Eco-friendly Wood-biofungicidal and Antibacterial Activities of Various *Coccoloba uvifera* L. Leaf Extracts: HPLC Analysis of Phenolic and Flavonoid Compounds

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Aqueous, acetone, and ethanol extracts of *Coccoloba uvifera* L. (Polygonaceae) leaves were assessed for their antibacterial and antifungal activities. The fungal pathogens *Fusarium culmorum*, *Rhizoctonia solani,* and *Botrytis cinerea* were isolated from strawberry plants, and they were molecularly identified through internal transcribed spacers (ITS) sequence analysis. Wood treated with ethanol extract at 3% showed the highest inhibition of *R. solani*, *B. cinerea,* and *F. culmorum* growth, with mycelial growth inhibited by 64.4%, 100%, and 38.5%, respectively. Moderate growth inhibition was found against the plant pathogenic bacteria *Agrobacterium tumefaciens*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Erwinia amylovora*, *Ralstonia solanacearum*, *Pectobacterium atrosepticum*, and *Dickeya solani*. High-performance liquid chromatography analysis identified the phenolic and flavonoid compounds in the extracts. Regarding phenolic acid compounds, benzoic, ellagic, gallic, and *o*-coumaric acids were found as the main compounds in ethanol, acetone, and aqueous extracts. Regarding flavonoids, rutin, myricetin, and quercetin were identified in aqueous, acetone, and ethanol extracts. The results suggesting that the extracts can be used as environmentally friendly bioagents.

Keywords: Coccoloba uvifera leaves; Phenolic compounds; Flavonoid compounds; HPLC analysis; Antimicrobial activity

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INTRODUCTION

Natural extracts from various species of the genus *Coccoloba* (approximately 120 to 150 species), have been reported to have antimicrobial activities (Li *et al.* 1999; Perez *et al.* 2001; Cota *et al.* 2003; Meléndez and Capriles 2006; Sharma *et al.* 2017). These biological activities have been revealed to be due to the presence of phenolic or flavonoid-type compounds (Compagnone *et al.* 1995; Li *et al.* 1999; Campos *et al.* 2015; Povi *et al.* 2015), terpenoids (Cota *et al.* 2003), benzenoids (Li *et al.* 1999), and carboxylic acids and esters (Shaw *et al.* 1992). EL-Hefny *et al.* (2019) suggested the potential uses of essential oil and recovery oil from the fresh flowers *Matricaria chamomilla* as environmentally

friendly bio-fungicides against *Aspergillus niger*, *A. flavus*, *A. terreus*, and *Fusarium culmorum*.

Coccoloba uvifera L. belongs to the Polygonaceae family, and it is found naturally in the Antilles, the Bahamas, the South American tropical places, and on the Venezuelan coast, where it is commonly known as "sea grape". Its leaves have been used to treat dysentery, diarrhea, asthma, wounds, and skin diseases (Adonizio *et al*. 2006; Boulogne *et al*. 2011). The ethyl acetate fraction from the methanolic extract of *C. uvifera* L. seeds contains a tannic compound (gallic acid), an organic acid (hexenedioic acid), and a benzopyran (1,3,4,6,7,8–hexahydro-4,6,6,8,8,8-hexamethylcyclopenta-2-benzopyran) having antifungal activities against *Candida albicans*, *Fusarium oxysporum*, and *F. decencellulare* as well as antibacterial activities against *Salmonella typhimurium* and *Staphylococcus aureus* (Moreno-Morales *et al*. 2008).

Anthocyanins, ascorbic acid, phenolic compounds, and flavonoids with free radical scavenging and antioxidant properties have been identified in fruit extracts of *C. uvifera* (Campos *et al*. 2015). In addition, the ethanol and water extracts of *C. uvifera* leaves have effective antioxidant agent, as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and weak antibacterial properties (Kaewpiboon *et al*. 2012). Emodin, chrysophanol, physcion, rhein, royleanone, α-amyrin, and β-sitosterol have also isolated from the extracts of shade-dried *C. uvifera* leaves (Malathi *et al*. 1995).

Potato bacterial pathogens are responsible for serious plant and tuber damages. *Dickeya* and *Pectobacterium* bacterial species are characterized as potato pathogens, and cause soft rot disease in tubers, as well as blackleg and wet rot diseases in stems (Van der Wolf and De Boer 2007; Ashmawy *et al*. 2014, 2015a, 2020; Behiry *et al*. 2018a). *Pectobacterium atrosepticum* and *Dickeya* blackleg symptoms appear to spread as slim wet and rotted-black lesions from the parent tuber to the stems under humid conditions (Pitman *et al*. 2010; Ashmawy *et al*. 2015a). Furthermore, *Ralstonia solanacearum*, a bacterial wilt and potato brown disease agent, is classed as one of the most severe Egyptian bacterial plant diseases (Behiry *et al*. 2018b; Mohamed *et al*. 2019).

Erwinia amylovora, the causal agent of fire blight disease, is one of the most destructive bacteria that can attack apple and pear fruit trees, and pear plantations in Egypt (Ashmawy *et al*. 2015b). *Agrobacterium tumefaciens* (synonym *Rhizobium radiobacter*) is the causal agent of crown gall disease in over 140 species of dicots (Young *et al*. 2001), including many trees, as well as grassy plants (DeCleene and DeLey 1976).

Black root rot is a serious disease triggered by one or more fungal genera, including *F. oxysporum* (Juber *et al*. 2014), *Pythium* spp. (Abdel-Sattar *et al*. 2008), *Phytophthora* spp. (Mingzhu 2011), and *Rhizoctonia* spp. (Fang *et al*. 2013).

Several synthetic chemical substances that are deemed to efficiently and effectively control many plant pathogens can cause serious injury to crops, particularly citrus. The continued use of these residual toxic synthetic bactericides leads to soil and water pollutions (Pimentel and Levitan 1986). Consequently, the use of plant extracts or the essential oils to combat bacterial and fungal plant diseases has become a significant component of integrated pest management, as they are environmentally friendly natural bactericides (EL-Hefny *et al*. 2017a, 2017b; Ashmawy *et al*. 2018a, 2018b; Behiry *et al*. 2019a; Okla *et al*. 2019; Behiry *et al*. 2020; Mohamed *et al*. 2020).

Although the application of chemical compounds has serious detrimental effects on environmental and human health, it can sometimes accomplish significant results. This is why manufacturers struggle to stop and substitute these hazardous chemicals with less harmful products (Ahmed and El-Fiki 2017). Synthesized substances are limited in their

usefulness because of their excessive toxicity and because grey mold fungicides are usually applied at least one week before harvest, and this is deemed unacceptable. As a potential solution, it is possible to control strawberry grey mold disease with natural products and immunity inducers, which can increase plant defense (Awad 2017).

