparable against *R. stolonifer* than the carbendazim treatment. Based on these results the effect of EWCW on spore germination was studied using 400 μg/mL for *A. niger* and 2000 μg/mL for *B. cinerea* and *R. stolonifer*. Table 5 shows that independently of the fungi strain, spore germination was significantly lower for EWCW than for carbendazim after either 5 or 20 h. In order to quantify the rate of change in spore germination, the slope of (Δ spore germination = $\%/\Delta t_{5-20h}$) was determined. Interestingly, it can be appreciated that for all the fungi the slope was smallest for EWCW than for carbendazim, which were for *A. niger* (0.34 and 0.68 %/h), for *B. cinerea* (0.35 and 0.65 %/h), and for *R. stolonifer* (0.87 and 3.15 %/h), respectively.

The antifungal activity of EWCW seems to be due to the phenolic compounds identified in this work (Sub-section 3.2.4). These compounds have been reported as having the capability of breaking-down the cell wall of pathogenic fungi (Sung and Lee, 2010). It has been shown what a high concentration of phenolic compounds and a consequent high antioxidant activity of the extracts, promotes in turn a high antifungal activity (de Rodríguez *et al*., 2017; Mirón-Mérida *et al*., 2019). Moreover, the phenolic compounds found in higher concentration in the extract are considered as being responsible for the antifungal activity (Martínez *et al*., 2017; Sung and Lee, 2010). Specifically, chlorogenic acid, our major phenolic compound, affects fungi cells by injuring their membranes, thus dissipating the electrical potential of the membrane (Sung and Lee, 2010). Martínez *et al*. (2017) reported that chlorogenic induced a fast membrane permeabilization in fungal spores, which caused loss of viability. Likewise, Ahmad and Matsubara (2019) found that the antifungal activity of caffeic acid against *Fusarium oxysporum* f. sp. *asparagi* was exerted through the breakage of intercept

Table 4. Effects of different concentrations of the extract (EWCW) on mycelial growth inhibition.

Fungal strains	Concentration (μg/mL)	Mycelial Growth Inhibition (%)
A. niger	$EWCW_{400}$	89.1 ± 7.3^{a}
	$EWCW_{600}$	99.3 ± 10.3^{b}
	$EWCW_{1500}$	99.5 ± 11.3^{b}
	$EWCW_{2000}$	99.7 ± 12.3^{b}
	$Carbendazim_{1000}$	90.1 ± 6.3^{a}
B. cinerea	$EWCW_{400}$	62.0 ± 2.3^{a}
	$EWCW_{600}$	70.2 ± 3.4^{b}
	$EWCW_{1500}$	74.1 ± 4.4^{b}
	$EWCW_{2000}$	97.3 ± 10.1^{d}
	$Carbendazim_{1000}$	83.3 ± 4.1^{c}
R. stolonifer	$EWCW_{400}$	14.6 ± 1.1^{a}
	$EWCW_{600}$	51.0 ± 4.7^{b}
	$EWCW_{1500}$	71.0 ± 9.5^{c}
	$EWCW_{2000}$	88.7 ± 11.2^{d}
	$Carbendazim_{1000}$	81.1 ± 10.2^{d}

Subindexes in the treatment column refer to concentration used. ^{a,b,c,d}Different superscripts within the same column indicate that the means differ significantly ($p \leq 0.05$).

in the mycelia of fungi and cell surface damage through pilferage. de Rodríguez *et al*. (2017) stated that the mechanism of phenols against fungal pathogens is related with enzymatic inhibition through its oxidation. The presence of hydroxyl groups in phenolic compounds could link with sulphydryl groups in fungal proteins, changing the protein conformation of the cell wall (Elfirta *et al*., 2018). Other mechanism by which compounds exert their antifungal activity is through the degradation of cell walls of pathogenic fungi. In particular, gallic acid may produce the enzyme chitinase that degrades chitin, the major structural component of the cell walls of phytopathogenic fungi (Seo *et al*., 2013). Wang *et al*. (2016) demonstrated that caffeine destroys the fungal protective layers (cell wall and cell membrane) and subcellular organelles. Also, a synergistic or complementary effect between phenolic compounds may occur, so that the effect of these interactions could be very useful in the fight against pathogens (de Rodríguez *et al*., 2017; Elfirta *et al*., 2018).

