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The Potential Antibacterial and Antifungal Activities of Wood Treated with *Withania somnifera* Fruit Extract, and the Phenolic, Caffeine, and Flavonoid Composition of the Extract According to HPLC

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Abstract: In the present study, *Melia azedarach* wood blocks treated with different acetone extract concentrations from *Withania somnifera* fruits are assessed for their antibacterial and anti-fungal activities. Wood blocks of *M. azedarach* treated with *W. somnifera* fruit extract at concentrations of 0, 1, 2, and 3% are evaluated for in vitro antimicrobial activity against five genbank accessioned bacterial strains—*Agrobacterium tumefaciens*, *Dickeya solani*, *Erwinia amylovora*, *Pseudomonas cichorii*, and *Serratia pylumthica*—and two fungi, namely, *Fusarium culmorum* and *Rhizoctonia solani*. Through HPLC analysis we find that the most abundant quantified phenolic and flavonoid compounds of acetone extract (mg/100 g) are salicylic acid (9.49), vanillic acid (4.78), rutin (4702.58), and myricetin (1386.62). Wood treated with the extract at 2% and 3% show no growth of *A. tumefaciens*, *E. amylovora*, and *P. cichorii*. Use of the extract at 3% causes inhibition of fungal mycelia of *F. culmorum* and *R. solani* by 84.07% and 67.03%, respectively. In conclusion, potent antifungal and antibacterial activity against plant pathogens is found when an acetone extract of *W. somnifera* fruits is applied to wood samples.

Keywords: *Withania somnifera* fruits; antimicrobial activity; wood bio-fungicide; phenolic; flavonoid

1. Introduction

Plant extract compounds and their application in food industries have great value in preventing growth of fungi or bacteria, and can be involved in possible processing technologies in which they can be exploited as ideal preservative solutions.

The highlighted benefits and challenges of plant-derived products need further research for green society implementation and governmental regulation [1,2]. Increasing regulatory restrictions and negative consumer responses to chemical compounds and to the use of antibiotics in agriculture have contributed to pressure for the development of alternative compounds for use as antimicrobial agents [3,4].

In traditional medicine, *Withania somnifera* L. Dunal (Solanaceae) has been authenticated as having antitumor, anti-inflammatory, antiarthritic, antioxidant, immunomodulatory, and hepatoprotective effects [5–7].

Bioactive compounds such as ashwagandhine, cuscohygrine, anaferineisopelletierine, sitoindosides, anhygrine, withanamides, and tropine have been isolated and identified from *W. somnifera* [8–10]. In addition, the most important withanolides, withaferine A and withanolide A, have been isolated [11]. The plant has shown the presence of chemically active compounds related to flavonoids, alkaloids (tropine, hygrine, and anferine), terpenoids, steroidal lactones (anolides), and saponins in the extracts from different parts [12–16]. Fatty acids such as palmitic, oleic, linoleic, and linolenic acids have been isolated from n-hexane extracts of leaves and roots [8]. In some cases, *W. somnifera* extracts with multi-components have shown better medicinal effects than the purified compounds [17].

W. somnifera extracts have been demonstrated to possess strong antifungal and antibacterial activities [18–22]. Acetone root-bark extract from *Salvadora persica* has been shown to be very effective at stopping the bacterial growth of *Agrobacterium tumefaciens* and *Dickeya solani* [23], and leaf aqueous extract has been observed to inhibit the mycelial growth and spore germination of some plant pathogenic fungi [24].

During recent years, research manuscripts have introduced soft rot bacteria as a dangerous pathogen that could destroy many horticulture crops, and have developed methods to detect or characterize it, and even to provide control strategies [25,26]. *Dickeya* spp. strains have been isolated from diseased plants in Finland, Poland, France, the Netherlands, Switzerland, and other European countries, while Egypt and Israel have been associated with *Serratia pylumthica* [23,27–30]. *D. solani* strains are considered more aggressive than other blackleg-causing bacteria [31]. *Erwinia amylovora*, a Gram-negative bacterium, is the causal agent of fire blight [32].