The aim of the present study was to evaluate the antimicrobial activities of different solvent extracts from *C. uvifera* leaves against the growth of some phytopathogenic bacterial and fungal strains. Furthermore, to identify the phenolic/caffeine and flavonoid type of compounds in the leaf extracts using high-performance liquid chromatography (HPLC) analysis.

EXPERIMENTAL

Materials

Extraction and preparation of Coccoloba uvifera L. leaf extracts

Coccoloba uvifera L. leaves were collected from Alexandria, Egypt during January 2018, and were washed using tap water. The leaves were then air-dried for two weeks under laboratory room conditions before being ground into small pieces using a small laboratory mill. The ground leaf materials were divided into three groups, fifty grams for each; the first group was soaked with distilled water (200 mL), the second soaked with 90% acetone (200 mL), and the third soaked with 96% ethanol (200 mL) for one week (Salem *et al.* 2019b). At the end of the extraction process, the soaked materials were filtered using Whatman No.1 filter paper. The solvents were removed using a rotary evaporator at 45 °C (Salem *et al.* 2013). The crude extracts were stored in sealed vials at 4 °C until further use.

Standard chemicals used

Gallic acid, catechol, *p*-hydroxy benzoic acid, caffeine, vanillic acid, caffeic acid, syringic acid, vanillin, *p*-coumaric acid, ferulic acid, ellagic acid, benzoic acid, *o*-coumaric acid, salicylic acid, and cinnamic acid were used as the standard compounds for the phenolics/caffeine, and rutin, myricetin, quercetin, naringenin, kaempferol, and apigenin were used for flavonoid compounds. All the chemical compounds were provided from Sigma-Aldrich (Darmstadt, Germany), and the analyses were performed at FSQC Laboratory (Cairo University, Faculty of Agriculture, Giza, Egypt).

Preparation of wood blocks

Pinus roxburghii wood blocks with dimensions of 1 × 1 × 0.5 cm^3 were prepared at the Department of Forestry and Wood Technology, Alexandria University (Alexandria, Egypt). The blocks were autoclaved at 121 °C for 20 min and then cooled.

Methods

Analytical HPLC of phenolic/caffeine and flavonoid compounds

Phenolic/caffeine-type compounds were identified using an Agilent 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) HPLC series (Agilent Technologies, Santa Clara, CA, USA), equipped with a Quaternary pump and a Zorbax Eclipse plus C18 column (100 mm × 4.6 mm i.d.). An HPLC Smartline (Knauer, Berlin, Germany) equipped with a binary pump and a Zorbax Eclipse plus C18 (column 150 mm × 4.6 mm i.d.) (Agilent Technologies, Santa Clara, CA, USA) was used for identifying flavonoid compounds. The

conditions used to operate the apparatus can be found in the authors' previous published works (Al-Huqail *et al.* 2019; Behiry *et al.* 2019b; Salem *et al.* 2019b).

Antifungal Activities of *Pinus roxburghii* Wood Treated with Leaf Extracts

Isolation of the root rot and grey mold pathogens

Fungal pathogens isolated from infected plant samples were retrieved from the most vital strawberry-producing region in the district of Bader, Behiera Governorate, Egypt. The strawberry root and fruit tissues that were symptomatic parts of root rot and grey mold fungus were isolated on potato dextrose agar (PDA) medium. The resultant cultures were purified using single spore culture or hyphal tip techniques (Dhingra and Sinclair 1985). The fungal isolates were transferred to slant tubes containing PDA medium and were incubated for one week at room temperature. The pure cultures were examined microscopically, and they were morphologically identified at the Agricultural Botany Department, Faculty of Agriculture Saba Basha, and Plant Pathology Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt. Samples were further molecularly identified.

Identification of tested fungi through internal transcribed spacers (ITS) gene sequencing

Isolates were grown for one week on PDA at 25 °C. Total DNA was extracted from fresh mycelia using the QIAquick PCR purification Kit (QIAGEN, Manchester, England). Amplicons of the internal transcribed spacer region of the rDNA (ITS genes) were generated using ITS1/ITS4 primers and were sequenced (White *et al.* 1999; Geiser *et al.* 2004). Forward sequences were assembled at Macrogen Co., Seoul, Korea, and were then accessioned and deposited in GenBank.

Antifungal activity tests

Extracts were dissolved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Darmstadt, Germany) and were prepared at concentrations of 1%, 2%, and 3% solutions. The antifungal activities of *C. uvifera* leaf extracts (aqueous, acetone, and ethanol extracts) were assayed against the growth of the three isolated phytopathogenic fungi (*Rhizoctonia solani*, *Fusarium culmorum*, and *Botrytis cinerea*). Wood samples of *Pinus roxburghii* were treated with different concentrations (1%, 2%, and 3%) of the various *C. uvifera* leaf extracts. Three wood samples were used to treat with each fungus (Mansour and Salem 2015), and each wood sample received approximately 100 µL of the concentrated extracts (Salem *et al.* 2019a). The wood samples treated with 10% DMSO were used as a negative control.

Treated wood samples were placed directly on PDA medium in petri dishes inoculated with 5-mm diameter discs of 15-day-old PDA culture from each fungus. The petri dishes were incubated for one week at 25 ± 1 °C. The linear fungal growth was measured and compared to control treatments using the margin around the wood samples with no fungal growth (Povi *et al.* 2015; Mansour *et al.* 2015; Salem *et al.* 2016a,b, and 2019b). Mycelial growth inhibition (%) was calculated using Eq. 1,

$$\text{Mycelial growth inhibition (\%)} = \left(\frac{A0 - At}{A0}\right) \times 100 \quad (1)$$

where A_0 and A_t are the average diameters (mm) of fungal colonies under the control and experimental treatments, respectively.

Antibacterial activity assays

Six plant pathogenic bacteria were provided by the Bacterial Plant Diseases Laboratory, Plant Pathology Department, Faculty of Agriculture, Alexandria University, Alexandria, Egypt. The bacterial strains *Agrobacterium tumefaciens* (MG706145), *Erwinia amylovora* (HG423347), *Ralstonia solanacearum* (GH425351), *Pectobacterium carotovorum* subsp. *carotovorum* (HF674984), *Pectobacterium atrosepticum* (MG706146), and *Dickeya solani* (HF569035) were previously identified using the 16S rRNA gene, and were deposited into GenBank under the accession numbers listed above (Ashmawy 2015b; Salem *et al.* 2018). These bacterial strains were used to evaluate the antibacterial activities of *C. uvifera* leaf extracts.

The antibacterial activities of aqueous, acetone, or ethanol *C. uvifera* leaf extracts were assayed using the agar disk diffusion method (Kiehlbauch *et al.* 2000). Extracts with concentrations of 50, 125, 250, 500, 1250, and 2500 µg/mL were made by dissolving extracts in 10% DMSO, and three discs were used for each concentration. Each disc received 20 µL of a concentrated extract, while discs also received 20 µL of the solvent used (10% DMSO) as negative controls. The antibacterial activities of the extracts were compared with positive controls of amoxicillin (25 µg/disc), chloramphenicol (30 µg/disc), and tobramycin (10 µg/disc). All discs were placed directly onto the solid media plates that were inoculated with the bacterium suspension (0.1 mL of 10^8 CFU/mL) and were incubated at 30 °C for three days before comparisons were made. The inhibition zones around the treated discs were recorded in mm.

