



Bio-based chemical analysis of extracts from the biomass residues of *Ceratonia siliqua* and *Ziziphus spina-christi* with their bioactivities against molecularly identified fungi

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Abstract

Carob (*Ceratonia siliqua* L.) and Sider (*Ziziphus spina-christi* (L.) Desf.) are evergreen trees whose plant parts are rich in bioactive polyphenol compounds with potential for application in functional food production. Extracts from leaves and branches of *C. siliqua* and *Z. spina-christi* were applied to *Pinus sylvestris* wood blocks and subjected to fungal infestation with *Alternaria alternata* (PQ326444), *Fusarium oxysporum* (OR116513), and *Rhizoctonia solani* (OR116533). The methanol extracts from each part were analyzed for their chemical compounds using the HPLC apparatus. In the methanol extract of *C. siliqua* leaves, the most abundant compounds were catechin, syringic acid, gallic acid, and coumaric acid, with values of 153820.84, 33583.13, 11404.34, and 6714.78 µg/g extract, respectively. The abundant compounds in the methanol extract from *C. siliqua* branches were gallic acid, syringic acid, and methyl gallate with concentrations of 8461.39, 7779.52, and 2424.98 µg/g extract, respectively. The main phytochemical compounds identified in the methanol extract from *Z. spina-christi* leaves were ellagic acid (3173.65 µg/g extract), gallic acid (2479.39 µg/g extract), rutin (2076.35 µg/g extract), and catechin (1873.36 µg/g extract) and from branches were gallic acid (5999.97 µg/g extract), catechin (1507.08 µg/g extract), and chlorogenic acid (460.64 µg/g extract). The methanol extracts of the leaves and branches from *C. siliqua* at 4% showed the highest activity against the growth of *A. alternata* with inhibition percentage values of 72.22 and 71.48%, respectively, with the extract from leaves observed the highest fungal inhibition (77.77%) against *F. oxysporum*. The methanol extracts from *Z. spina-christi* branches and leaves showed the highest fungal inhibition percentages against *Rhizoctonia solani* with values of 59.26 and 42.22%, respectively. The study's findings suggest that methanolic extracts may be used to treat fungal infestations because they possess interesting antifungal characteristics.

Keywords Antifungal activity · Branches extract · *Ceratonia siliqua* · Leaves extract · *Ziziphus spina-christi*

1 Introduction

Carob (*Ceratonia siliqua* L.), belongs to the Caesalpinaceae subfamily to the family of Leguminosae (syn. Fabaceae) and is a typical Mediterranean plant, mainly used in food and Tunisian traditional folk medicine [1–4]. The leaves and

fruit of this plant are used to cure various diseases. Carob pods have traditionally been used as animal and human food, and the seed is used primarily for gum extraction. Bark and leaves have been used in Tunisian folk medicine as laxatives, diuretics, antidiarrheal, and for treating gastroenteritis in lactating babies [5]. Promising biological activity (particularly as an antioxidant, hypoglycemic, and antibacterial with minimal toxicity) is demonstrated by the polyphenol-rich leaf extract of *C. siliqua* [6].

The growing interest in plant extracts and natural products stems from their safety, eco-friendliness, and cost-effectiveness compared to synthetic chemicals. These natural alternatives minimize health risks, environmental pollution, and adverse side effects on hosts. Unlike chemical pesticides, plant extracts avoid high toxicity, prolonged degradation,

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bioaccumulation, and the indiscriminate destruction of beneficial organisms alongside harmful pests [7].

Because the extracts from carob have very significant antibacterial action against various bacterial and fungal species, carob has attracted a lot of attention [5, 8, 9]. Carob leaves are rich in polyphenols and flavonoids [10]. In this respect, Kivçak et al. [5] have shown that the various extracts (*n*-hexane, methanol, ethyl acetate, and water extracts) of *C. siliqua* leaves (mg of extract/disc) inhibited *Escherichia coli*. Methanolic extracts of *C. siliqua* fruits demonstrated strong antibacterial activity, outperforming *Plantago major* extracts and showing enhanced effects when combined with antibiotics like ampicillin and gentamicin [11].