A 16S rRNA gene PCR-based assay has been developed as a fast-molecular diagnostic method to differentiate between phylogenetically closely related species, such as the Crown gall bacterial pathogen caused by *Agrobacterium tumefaciens* and other species causing plant bacterial diseases which have different symptoms, in order to identify and discriminate the strains belonging to all bacterial species [33,34].

Molds (*Penicillium selerotigenum*, *Paecilomyces variotii,* and *Aspergillus niger*) show different growths on *Citharexylum spinosum* and *Morus alba* woods, reflecting their natural durability [35]. On the other hand, and during storage conditions with moist and poor environments, wood, wood containers, and wood-boxes may deteriorate or become stained with the growth of molds [36], which colonize these wood types and use simple sugars and starches for growth [37,38]. Previous works have shown that extracts or essential oils have potential antimicrobial activities against several fungi and bacteria in treated wood of *Pinus sylvestris*, *P. rigida* and *Fagus sylvatica*, *Leucaena leucocephala*, *Melia azedarach*, and *Acacia saligna* [39–46].

Hence, in this work we assess the antimicrobial activity of *Melia azedarach* wood treated with *W. somnifera* fruit extract against some plant pathogenic bacteria and fungi. This study also analyzes the chemical compositions of phenolic and flavonoid compounds using HPLC analysis.

2. Materials and Methods

2.1. Isolation Procedure

Bacterial isolation trials were carried out on infected pear and cabbage leaves, guava root galls, and potatoes that showed symptoms collected from Beheira Governorate during 2018 in Egypt. The plant samples were rinsed with water, placed in 1% sodium hypochlorite solution for 3 min, washed in sterile distilled water, and left to dry on sterilized paper. The infected tissues were milled in a mortar with sterile NaCl 0.8% solution, and then 5% sucrose nutrient agar plates for the pear leaves bacteria and glycerol nutrient agar for the other studied bacteria were inoculated with a loop of the

previous suspension [47]. Pure colonies were picked and reserved in a refrigerator until use. The two fungal cultures used in this study had been previously isolated and molecularly identified as *Fusarium culmorum* and *Rhizoctonia solani*, and were obtained from Dr. Said Behiry, Head of the Molecular Plant Pathology Laboratory, Agricultural Botany Department, Faculty of Agriculture Saba Basha, Alexandria University, Alexandria, Egypt [3]. All the isolates were reserved on glycerol nutrient agar (for bacteria) and potato dextrose agar (for fungi) for later use.

2.2. Bacterial Phenotypic Characterization

The morphological, physiological, and biochemical features of the bacterial isolates were characterized and described according to the Schaad et al. [47] laboratory guide.

2.3. DNA Extraction, PCR Analyses, and Sequencing

DNA isolation from the bacterial cultures was performed according to the protocol cited by Ausubel et al. [48]. Partial amplification of the 16S rRNA gene using P0 (5′-GAAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′) primers was analyzed by a thermal cycler (Techne prime, Cambridge, UK). The partial amplicons were purified and sequenced with a ABI3730XL DNA sequencer at Macrogen Inc. (Seoul, South Korea) [45].

2.4. Preparation of Wood Blocks

Air-dried *Melia azedarach* wood samples were prepared with dimensions of 1 × 1 × 0.5 cm at the Department of Forestry and Wood Technology in January 2018. The samples were autoclaved at 121 °C for 20 min and then cooled.

2.5. Extraction and Preparation of Concentrations of Acetone Extract from Withania somnifera fruits

Fruits of *Withania somnifera* (Alexandria, Egypt) were thoroughly washed using tap water, air-dried, and then crushed into small pieces using a small laboratory Willy mill. Each flask contained 200 mL of acetone solvent; 50 g were soaked for three days at room temperature [44] and then filtered using Whatman No. 1 filter paper and microfiltered using a Millipore (0.45 µm pore size membrane). The acetone solvent was evaporated using a rotary evaporator at 45 °C and then the crude acetone extract was stored in sealed vials at 4 °C until further use [49]. The extract was prepared at concentrations of 1%, 2%, and 3% (*w*/*v*) as dissolved in 10% dimethyl sulfoxide (Sigma-Aldrich, Darmstadt, Germany) prior to treatment of the wood samples. All the treatments were compared with a control treatment (10% DMSO).