Statistical Analysis

The mycelial growth inhibition percentages for fungi and the inhibition zones recorded for the studied bacterial phytopathogens were statistically analyzed using two-way analysis of variance (ANOVA) with SAS software (v.8.02, SAS Institute, Cary, NC, USA). Comparisons among means were compared against the negative and/or positive control treatments using least significant difference (LSD 0.05) test.

RESULTS AND DISCUSSION

Phenolic and Flavonoid-type Compounds

Table 1 lists the phenolic and flavonoid-type compounds identified in the aqueous, acetone, and ethanol extracts. In the aqueous extracts (Fig. $1A_1$), the main identified phenolic compounds were benzoic acid, gallic acid, ellagic acid, caffeine, and *o*-coumaric acid. In the acetone extracts (Fig. $1B_1$), the predominant phenolic compounds were benzoic acid, ellagic acid, gallic acid, *o*-coumaric acid, *p*-coumaric acid, caffeine, salicylic acid, and *p*-hydroxy benzoic acid. Finally, the primary phenolic compounds in the ethanol extracts (Fig. $1C_1$) were benzoic acid, ellagic acid, gallic acid, *o*-coumaric acid, and *p*-coumaric acid.

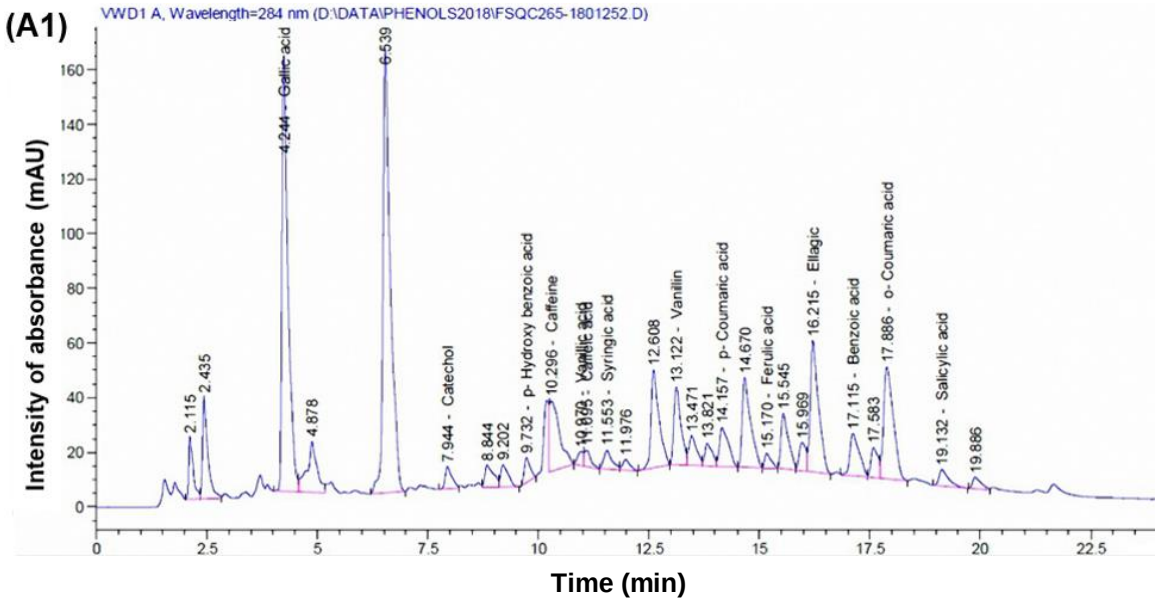
In terms of flavonoid-type compounds, all three extracts contained rutin (816 mg, 12054 mg, and 53061 mg, in 100 g of aqueous, acetone, and ethanol extracts, respectively), myricetin (489 mg, 1753 mg, and 10271 mg, in 100 g of aqueous, acetone, and ethanol extracts, respectively), and quercetin (41.9 mg, 118 mg, and 477 mg in 100 g of aqueous, acetone, and ethanol extracts, respectively). The flavonoid compounds found in aqueous, acetone, and ethanol extracts are summarized in Figs. $1A_2$, $1B_2$, and $1C_2$, respectively.

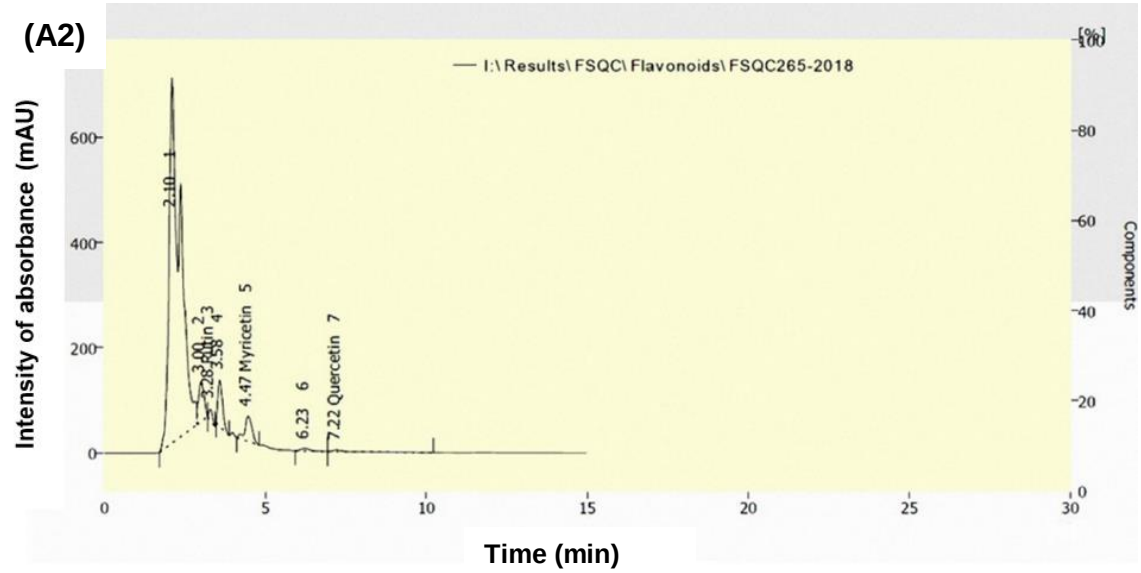
The above results showed that ethanol extracts contained the greatest amounts of gallic acid, ellagic acid, hydrolysable tannin, and flavonoid-types of compounds. In contrast, benzoic acid was observed in the highest quantities in acetone extracts, followed by ethanol extracts.