Phytochemicals present in the extracts can be evaluated and identified by several spectroscopic analyses using chromatography like gas chromatography-mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) [12–14]. HPLC analysis identified bioactive compounds, including epigallocatechin-3-gallate, which exhibited significant activity against pathogens such as *Listeria monocytogenes* and *Pectobacterium atrosepticum* [15]. Carob leaves have been investigated for their antifungal potential against phytopathogenic fungi, including *Geotrichum candidum*, a significant agent of citrus sour rot. Extracts prepared using various solvents, such as hexane, ethyl acetate, chloroform, and methanol, have shown differing activity levels. The methanol extracts exhibited the highest antifungal efficacy [16]. Leaves contain a range of biologically active compounds, including polyphenols, flavonoids, and tannins, and have been traditionally used for medicinal purposes [2, 17, 18].

According to earlier research, total phenolic compounds in *C. siliqua* leaf and pod extracts grown in Algeria ranged from 0.61 to 22.98 mg GAE/g. Regardless of solvent, carob leaf extracts had substantially greater ($\times 10$) phenolic concentrations than pod extracts [19]. The ethyl acetate fraction from the extract of *C. siliqua* fresh leaves showed the presence of phenolic compounds 1,6-di-O-galloyl-glucose, 1,2,6-tri-O-galloyl-glucose, myricetin glucoside, 1,2,3,6-tetra-O-galloyl-glucose, myricetin rhamnoside, and syringic acid [1]. However, there is limited research on the potential uses of carob leaves, and they are often considered a byproduct of carob production.

Ziziphus spina-christi (L.) Desf. belongs to the family Rhamnaceae, locally known as Sider, is readily distributed in the Sahara and Sahel, from Senegal to the Sudan and Arabia, where the annual rainfall is about 50–300 mm or on periodically inundated sites [20]. It is an important multipurpose cultivated tree and one of the few truly native species of Arabia still growing along with many newly introduced exotic plants [21]. Sider has been used in folk medicine as a demulcent depurative, anodyne, emollient, stomachic for toothaches, astringents, and mouthwash agent to prevent

and treat *Candida* infections [22]. Antifungal activity of the ethanol extracts from *Z. spina-christi* floral parts was reported [23]. The aqueous extract of *Z. spina-christi* demonstrated significant antifungal activity against *Fusarium*, *Helminthosporium*, *Alternaria*, and *Rhizoctonia* species. It effectively suppressed the sporogenesis of *Fusarium* and *Alternaria* while impacting fungal growth, conidial germination, and structural integrity [24].

Z. spina-christi extracts are employed as an active ingredient in the defense against various plant diseases that have affected certain other significant crops [24, 25]. *Z. spina-christi* leaves have an abundance of flavonoids and phenolic chemicals in their aqueous extract [26, 27]. The extracts were reported to have potential antifungal and antibacterial bioactivities [28–30].

To maximize the uses of the bio-waste from trees and add significant values, this study aimed to use high-performance liquid chromatography (HPLC–DAD) to screen the polyphenolic compounds in the leaves and branches of *C. siliqua* and *Z. spina-christi*. Furthermore, it aimed to evaluate the antifungal efficacy of the extracts on wood blocks against molecularly characterized fungal isolates. Additionally, the study described the active extract and its potential health benefits.

2 Materials and methods

2.1 Preparation of botanical materials and the extracts

Ziziphus spina-christi and *Ceratonia siliqua* leaves and branches were gathered at the Faculty of Agriculture Garden at Alexandria University in Alexandria, Egypt. First, branches and leaves were divided and air-dried at room temperature (Fig. 1). The branches were oven-dried at 45 °C to shorten the drying process. After that, a lab mill was used to pulverize all ingredients. Approximately 100 g of each powdered material was steeped in 200 mL of methanol solvent for 7 days throughout the extraction process. Following this time frame, the extracted biochemical substances were filtered using Whatman filter paper No. 1 and a cotton plug to produce the methanol extracts. The rotary evaporator was used to concentrate the extracts under vacuum. Before additional analysis, the concentrated extracts were put into Petri dishes and allowed to air dry. The yield of extract was calculated from branches and leaves using the following formula [31]: Extract percentage = [extract amount (g)/air-dry sample amount (g)] $\times$ 100.

All the extracts were prepared at concentrations of 1, 2, and 4% by dissolving the respective amounts of extract in 10% dimethyl sulfoxide (DMSO).