2.6. Treatment of M. azedarach Wood using the Soaking Method

Each wood sample of *M. azedarach* received 100 µL of the concentrated extract, and the control received 100 µL of 10% DMSO. All the treated woods were kept for 6 h in flasks. Three wood samples were used for each treatment [50].

2.7. Antimicrobial Activity of Wood Treated with Acetone Extract

The antibacterial activity of wood treated with an acetone extract of *W. somnifera* was tested against *Agrobacterium tumefaciens*, *Dickeya solani*, *Erwinia amylovora*, *Pseudomonas cichorii*, and *Serratia pylumthica*, while the antifungal activity was evaluated using the fungal strains *Fusarium culmorum*, MH352452 and *Rhizoctonia solani*, MH352450.

The antibacterial activity of wood treated with an acetone extract of *W. somnifera* fruits was measured according to National Committee for Clinical Laboratory Standards (NCCLS) [51] with minor modifications, wherein the treated wood samples were placed over the inoculated medium with each bacterium at 30 °C for three days and compared with the control treatment. For antifungal activity, the fungal isolates were grown at 28 °C and the wood-treated samples were assayed according to

previous studies [41–43,50], with measurement of the mycelial growth inhibition percentage performed. The inhibition zone (IZ) was measured from the outer margin of the IZ to the inner margin of the surrounding pathogens, including the treated wood blocks for bacteria, and the IZ was measured from the edge of the treated wood blocks to the outer margin of the surrounding pathogens for the fungi.

2.8. Analytical HPLC of Phenolic Compounds/Caffeine and Flavonoids for the Acetone Extract

The HPLC apparatus and conditions used (Table 1), as well as all the standards used, can be found in previous works [39,44,45,52]. The minimum concentration that the detector was able to measure was 0.1 µg/mL.

Table 1. HPLC conditions for phenolic/caffeine and flavonoid compounds.

HPLC Conditions	Phenolic/Caffeine Compounds	Flavonoid Compounds
Instrument	Agilent 1260 Infinity HPLC Series equipped with a Quaternary pump and a Zorbax Eclipse plus C18 column (100 mm × 4.6 mm i.d.) (Agilent, Santa Clara, CA, USA)	Smart line (Knauer, 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)
Temperature of operation	30 °C	35 °C
Separation elution gradient	A: HPLC grade water 0.2% H_3PO_4 (*v/v*) B: Methanol C: Acetonitrile	Methanol: H_2O with 0.5% H_3PO_4 (50:50) Flow rate 0.7 mL/min
Injected volume	20 µL	20 µL
Detector	Variable wavelength detector (VWD) at 284 nm	UV detector at 273 nm

2.9. Statistical Analysis

The percentages of mycelial inhibition growth of the three fungi and two bacterial strains as affected by the three concentrations (1, 2, and 3%) of *W. somnifera* fruit extract were statistically analyzed and compared with the control treatment (10% DMSO) using one-way ANOVA [53]. Comparisons among means were performed using the least significant difference at the 0.05 level of probability ($LSD_{0.05}$).

3. Results

3.1. Bacterial Isolation and Characterization

Five bacterial isolates were isolated from infected cabbage, guava, pear, and potato plants. The morphological and biochemical characteristics and the partial sequence of 16S rRNA revealed that the isolates were *Erwinia amylovora*, *Agrobacterium tumefaciens*, *Pseudomonas cichorii*, *Dickeya solani*, and *Serratia pylumthica* (Table 2).

3.2. In Vitro Visual Observations of the Antibacterial Activity of Extract-Treated Wood

The antibacterial activity of the treated wood with acetone extract from *W. somnifera* fruits is shown in Table 3 and Figure 1. No growth of *A. tumefaciens*, *E.a amylovora*, and *Pseudomonas cichorii* was found when using the extract at 2% and 3%, and the inhibition zone (IZ) reached 90 mm. No significant differences among the three concentrations of extract were shown against the growth of *D. solani*, where the IZ reached 31.66 mm and was higher than the value for the control treatment. The IZ values found against the growth of *S. pylumthica* were 76 mm and >90 mm for wood treated at 2% and 3% of the extract, respectively.