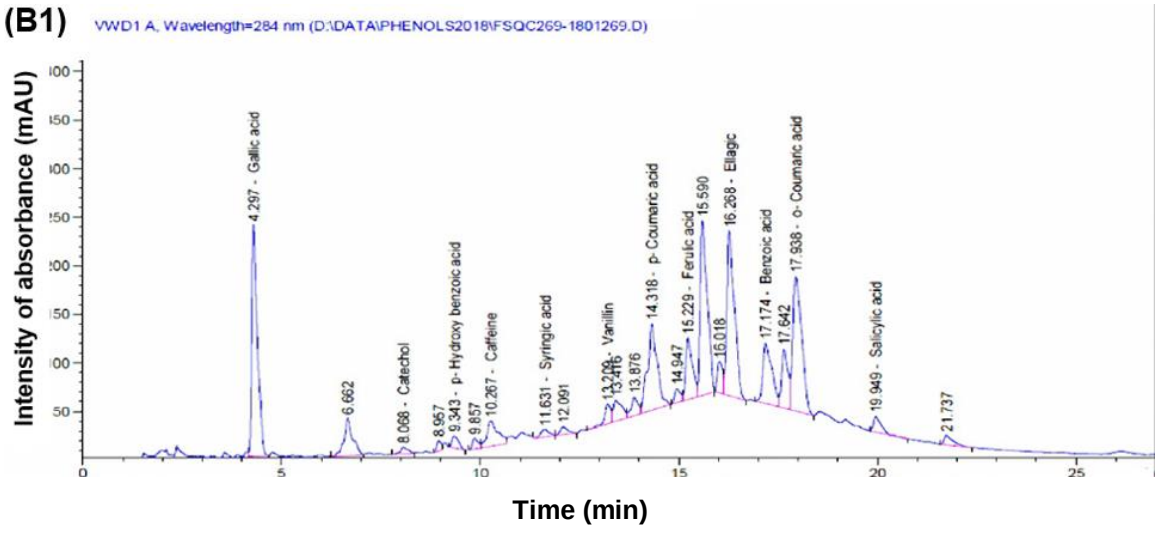
Table 1. HPLC Chemical Composition Analysis of Phenolic and Flavonoid Compounds in Aqueous, Acetone, and Ethanol *C. uvifera* Leaf Extracts

Compound	Extract (mg/100 g)		
	Aqueous	Acetone	Ethanol
Phenolic/Caffeine			
Gallic acid	90.68	139.68	213.30
Catechol	17.61	19.82	22.65
p-Hydroxy benzoic acid	18.74	33.73	26.55
Caffeine	32.96	41.07	15.32
Vanillic acid	3.69	ND	7.94
Caffeic acid	2.49	ND	ND
Syringic acid	5.27	6.80	6.58
Vanillin	16.01	10.80	15.88
p-Coumaric acid	5.84	43.19	33.76
Ferulic acid	2.71	38.87	28.65
Ellagic acid	89.27	322.51	327.25
Benzoic acid	180.39	777.16	694.16
o-Coumaric acid	20.77	70.34	66.09
Salicylic acid	14.39	36.57	32.27
Cinnamic acid	ND	ND	ND
Flavonoid			
Rutin	816.66	12054.91	53061.54
Myricetin	489.14	1753.72	10271.96
Quercetin	41.87	118.22	476.88
Naringenin	ND	ND	ND
Kaempferol	ND	ND	ND
Apigenin	ND	ND	ND

ND: Not determined


(A1)
VWD1 A, Wavelength=284 nm (D:\DATA\PHENOLS2018\FSQC265-1801252.D)
Intensity of absorbance (mAU)
Time (min)
2.115
2.435
4.244 - Gallic acid
4.878
6.539
7.944 - Catechol
8.844
9.202
9.732 - p- Hydroxy benzoic acid
10.296 - Caffeine
11.553 - Syringic acid
11.976
12.608
13.122 - Vanillin
13.471
13.821
14.157 - p- Coumaric acid
14.670
15.170 - Ferulic acid
15.545
15.969
16.215 - Ellagic
17.115 - Benzoic acid
17.583
17.886 - o- Coumaric acid
19.132 - Salicylic acid
19.886


(A2)
I:\Results\FSQC\Flavonoids\FSQC265-2018
Intensity of absorbance (mAU)
Time (min)
Components
2.10 1
3.00 2
3.28 Rutin 3
3.58 4
4.47 Myricetin 5
6.23 6
7.22 Quercetin 7


(B1)
VWD1 A, Wavelength=284 nm (D:\DATA\PHENOLS2018\FSQC269-1801269.D)
Intensity of absorbance (mAU)
Time (min)
4.297 - Gallic acid
6.662
8.068 - Catechol
8.957
9.343 - p- Hydroxy benzoic acid
9.857
10.267 - Caffeine
11.631 - Syringic acid
12.091
13.206 - Vanillin
13.476
13.876
14.318 - p- Coumaric acid
14.947
15.229 - Ferulic acid
15.590
16.018
16.268 - Ellagic
17.174 - Benzoic acid
17.642
17.938 - o- Coumaric acid
19.949 - Salicylic acid
21.737