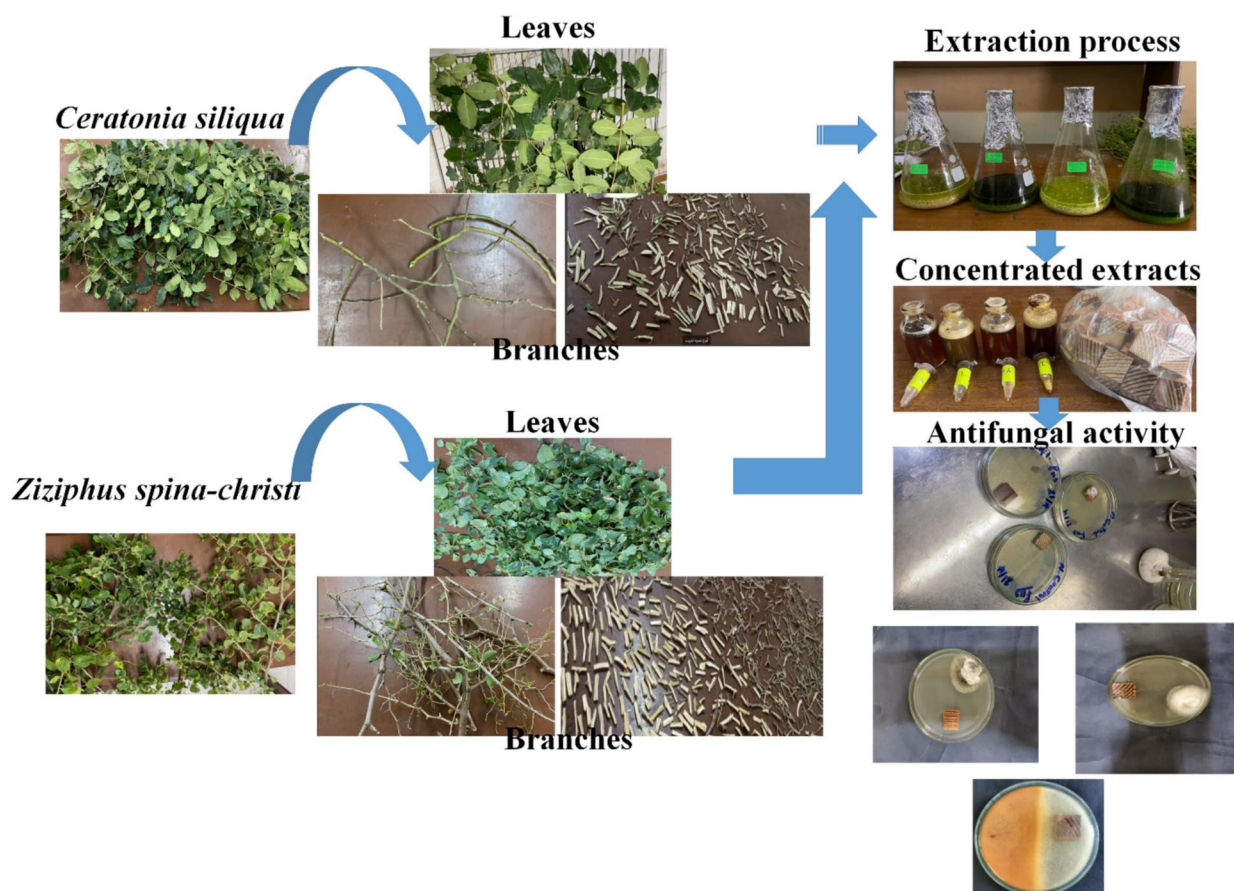


Fig. 1 Scheme summarizing the extraction process of the methanol extracts

2.2 HPLC conditions

An Agilent 1260 series was used for the HPLC analysis. A Zorbax Eclipse Plus C8 column (4.6 mm × 250 mm, i.d., and a film size of 5 μm) was used to separate the phytochemicals in the extracts. Water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) were the components of the mobile phase, which was flowing at a rate of 0.9 mL/min. The following was the sequential linear gradient programming for the mobile phase: 0 min (82% A); 0–1 min (82% A); 1–11 min (75% A); 11–18 min (60% A); 18–22 min (82% A); 22–24 min (82% A). At 280 nm, the multi-wavelength detector was observed. Each sample solution had an injection volume of 5 μL.