Table 2. Morphological traits, physiological traits, biochemical reactions, and accession numbers of bacterial isolates obtained from infected samples.

Characteristic	Bacterial Isolate				
	Erwinia amylovora	*Agrobacterium tumefaciens*	*Pseudomonas cichorii*	*Dickeya solani*	*Serratia plymuthica*
GenBank accession number of 16S rDNA sequence	MK720288	MK720285	MK720283	MK720279	MK720275
Shape (rods)	-	-	-	-	-
Gram staining	+	+	+	+	+
Motility	+	+	+	+	+
Anaerobic growth	+	+	+	+	+
3-Ketolactose production	+	-	+	+	+
Growth at 37 °C	-	-	+	+	+
Catalase test	+	+	-	+	+
Mucoid growth	+	+	-	+	+
Oxidase reaction	-	+	+	-	-
Pigment on kingsB	nd	nd	+	+	+
Indole production	-	-	-	+	-
R. substance from sucrose	-	-	-	-	-
Urease production	-	-	-	-	-
Growth in 5% NaCl	-	-	+	-	+
Citrate utilization	-	+	-	+	-
Malonate utilization	-	-	+	+	-
Alkali from tartaric acid	nd	+	+	+	-
Glucose	a	-	-	+	+
α-methyl glucoside	-	-	a	a	a
Maltose	-	-	-	-	a
Sucrose	a	a	a	a	a
Lactose	a	a	-	a	a
Dulcitol	a	a	-	a	a
Manitol	a	a	a	a	a
Trehalose	a	a	-	a	a

+ = More than 80% of isolates gave a positive reaction. - = Less than 20% of isolates gave a negative reaction. Legend: a, acid; nd, not determined.

Table 3. Antibacterial activity of wood treated with acetone extract from *Withania somnifera* fruits.

Conc.	Inhibition Zone (mm ± SD)				
	Agrobacterium tumefaciens	*Dickeya solani*	*Erwinia amylovora*	*Pseudomonas cichorii*	*Serratia pylumthica*
Control (10% DMSO)	0.00 ^{c} *	0.00 ^{b}	0.00 ^{c}	0.00 ^{c}	0.00 ^{c}
1%	60.33 ^{b} ± 3.05	31.66 ^{a} ± 1.15	61.33 ^{b} ± 1.15	79 ^{b} ± 1.73	0.00 ^{c}
2%	>90 ^{a}	31.66 ^{a} ± 0.57	>90 ^{a}	>90 ^{a}	76 ^{b} ± 1.73
3%	>90 ^{a}	31.66 ^{a} ± 0.57	>90 ^{a}	>90 ^{a}	>90 ^{a}
$LSD_{0.05}$	2.87	1.33	1.08	1.63	1.63

* No significance between means within the same column and have the same letters. Legend: LSD, least significant difference at the 0.05 level of probability.