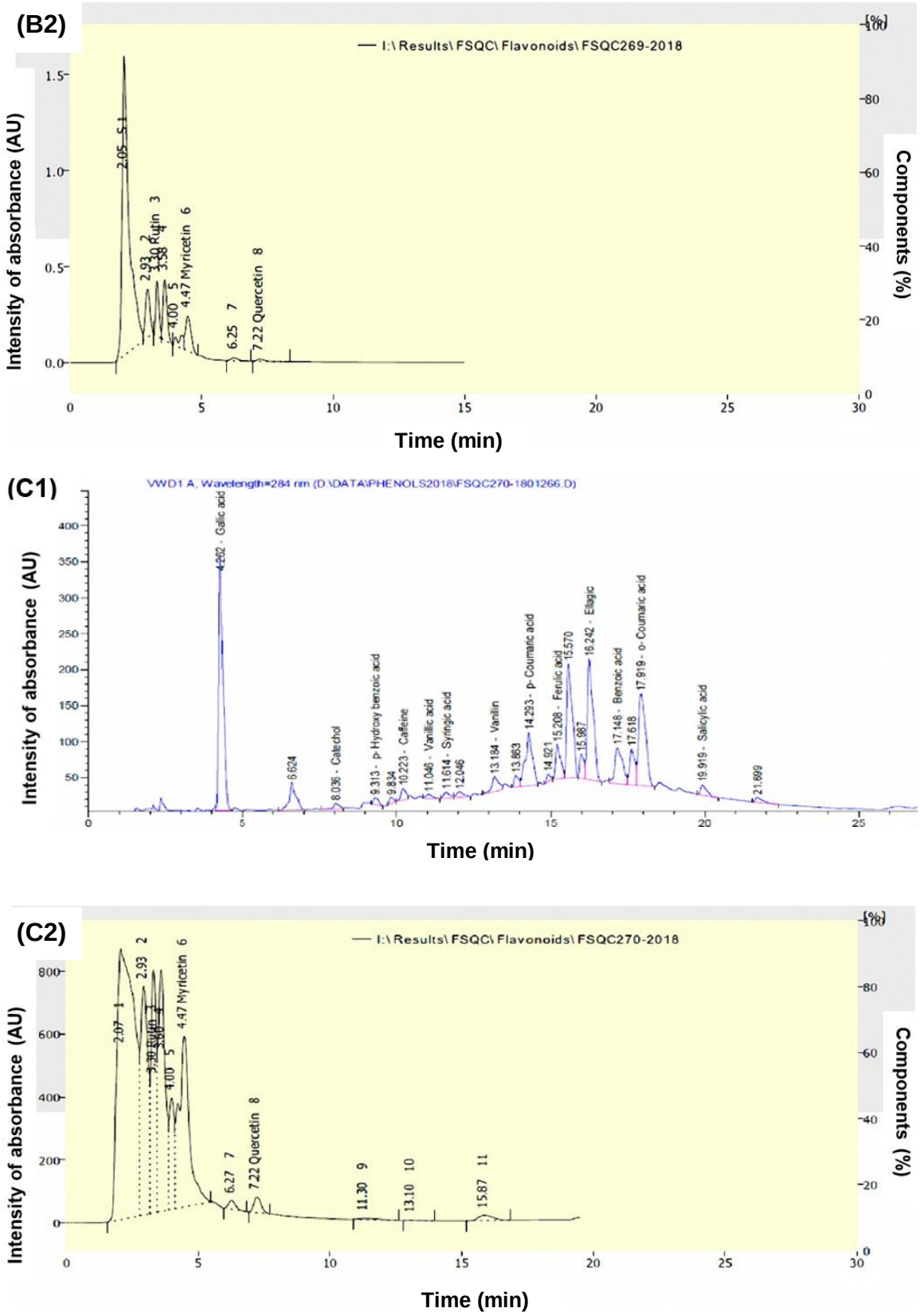


Fig. 1. HPLC chromatograms of *C. uvifera* leaf extracts: A_1, B_1, and C_1 are phenolic compounds and A_2, B_2, and C_2 are flavonoid compounds from aqueous, acetone, and ethanol extracts, respectively.

Antifungal Activity

Isolation and initial identification

Three fungal isolates were recovered from infected strawberry plants using the methodology outlined in the 'Materials' and 'Methods' sections. Cultures that possessed typical morphological characteristics of *F. culmorum*, *R. solani*, and *B. cinerea* were purified.

ITS identification

The rDNA regions of the ITS were amplified and sequenced for all fungal isolates. The nucleotide sequences blasted in NCBI confirmed that the three isolates were identical to the initial identifications of *F. culmorum*, *R. solani*, and *B. cinerea*. The sequences were deposited in GenBank under accession numbers MN398395, MN398397, and MN398399, respectively.

Antifungal activity and bioactivity of extracts

Table 2 shows the mycelial growth inhibition (MGI%) for *R. solani*, *Botrytis cinerea*, and *F. culmorum* caused by wood treated with aqueous, acetone, and ethanol *C. uvifera* extracts at concentrations of 1%, 2%, and 3% levels.

Ethanol extracts at 3%, 2%, and 1% concentrations indicated the greatest growth inhibition of *R. solani*, with MGIs of 64.4%, 61.8%, and 58.1%, respectively. This was followed by acetone extracts at 3% and 2%, with MGIs of 52.2% and 49.2%, respectively. Furthermore, inhibition of 43.7% was achieved by aqueous extract applied to wood at a concentration of 3%.

Complete inhibition (MGI 100%) of *B. cinerea* growth was achieved using wood treated with 3% ethanol extract, when compared to concentrations of 2% and 1% that achieved only some inhibition (MGIs of 61.8% and 58.1%, respectively). Wood treated with 2% and 1% acetone extracts reached MGI values of 52.2% and 49.2% against the growth of *B. cinerea*, respectively, while 43.7% inhibition was reached by aqueous extracts applied to wood at 3%.

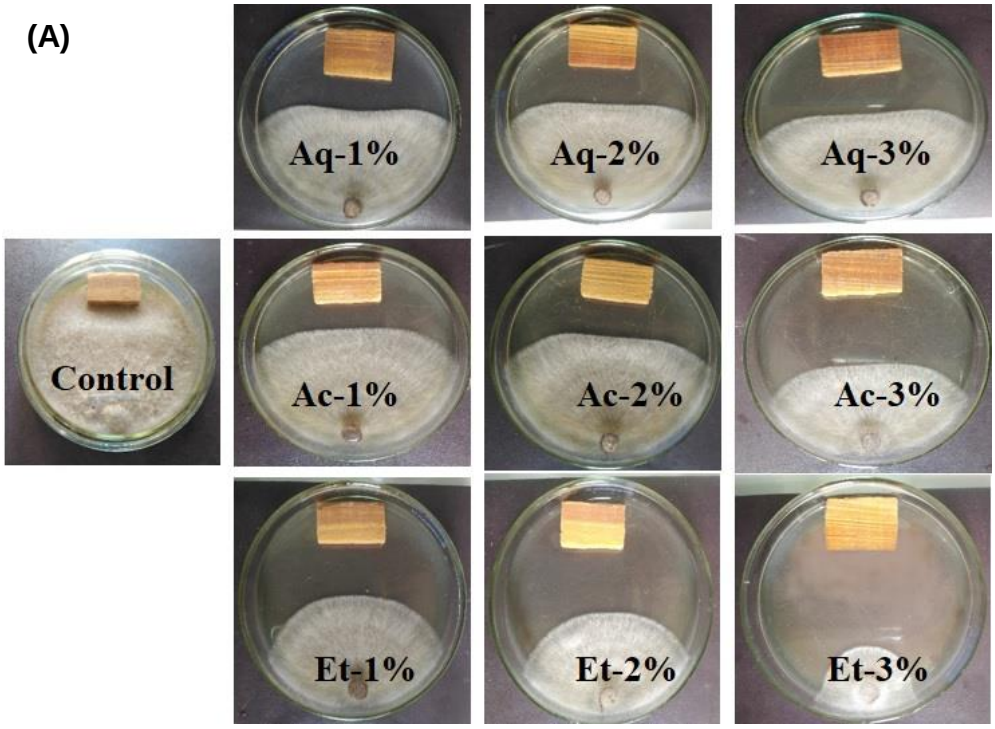
Ethanol extracts at concentrations of 3% and 2% accomplished MGI values of 38.5% and 38.1%, respectively, while acetone extracts at concentrations of 3% and 2% achieved 27.8% and 24.1% inhibition of *F. culmorum* growth, respectively.