The column temperature was kept constant at 40 °C [32]. Standard HPLC-grade phenolic and flavonoid compounds were employed, including gallic acid, chlorogenic acid, catechin, methyl gallate, caffeic acid, syringic acid, pyrocatechol, rutin, ellagic acid, *p*-coumaric acid, vanillin, ferulic acid, naringenin, rosmarinic acid, daidzein, quercetin, cinnamic acid, kaempferol, and hesperetin.

2.3 Morphological and molecular characterization of fungi

In this study, plant samples were collected from agricultural tomato fields, which showed typical symptoms of fungal infection. Each infected plant was carefully removed and immediately placed into sterile plastic bags to prevent contamination. The bags were clearly labeled with location and sample identification and promptly transported to the laboratory for further processing. To ensure accurate isolation of the fungal pathogens, the infected plant tissues were surface-sterilized using a 1% sodium hypochlorite solution for 2 min, followed by thorough rinsing with sterile distilled water to eliminate any residual disinfectant. Sterilized tissue sections were then placed onto potato dextrose agar (PDA) plates and incubated at 25 °C for 5–7 days. The emerging fungal colonies were carefully sub-cultured onto fresh PDA plates to obtain pure isolates [33].

Both macroscopic and microscopic examinations of the fungal isolates were conducted for the morphological identification of fungi. Macroscopically, the colonies were

observed for their color, texture, and growth patterns. Microscopic evaluation of spores and hyphae was performed using a light microscope after staining with lactophenol cotton blue. The morphological features, including conidial structure, septation, and hyphal characteristics, were used to tentatively identify the fungal species based on standard taxonomic keys [33–36].

In parallel with morphological identification, molecular methods were employed for more accurate species identification. Total genomic DNA was extracted from the pure fungal cultures using the cetyltrimethylammonium bromide (CTAB) method [37]. The DNA samples were then subjected to polymerase chain reaction (PCR) amplification using species-specific primers that targeted the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). Universal ITS1 and ITS4 primers were used for this purpose. The PCR amplification was performed under standard conditions with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A final extension step at 72 °C for 10 min ensured complete amplification. The resulting PCR products were separated on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light to confirm successful amplification [38].

Following PCR, the amplified ITS fragments were purified and sequenced. The obtained sequences were compared with reference sequences from the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed by MEGA 11 software [39] using the maximum likelihood method, with bootstrap values (1000 replicates). This allowed for precise species identification by comparing the genetic sequences of the isolates with known sequences in the database.

2.4 Antifungal activity

On PDA medium, a culture of 7-day-old *Alternaria* spp., *Fusarium* spp., and *Rhizoctonia* spp. was grown. The Petri dishes were filled with the PDA medium and let to settle in the incubator. *Pinus sylvestris* wood blocks were prepared at the dimensions of 2 × 2 × 0.7 cm³ and then autoclaved at 121 °C for 20 min and left to cool. In 150 µL of the concentrated extracts, positive and negative samples were provided to each wood sample in conditions of surface sterilization using Laminar Flow Sterile Cabinets. Each treated wood block was added to the PDA medium, and then a 5-mm-diameter disc was used to inoculate each fungus onto a Petri dish. After that, the dishes were cultured at 25 ± 1 °C for 10 days. Each treatment was assessed three times. The samples were treated with 10% DMSO (negative control) and Carbendazim 50 WP (250 µg/mL) (positive control). The percentage of fungal growth inhibition was measured

with the following formula [40]; $FGI = [(A_c - A_t) / A_c] \times 100$, where FGI is the fungal growth inhibition (%) and A_c and A_t are average diameters of the fungal colony of the control and treatment, respectively.

2.5 Statistical analysis

The fungal growth inhibition measured from the treated wood samples with the methanol extracts from leaves and branches of *Ceratonia siliqua* and *Ziziphus spina-christi* at 1%, 2%, and 4% was statistically analyzed in a completely randomized design (CRD). The statistical method was performed using a two-way analysis of variance (ANOVA) (SAS software (SAS Institute, Release 8.02, Cary, NC, USA). The comparison among means was done against the control treatments using Duncan's multiple range test at the 0.05 level of probability. All values are expressed as mean ± standard deviation of three parallel measurements.