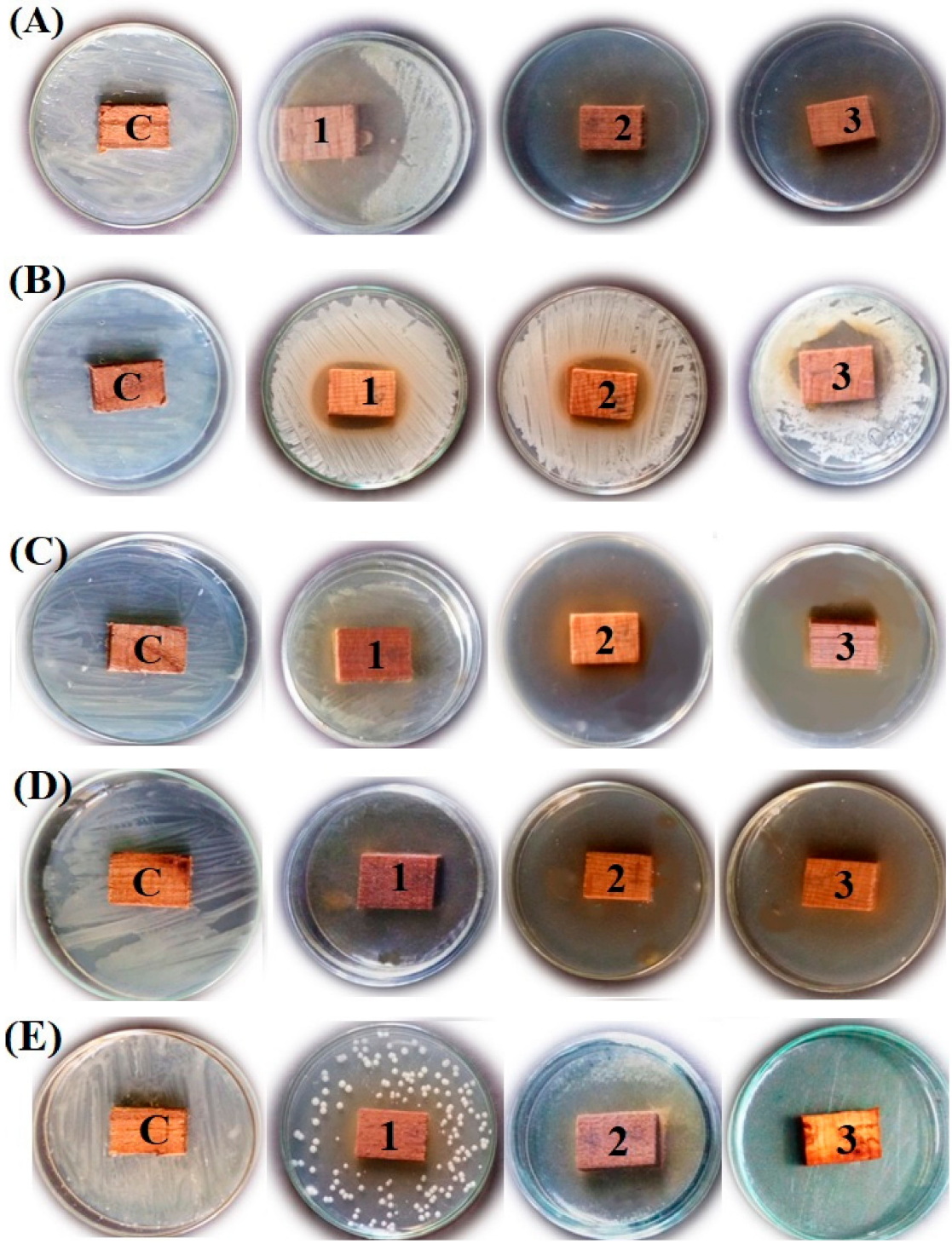

Figure 1. Antibacterial activity of wood treated with acetone extracts of *Withania somnifera*. (**A**) *Agrobacterium tumefaciens*; (**B**) *Dickeya solani*; (**C**) *Erwinia amylovora*; (**D**) *Pseudomonas cichorii*; (**E**) *Serratia pylumthica*. C indicates control treatment (10% DMSO); 1, 2, and 3 indicate 1%, 2%, and 3% concentrated acetone extract.

3.3. In Vitro Visual Observations of the Antifungal Activity of Extract-Treated Wood

Table 4 and Figure 2 present an antifungal bioassay of wood treated with acetone extracts of *W. somnifera* fruits against two fungi (*Fusarium culmorum* and *Rhizoctonia solani*). Compared to control treatments with complete growth of fungi and when increasing the concentrations of the extract,

fungal mycelia inhibition (FMI) was observed. At 3% of the extract, the FMI reached 84.07% and 67.03% for growth of *F. culmorum* and *R. solani*, respectively. On the other hand, the lowest values of 49.62% and 49.25% were observed at the concentration of 1% against the growth of *F. culmorum* and *R. solani*, respectively.

Table 4. Antifungal activity of wood treated with acetone extracts from *Withania somnifera* fruits.

Conc.	Inhibition Percentage (%)	
	Fusarium culmorum	***Rhizoctonia solani***
Control (10% DMSO)	0.00 ^{d} *	0.00 ^{d}
1%	49.62 ^{c}	49.25 ^{c}
2%	65.92 ^{b}	53.33 ^{b}
3%	84.07 ^{a}	67.03 ^{a}
$LSD_{0.05}$	1.81	1.35

* No significance between means have the same letters within the same column.