Table 2. Antifungal Activities of Wood Treated with *C. uvifera* Leaf Extracts

Treatments		Concentration (%)	Mycelial Growth Inhibition (%)±SD*		
			Rhizoctonia solani	*Botrytis cinerea*	*Fusarium culmorum*
	Control	10% DMSO	0.00± 0.00	0.00± 0.00	0.00± 0.00
C. uvifera Leaf Extracts	Aqueous	1%	35.18 ± 0.64	31.85 ± 1.69	0.00± 0.00
		2%	36.29 ± 0.64	36.66 ± 0.00	0.00± 0.00
		3%	43.71 ± 1.28	38.14 ± 1.28	0.00± 0.00
	Acetone	1%	35.92 ± 1.28	37.77 ± 1.92	0.00± 0.00
		2%	49.25 ± 1.28	38.88 ± 2.22	24.07 ± 0.64
		3%	52.22 ± 0.00	54.07 ± 0.64	27.77 ± 1.11
	Ethanol	1%	58.14 ± 0.64	53.33 ± 0.00	0.00± 0.00
		2%	61.85 ± 0.64	70.37 ± 6.11	38.14 ± 1.28
		3%	64.44 ± 0.00	100 ± 0.00	38.51 ± 0.64
	P-value		***	***	***

*SD, standard deviation

(A)



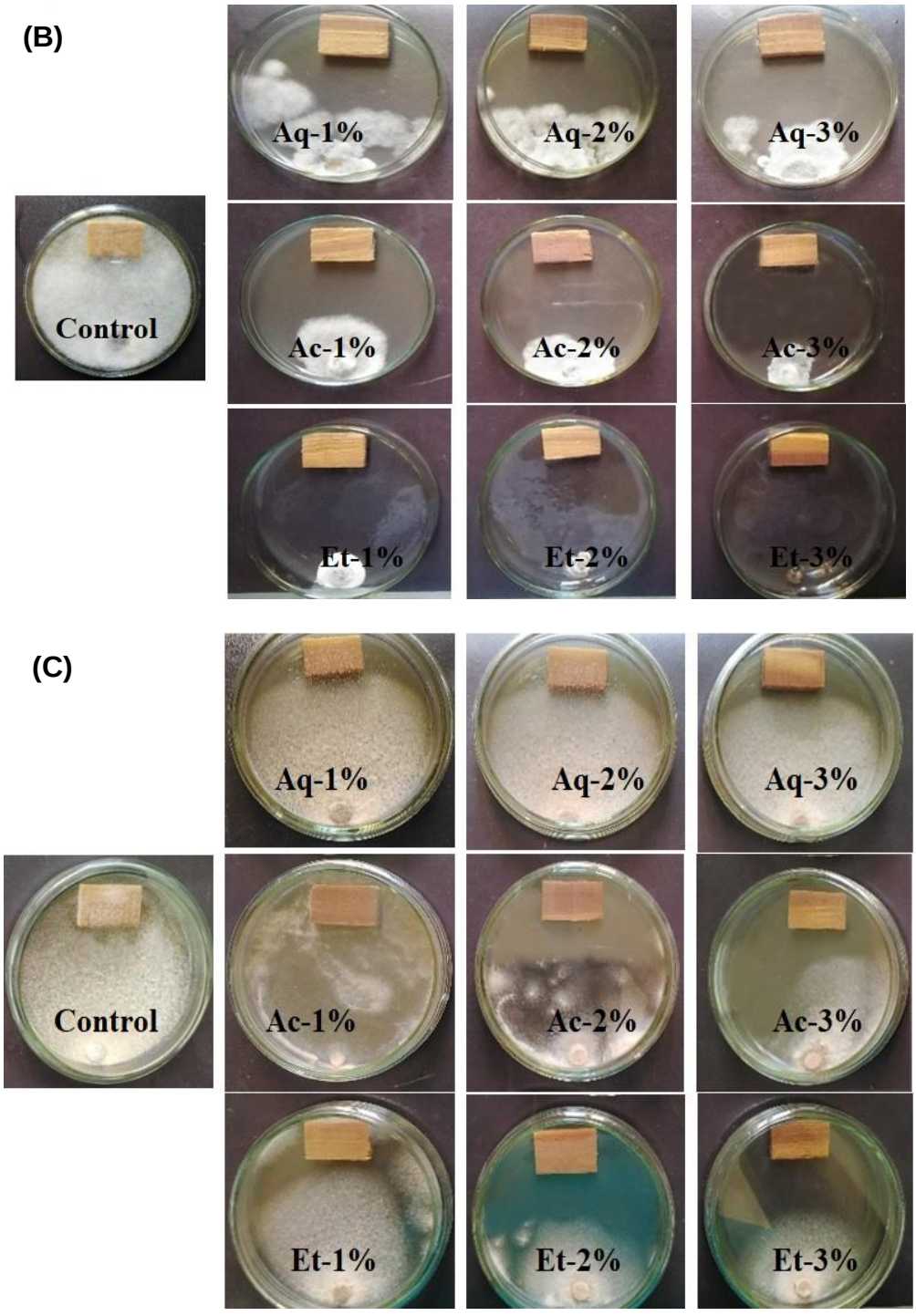


Fig. 2. Antifungal activities of wood treated with aqueous (Aq), acetone (Ac), and ethanol (Et) *C. uvifera* leaf extracts *vs. Rhizoctonia solani* (A), *Botrytis cinerea* (B), and *Fusarium culmorum* (C)

Antibacterial activities of extracts

Table 3 presents the antibacterial activities of *C. uvifera* extracts against the growth of six phytopathogenic bacteria. Acetone extracts were moderately active against *R. solanacearum*, with an inhibition zone of 10 mm at the concentrations of 250 µg/mL, 500 µg/mL, 1250 µg/mL, and 2500 µg/mL, while the chloramphenicol positive control (30 µg/disc) reached an inhibition zone (IZ) of 28.8 mm. Ethanol extract at 2500 µg/mL achieved an IZ value of 14.0 mm against the growth of *Erwinia amylovora*, while the positive control (Chloramphenicol 30 µg/disc) reached an IZ of 26.7 mm. Acetone and ethanol extracts at 2500 µg/mL observed IZ values of 10 mm against the growth of *Dickeya solani*, compared to 20 mm for the positive control (Chloramphenicol 30 µg/disc). At 2500 µg/mL, acetone and ethanol extracts showed IZ values of 12 mm against the growth of *Pectobacterium carotovorum* subsp. *carotovorum*; whilst the amoxicillin, chloramphenicol, and tobramycin positive controls produced IZ values of 15.3 mm, 19.3 mm, and 15.0 mm, respectively. Aqueous extract at 2500 µg/mL reached an IZ value of 10.00 mm against *Pectobacterium atrosepticum*, while other extracts with their concentrations indicated only weak activity, with inhibition zones that ranged from 6.0 mm to 8 mm. The positive controls of amoxicillin, chloramphenicol, and tobramycin reached IZ values of 10.0 mm, 12.3 mm, and 15.0 mm, respectively. Ethanol extracts at 1250 µg/mL and 2500 µg/mL showed IZ values of 11.0 mm and 12.0 mm, respectively, against the growth of *Agrobacterium tumefaciens*, while the amoxicillin, chloramphenicol, and tobramycin positive controls produced IZ values of 5.0 mm, 8.3 mm, and 13.7 mm.