3.4 In vivo experiments

Strawberries were wounded in four sites along the equatorial diameter and inoculated with *A. niger*, *B. cinerea* and *R. stolonifer*, and stored for 5 days at 25 °C. At the end of storage, the percentage of wounds presenting mold growth was determined. The percentage of wounds presenting mold growth with the sterile water control was significantly higher than for EWCW and carbendazim, independently of the fungi strain. In the case of *A. niger*, the wounds with mold growth was 65.7 ± 4.1% for sterile water, which was significantly lowered to 42.3 ± 0.5% for carbendazim and to 16.7 ± 1.1% for EWCW (400 μg /mL). For *B. cinerea*, the percentage of wounds was also significantly lowered to 36.1 ± 1.1% when using EWCW (2000 μg /mL) and to 38.2 ± 4.1% with carbendazim, compared to the 74.2 ± 8.1% sterile water control. Lastly, for *R. stolonifer* the percentage of wounds (70.4 ± 0.25%) occurring with sterile water were lowered to 50.1 ± 1.3% EWCW (2000 μg/mL) and to 51.2 ± 0.05% with carbendazim. These results are indicative of the efficacy of OE for inhibiting mold growth of different fungi strains.

B. cinerea is considered the primary pathogen of harvested strawberries, which causes grey mold in fruit (Petrasch *et al*., 2019). Often, *B. cinerea* can develop from rotted fruit to nearby healthy fruit, causing extensive breakdown of a commodity, and sometimes spoilage of entire batches (Feliziani and Romanazzi, 2016). *R. stolonifer* is recognized as one of the fastest growing and most destructive fungi. The postharvest diseases caused by it are known as soft rot, black rot and leak. In strawberry, the main symptom of soft rot is the deliquescence of the tissues, which lose their consistency, become water soaked, and exude a leachate (Feliziani and Romanazzi, 2015). *Aspergillus spp*., *Alternaria spp*. and *Cladosporium spp*. are the causal agents of postharvest diseases that generally show negligible levels; however, their incidence has

Table 5. Effects of different concentrations of the extract (EWCW) on spore germination (%).

Fungal strains	Treatments (μg/mL)	Time (h)	Spore germination (%)
A. niger	$EWCW_{400}$	5	2.4 ± 0.3^{b}
		20	7.5 ± 0.2^{c}
	$Carbendazim_{1000}$	5	2.8 ± 0.0^{b}
		20	13.0 ± 1.5^{b}
B. cinerea	$EWCW_{2000}$	5	3.6 ± 0.4^{b}
		20	10.2 ± 0.2^{c}
	$Carbendazim_{1000}$	5	5.0 ± 0.5^{a}
		20	14.7 ± 1.3^{b}
R. stolonifer	$EWCW_{2000}$	5	4.9 ± 0.2^{c}
		20	18.1 ± 1.1^{c}
	$Carbendazim_{1000}$	5	8.0 ± 0.8^{b}
		20	58.2 ± 0.7^{b}

^{a,b,c}Different superscripts in the same column indicate significant difference ($p \leq 0.05$) between means.

increased among cultivars that show resistance to gray mold (Feliziani and Romanazzi, 2016). *A. niger* is considered one of the fungi that causes black rot, a postharvest disease that invades and destroys the plant tissue of the fruit (Guédez *et al*., 2009).

Conclusions

In vitro and *in vivo* results demonstrated the efficacy of EWCW against *A. niger*, *B. cinerea* and *R. stolonifer*. Chlorogenic and caffeic acids were the main compounds identified in the extract, which showed antifungal activity. In this work, for the first time, the antifungal and antioxidant properties of wet processing coffee waste extracts are described.

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