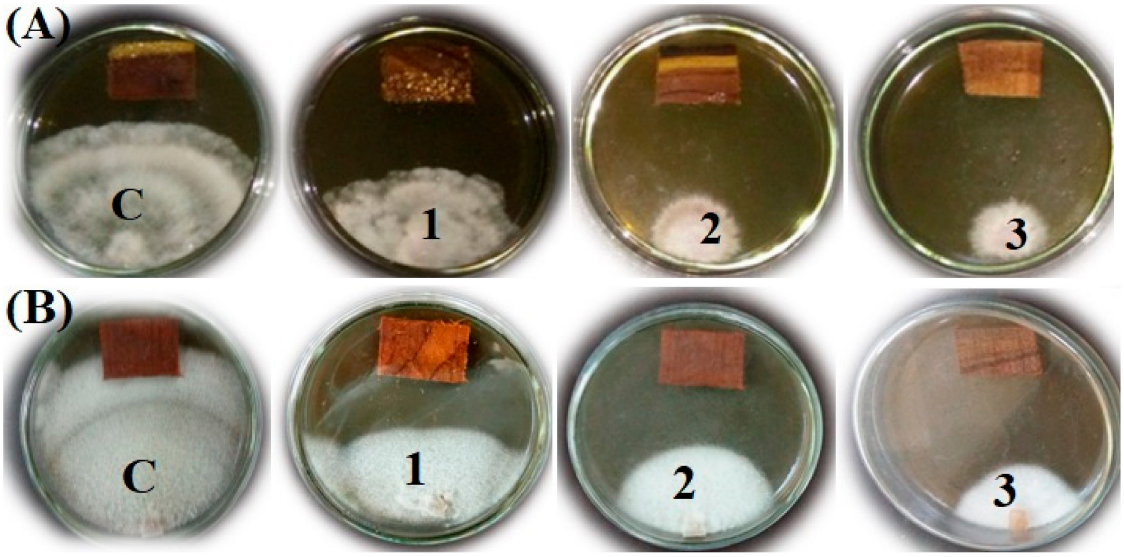

Figure 2. Antifungal activity of the treated wood with *Withania somnifera* fruit acetone extract. (**A**) *Fusarium culmorum;* (**B**) *Rhizoctonia solani.*

3.4. Phenolic/Caffeine and Flavonoid Compounds of the Acetone Extract

HPLC chromatograms of the phenolic/caffeine and flavonoid compounds in the acetone extract of *W. somnifera* fruits (Table 5) are presented in Figure 3a,b, respectively. In the mg/100 g extract, the most abundant phenolic compounds were found to be salicylic acid (9.49), vanillic acid (4.78), and *o*-coumaric acid (1.22), while the identified flavonoid compounds were found to be rutin (4702.58), myricetin (1386.62), and kaempferol (8.29).

Table 5. Phytochemical composition of the acetone extracts from *Withania somnifera* fruits by HPLC.

Compound	Conc. (mg/100 g extract)
Phenolic/caffeine compounds	
Gallic acid	0.58
Catechol	ND *
p-Hydroxy benzoic acid	ND
Caffeine	0.31
Vanillic acid	4.78
Caffeic acid	0.75
Syringic acid	ND
Vanillin	0.82
p-Coumaric acid	0.34
Ferulic acid	0.54
Ellagic acid	0.93
Benzoic acid	ND
o-Coumaric acid (*trans*-2-Hydroxycinnamic acid)	1.22
Salicylic acid	9.49
Cinnamic acid	ND
Flavonoid compounds	
Rutin	4702.58
Myricetin	1386.62
Quercetin	ND
Naringenin	ND
Kaempferol	8.29
Apigenin	ND

* ND, not detected.