Table 3. Antibacterial Activities of Extracts from Leaves of *C. uvifera*

Tested Material	Concentration (µg/mL)	Inhibition zone (Diameter ± SD*)					
		Ralstonia solanacearum	*Erwinia amylovora*	*Dickeya solani*	*Pectobacterium carotovorum* subsp. *carotovorum*	*Pectobacterium atrosepticum*	*Agrobacterium tumefaciens*
Negative control	0	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00	0.00± 0.00
Aqueous extract	50	7.00 ± 2.00	7.00 ± 1.00	8.00 ± 1.00	7.00 ± 1.00	6.00 ± 0.00	7.00 ± 0.00
	125	8.00 ± 2.00	8.00 ± 0.00	8.00 ± 1.00	7.00 ± 0.00	7.00 ± 0.00	8.00 ± .00
	250	8.00 ± 1.00	8.00 ± 0.00	9.00 ± 2.00	7.00 ± 0.00	7.00 ± 0.00	9.00 ± 0.00
	500	8.00 ± 1.00	9.00 ± 1.00	9.00 ± 1.00	8.00 ± 0.00	7.00 ± 0.00	9.00 ± 0.00
	1250	9.00 ± 1.00	9.00 ± 0.00	9.00 ± 1.00	10.00 ± 1.00	7.00 ± 1.00	8.00 ± 1.00
	2500	9.00 ± 0.00	8.00 ± 1.00	9.00 ± 1.00	10.00 ± 1.00	10.00 ± 1.00	10.00 ± 1.00
Acetone extract	50	8.00 ± 2.00	8.00 ± 1.00	6.00 ± 0.00	8.00 ± 1.00	7.00 ± 1.73	6.00 ± 1.00
	125	9.00 ± 1.00	8.00 ± 1.00	7.00 ± 0.00	10.00 ± 0.00	7.00 ± 1.00	7.00 ± 1.00
	250	10.00 ± 0.00	8.00 ± 1.00	8.00 ± 0.00	10.00 ± 1.00	7.00 ± 1.00	8.00 ± 1.00
	500	10.00 ± 1.00	9.00 ± 1.00	8.00 ± 0.00	10.00 ± 1.00	8.00 ± 0.00	8.33 ± 1.52
	1250	10.00 ± 1.00	10.00 ± 1.00	8.00 ± 1.00	10.00 ± 0.00	8.00 ± 1.00	8.00 ± 1.00
	2500	10.00 ± 0.00	10.00 ± 1.00	10.00 ± 0.00	12.00 ± 0.00	8.00 ± 1.00	8.00 ± 1.00
Ethanol extract	50	7.00 ± 0.00	8.00 ± 1.00	8.00 ± 1.00	8.00 ± 1.00	7.00 ± 1.73	9.00 ± 1.00
	125	8.00 ± 0.00	9.00 ± 1.00	8.00 ± 0.00	8.00 ± 0.00	7.00 ± 0.00	9.00 ± 1.00
	250	9.00 ± 1.00	10.00 ± 0.00	9.00 ± 1.00	8.00 ± 0.00	8.00 ± 0.00	10.00 ± 0.00
	500	9.00 ± 1.00	9.00 ± 0.00	9.00 ± 1.00	9.00 ± 0.00	8.00 ± 1.00	10.00 ± 2.00
	1250	9.00 ± 1.00	10.00 ± 2.00	9.00 ± 0.00	10.00 ± 1.00	8.00 ± 1.00	11.00 ± 1.00
	2500	9.00 ± 0.00	14.00 ± 0.00	10.00 ± 1.00	12.00 ± 2.00	8.00 ± 0.00	12.00 ± 1.00
Positive Controls (Antibiotics)							
Amoxicillin	25 µg/disc	6.00 ± 0.50	7.00 ± 0.50	5.33 ± 0.50	15.33 ± 0.86	7.00 ± 0.50	5.00 ± 0.00
Chloramphenicol	30 µg/disc	28.77 ± 1.48	26.66 ± 2.54	20.00 ± 0.71	19.33 ± 0.86	12.33 ± 0.86	8.33 ± 0.86
Tobramycin	10 µg/disc	10.00 ± 1.00	5.33 ± 0.50	5.00 ± 0.00	15.00 ± 0.50	15.00 ± 0.50	13.66 ± 0.86
P- value		**	**	**	**	**	**

*SD, standard deviation; ** Highly significant at 0.01 level of probability

Aqueous, acetone, and ethanol extracts from *C. uvifera* leaves grown in Egypt produced strong antifungal activities against three phytopathogenic fungi (*Rhizoctonia solani*, *Fusarium culmorum*, and *Botrytis cinerea*) when applied to wood samples of *Pinus roxburghii*. Moderate activity was found against the growth of six phytopathogenic bacteria (*Agrobacterium tumefaciens*, *Erwinia amylovora*, *Ralstonia solanacearum*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum*, and *Dickeya solani*). Furthermore, the extracts contained different compounds related to phenolic and flavonoid constituents.

Various phenolic and flavonoid types of compounds were identified in aqueous, acetone, and ethanol extracts of leaves from *C. uvifera* using HPLC analysis. Previously, aqueous extracts of *C. uvifera* leaves had total phenol and flavonoid contents of 346.15 ± 9.65 (μg gallic acid equivalent (GAE)/mL), and 360.44 ± 0.89 (μg catechin equivalent CE/mL), respectively, and exhibited weak antifungal activities against *Alternaria alternate*, *Fusarium oxysporum*, *F. verticillioides*, *Colletotrichum gloeosporioides*, and *C. capsici* (Rodríguez-García *et al.* 2019).

The antimicrobial activities observed in this study may have been due to the presence of phenolic and flavonoid types of compounds. Various medicinal plants containing phenolic and flavonoids in their different extracts have been reported to possess antimicrobial activities (Rahman and Moon 2007; Vaquero *et al.* 2007; Ayaz *et al.* 2008; Hendra *et al.* 2011; Al-Huqail *et al.* 2019; Behiry *et al.* 2019b).