3 Results

3.1 Extract percentages and the HPLC analysis

The percentages of the methanol extracts (MEs) from *Ceratonia siliqua* leaves and branches were 7.12 and 4.40%, respectively, and in *Ziziphus spina-christi* were 6.30 and 3.30%, respectively. Figure 2 presents the HPLC chromatograms of the identified phenolic and flavonoid compounds in the ME from *C. siliqua* leaves (Fig. 2a), *C. siliqua* branches (Fig. 2b), *Z. spina-christi* leaves (Fig. 2c), and *Z. spina-christi* branches (Fig. 2d).

The most abundant compounds in the MEs from *C. siliqua* leaves were catechin (153,820.84 µg/g extract), syringic acid (33,583.13 µg/g extract), gallic acid (11,404.34 µg/g extract), coumaric acid (6714.78 µg/g extract), methyl gallate (3547.44 µg/g extract), chlorogenic acid (1814.18 µg/g extract), caffeic acid (1492.04 µg/g extract), rutin (661.67 µg/g extract), and naringenin (590.76 µg/g extract) (Table 1). The abundant compounds identified in *C. siliqua* branch ME were gallic acid (8461.39 µg/g extract), syringic acid (7779.52 µg/g extract), methyl gallate (2424.98 µg/g extract), coumaric acid (1129.99 µg/g extract), catechin (387.56 µg/g extract), rutin (296.90 µg/g extract), chlorogenic acid (210.05 µg/g extract), and naringenin (205.29 µg/g extract) (Table 2).

The abundant phytochemicals identified by HPLC in the ME from *Z. spina-christi* leaves were ellagic acid (3173.65 µg/g extract), gallic acid (2479.39 µg/g extract), rutin (2076.35 µg/g extract), catechin (1873.36 µg/g extract), naringenin (873.84 µg/g extract), and syringic acid (243.39 µg/g extract) (Table 3). The ME from *Z. spina-christi* branches when analyzed by HPLC showed