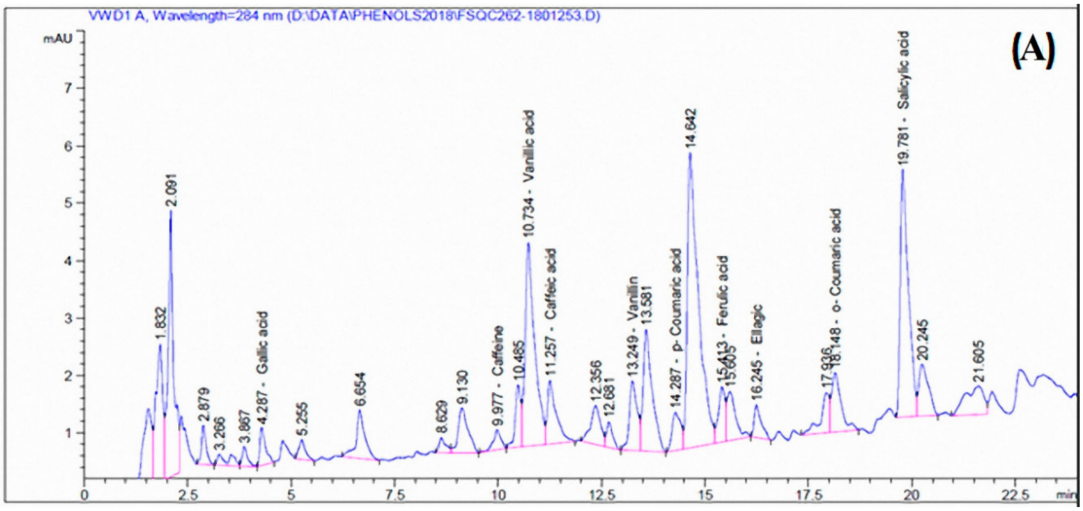

Figure 3. *Cont.*

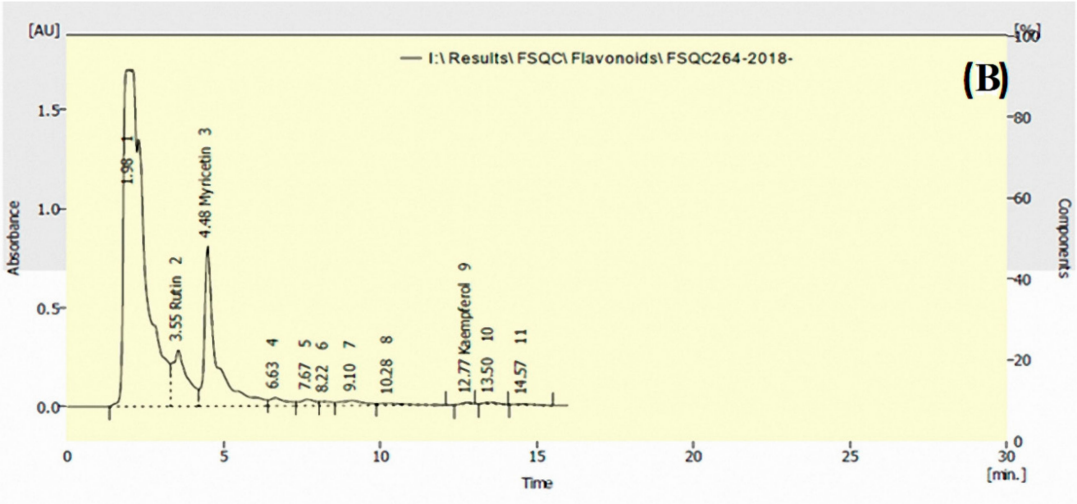

Figure 3. HPLC chromatogram of phenolic/caffeine (**A**) and flavonoid (**B**) compounds identified in acetone extracts of *Withania somnifera* fruits.

4. Discussion

In this work, acetone extracts of *W. somnifera* fruits applied to *M. azedarach* wood showed potent activity against *A. tumefaciens*, *E. amylovora*, and *P. cichorii,* as well as mycelia inhibition of *F. culmorum* and *R. solani*.