Previous studies by the authors gave promising information on the antibacterial and antifungal activities of soaking wood blocks with plant extracts. For instance, in the study of Al-Huqail *et al.* (2019) it was found that the high content of the phenolic and flavonoids, quercetin, benzoic acid, naringenin, caffeine, o-coumaric acid, and kaempferol in *Acacia saligna* flowers extract exhibited bioactivities against *F. culmorum*, *Rhizoctonia solani*, and *Penicillium chrysogenum* and several bacterial strains. Also, the peel extracts of *Musa paradisiaca* L. contained gallic acid, naringenin, rutin, ellagic acid, and myricetin compounds which presented antimicrobial activity against the fungal isolates *R. solani* and *F. culmorum*, and the bacterial isolate *A. tumefaciens* (Behiry *et al.* 2019b). According to the HPLC analysis of *Withania somnifera* fruits acetone extract, the most abundant quantified phenolic and flavonoid compounds are salicylic acid, vanillic acid, rutin, and myricetin. As a result, the applied concentrations of the extract at 2% and 3% totally inhibited the growth of *A. tumefaciens*, *E. amylovora*, and *Pseudomonas cichorii* bacteria and the concentration 3% caused fungal inhibition of *F. culmorum* and *R. solani* (EL-Hefny *et al.* 2020).

Gallic acid, caffeic acid, vanillic acid, rutin, and quercetin have been isolated from various wines and have exhibited strong antimicrobial properties against pathogenic microorganisms (Vaquero *et al.* 2007). Gallic and benzoic acids, as well as myricetin-3-O-rhamnoside (a flavonoid compound), have been isolated from *C. dugandiana* leaves (Compagnone *et al.* 1995; Li *et al.* 1999). Compounds of simiarenol, sitostenone, sitosterol, trans-phytol, and vanillic acid have been isolated from the leaves and stem of *C. mollis* (Oliveira *et al.* 2008).

Gallic acid, a phenolic compound, isolated from *C. uvifera* seeds has been shown to possess antibacterial properties against *Salmonella typhimurium* and *Escherichia coli* (Moreno-Morales *et al.* 2008). In addition, the methanolic extracts of *C. uvifera* seeds contained compounds with antifungal activities against *Candida albicans*, *Fusarium oxysporum*, and *Fusarium decencellulare* (Moreno-Morales *et al.* 2008). Aqueous extracts of *C. cozumelensis* were found to express antibacterial activities against *Staphylococcus*

aureus, *Bacillus punullus*, and *Pseudomonas aeruginosa*, while ethanol extracts of *C. pubescens* exhibited antimalarial activities (Coe and Anderson 1996).

To the best of the authors' knowledge, the present study is the first to evaluate the effects of *C. uvifera* leaf extracts on the growth of phytopathogenic bacteria. The moderate antibacterial activities of extracts from *C. uvifera* leaves observed in this study are consistent with the results of previous antibacterial evaluation trails using natural extracts. For example, alkaloidal extracts from *Conocarpus lancifolius* leaves are effective against *A. tumefaciens* and *E. amylovora* (Ali *et al.* 2013), while acetone and *n*-butanol extracts from *Callistemon viminalis* flowers, essential oils from the aerial parts of *Conyza dioscoridis*, and *n*-butanol extracts from the bark of *Eucalyptus camaldulensis* are all effective against *A. tumefaciens* (EL-Hefny *et al.* 2017b). Finally, essential oils or *n*-butanol fractions derived from cones of *Pinus halepensis* are effective against *D. solani*, *P. atrosepticum*, *R. solanacearum*, and *A. tumefaciens* (Ashmawy *et al.* 2018a).

Quercetin, a flavonoid compound, was found in all the extracts of *C. uvifera* leaves. Quercetin isolated from different plant extracts has been reported to have strong antifungal activities (Weidenbörner *et al.* 1990; Tempesti *et al.* 2012; Alves *et al.* 2014). For example, *Terminalia brownii* stem bark extract contained quercetin-7-*O*-diglucoside and demonstrated strong antifungal activities against some strains of *Aspergillus* and *Fusarium* (Salih *et al.* 2017), and dihydroquercetin isolated from barley suppressed the growth of *Fusarium* spp. (Mierziak *et al.* 2014).

Combinations of quercetin and morin, and quercetin and rutin, were more active as antibacterial agents than either flavonoid alone. While rutin has no antibacterial activities by itself, the antibacterial activities of quercetin and morin are enhanced in the presence of rutin against *Salmonella enteritidis* and *Bacillus cereus* (Arima *et al.* 2002). Quercetin and rutin are potentially effective as antifungal agents against *Candida* sp. and *Cryptococcus neoformans* strains (Oliveira *et al.* 2016).

The activities of ethanolic extracts from *Morinda citrifolia* fruit have been linked to the pure compounds of rutin and asperulosidic acid (Taechowisan *et al.* 2019). The antifungal activities of extracts from *Duguetia furfuracea* have been shown to be related to the presence of phenols and flavonoids such as caffeic acid, rutin, quercitrin, and isoquercitrin (Soares de Araújo Pinho *et al.* 2016). In addition, quercetin 3-O-methyl ether isolated from *Cistus laurifolius* leaves has antibacterial activities against *Helicobacter pylori* (Ustün *et al.* 2006), and quercetin-3-glucoside isolated from leaves of *Scutellaria oblonga* successfully kills *Staphylococcus aureus* (Rajendran *et al.* 2016). Naringin and quercetin have been found in the mesocarp and seed extracts of *Phaleria macrocarpa* and can have strong antifungal activities against *Aspergillus niger* (Hendra *et al.* 2011). Quercetin and kaempferol were identified in the bound flavonoids of *Euphorbia hirta* stem extracts, and have shown to be active against *Aspergillus flavus*, *A. niger*, *Trichophyton mentagrophytes*, and *Candida albicans* (Singh and Kumar 2013).

Myricetin has been shown to potentially possess antibacterial activities (Lopes *et al.* 2017). Myricetin and rutin were detected in the hydroalcoholic fractions of *Chrysobalanus icaco* extracts, and displayed potential antifungal activities against *Candida albicans* and *C. parapsilosis* (Silva *et al.* 2017). Therefore, *C. uvifera* leaf extract could be used as a source of phytochemical agent with potential antibacterial and antifungal activities.

CONCLUSIONS

1. Phytochemicals extracted from *C. uvifera* leaves using water, acetone, and ethanol as solvents were evaluated for their antimicrobial activities against some phytopathogenic bacterial and fungal strains.
2. The extracts showed strong antifungal activities against *R. solani*, *F. culmorum*, and *B. cinerea*, when applied to wood samples of *P. roxburghii*. Moderate activity was observed against the growth bacteria *A. tumefaciens*, *E. amylovora*, *R. solanacearum*, *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum*, and *D. solani*.
3. The HPLC analysis of the extracts showed the presence of various phenolic and flavonoid-type compounds, which exhibited the potential antimicrobial activity. It was concluded that *C. uvifera* leaves extracts are a good alternative source to phytochemicals for use as potential antifungal and antibacterial agents.

ACKNOWLEDGMENTS

The authors are grateful to the Deanship of Scientific Research, King Saud University, for funding through the Vice Deanship of Scientific Research Chairs. The authors also thank the Deanship of Scientific Research and RSSU at King Saud University for their technical support.

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Article submitted: November 5, 2019; Peer review completed: March 8, 2020; Revised version received and accepted: April 10, 2020; Published: April 15, 2020.
DOI: 10.15376/biores.15.2.4165-4187