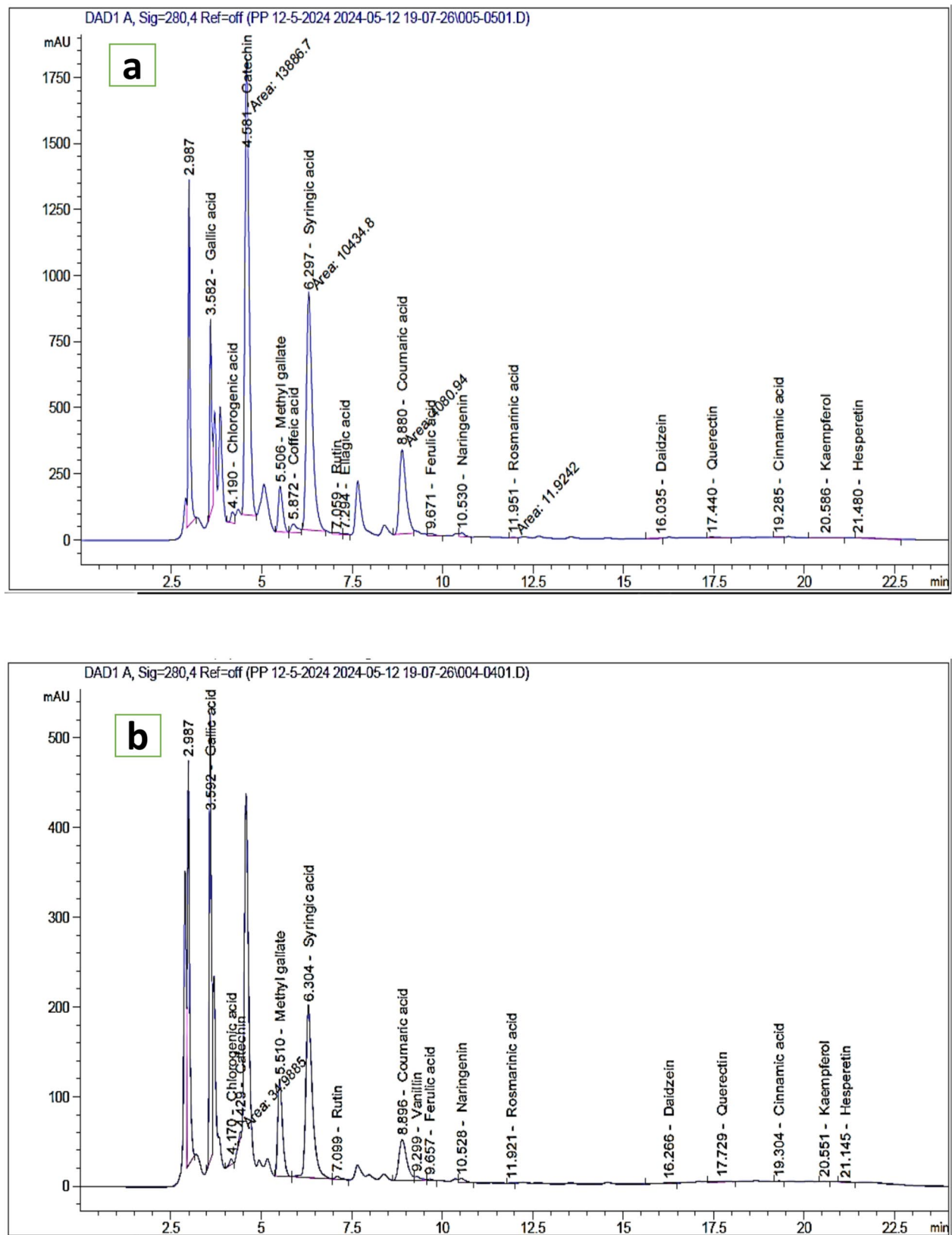


Fig. 2 The analysis of methanol extracts by HPLC. **a** *Ceratonia siliqua* leaves, **b** *Ceratonia siliqua* branches, **c** *Ziziphus spina-christi* leaves, and **d** *Ziziphus spina-christi* branches