Flavonoid compounds found in *W. somnifera* extract have been proven to be responsible for antimicrobial activity against certain pathogenic bacteria and fungi [54,55]. Previously, *W. somnifera* leaf methanol and acetone extracts have shown moderate antifungal activity against *F. oxysporum* and *Colletotrichum capsici* [56]. A methanol extract of *W. somnifera* has shown potential antifungal activity against the growth of *Alternaria alternata*, *Bipolaris oryzae*, *Colletotrichum capsici*, *C. lindemuthianum*, *Curvularia lunata*, *F. moniliforme*, *F. oxysporum*, *Macrophomina phaseolina*, *Rhizoctonia solani*, and *Pyricularia oryzae*, with the percentage of inhibition of mycelial growth ranging 54.44–78.88% [57].

The aqueous fruit extract of *W. somnifera* at 2% has exhibited potential antifungal action against *F. oxysporum f.* sp. *radicis-lycopersici* [58]. Phenolic compounds, flavonoid compounds, glycoside, fixed oils, tannins, alkaloids, and saponins, as well as withaferin A, ascorbic acid, and anthocyanin as polar compounds, are responsible for the potential antifungal activity of the extracts from this plant [18,59].

The black pointed disease caused by *A. alternata* in *Triticum aestivum* has been found to be reduced significantly when an aqueous extract of *W. somnifera* is begun to be used [60]. Methanol extract from fruit and leaf of *W. somnifera* has been observed to decrease *Ascochyta rabiei* biomass, the cause of chickpea blight disease [61]. Additionally, *F. oxysporum f.* sp. *cepae* biomass has been seen to be decreased by 93% when root methanolic extracts of *W. somnifera* are added [62].

Our previous works which have carried out studies of the effects of soaked or treated wood samples with natural products on the growth of pathogenic fungi and bacteria have shown some promising results. *Acacia saligna* wood treated with a combination treatment of Paraloid B-72 and *Cupressus sempervirens* wood methanolic extract has been found to have potent biocide activity against the mold fungus *Trichoderma harzianum* [40].

Several vital species of the pathogens *Fusarium*, *Phaeoacremonium, Phytophthora*, and *Uromycladium* are associated with diseases occurring in woody plant tissues. The best known of these include twig dieback in citrus, dieback and cankers on fruit trees (Petri and esca diseases), and collar rots and rusts of *Acacia* [63–65]. Species from many families are responsible for severe damage diseases of trees; for example *Diaporthales* and *Botryosphaeriaceae* cause diseases such as stem cankers, shoot, and twig blight of *Eucalyptus* [66,67].

Several studies have shown that wood can be protected by biopreservation chemicals using extracts or essential oils; for instance, the extent of mycelia of *A. alternata*, *F. subglutinans*, *Chaetomium*

globosum, *A. niger*, and *Trichoderma viride* have been found to decrease or have their growth prevented on the surfaces of some woods treated with extracts from *Pinus rigida* (heartwood essential oil and extract), *Eucalyptus camaldulensis* (leaf extract and essential oil), and rhizomes of *Costus speciosus* (extract) [42,43]. *Leucaena leucocephala* wood treated with a combination of concentrations of extracts from the inner and outer bark of *A. saccharum var. saccharum* with citric acid has shown good inhibition against the growth of *T. viride*, *F. subglutinans*, and *A. niger* [44], which is related to the presence of phenolic compounds such as *p*-hydroxy benzoic acid, gallic acid, salicylic acid, and caffeine. *M. azedarach* wood treated with *A. saligna* flowers has shown antifungal and antibacterial activities with a high content of quercetin, benzoic acid, naringenin, caffeine, o-coumaric acid, and kaempferol [45]. Additionally, *M. azedarach* wood samples treated with peel extracts of *Musa paradisiaca* L. in the presence of ellagic acid, gallic acid, rutin, myricetin, and naringenin have shown good antifungal activity against *R. solani* and *F. culmorum,* as well as antibacterial activity against *A. tumefaciens* [39].

5. Conclusions

The potent antifungal and antibacterial activities exhibited by *W. somnifera* extract might be attributed to the presence of either the single or synergetic effects of more than one compound, or the high amount of flavonoid compounds, and may help us to discover new antibiotic substances that could serve as alternative treatments for plant diseases and their control. Hence, *W. somnifera* extract might be used as a bioactivity agent (antibacterial or antifungal) against plant pathogens. Further field studies are required to generalize the activity of this plant extract in treating various plant diseases.

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