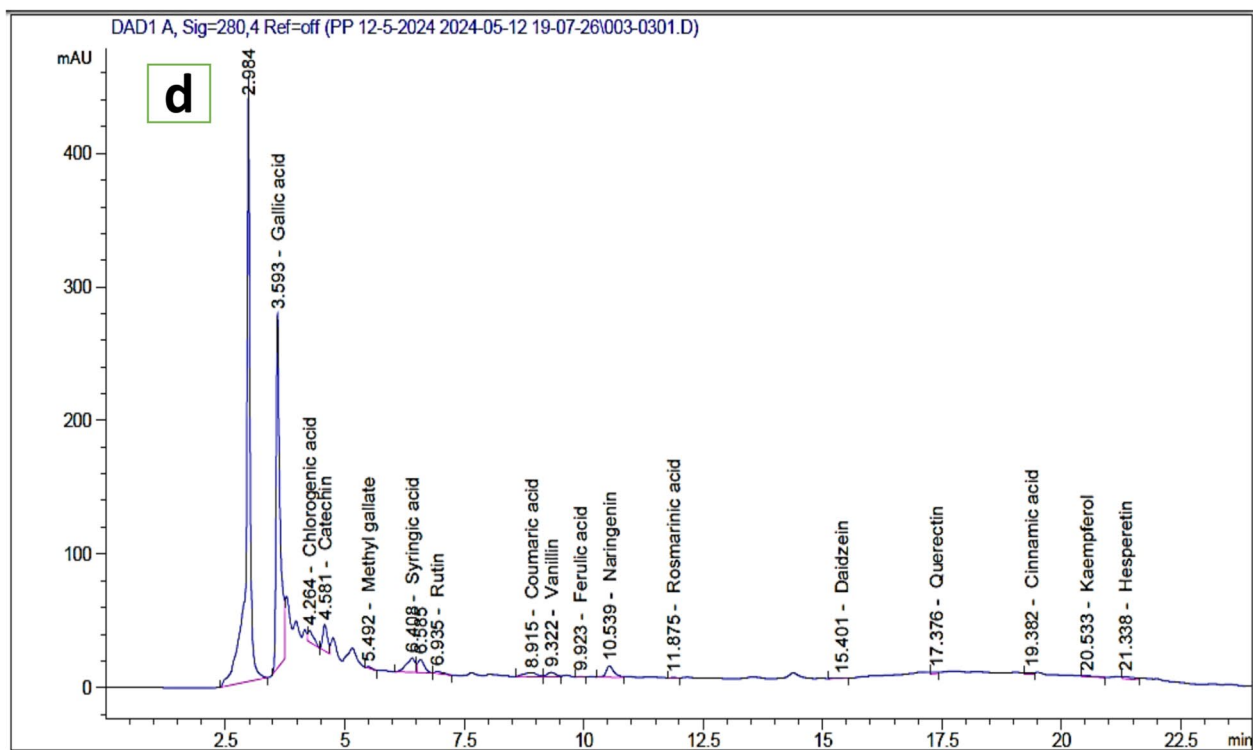
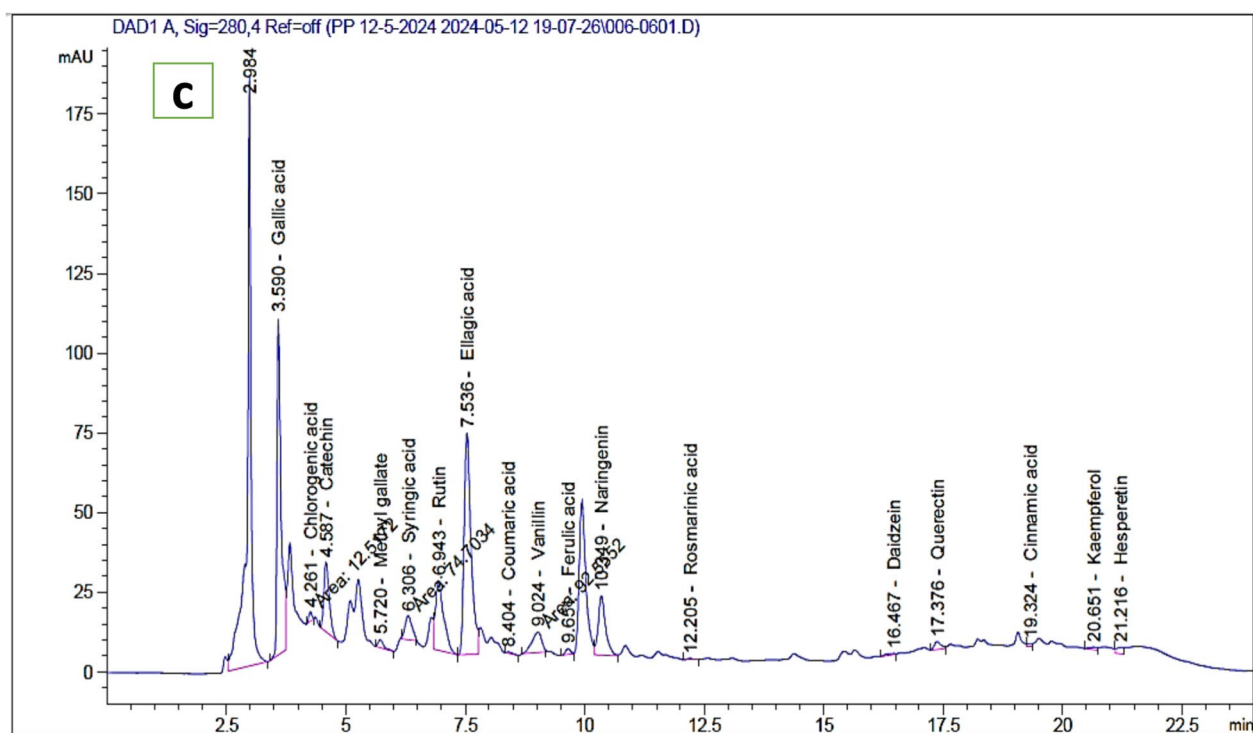


Fig. 2 (continued)

Table 1 Phenolic profile of the methanolic extract in *Ceratonia siliqua* leaves using HPLC–DAD

Compounds	Formula	Group	RT (min)	Area (mAU*s)	%Area	Concentration (µg/g extract)
Unknown	-	-	2.987	4753.594	12.278	-
Gallic acid	C ₇ H ₆ O ₅	Phenolic acids	3.582	2906.048	7.506	11,404.34
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Hydroxycinnamic acids	4.190	291.542	0.753	1814.18
Catechin	C ₁₅ H ₁₄ O ₆	Flavan-3-ols	4.581	1.3886	35.868	153,820.84
Methyl gallate	C ₈ H ₈ O ₅	Methyl ester of gallic acid	5.506	1440.179	3.720	3547.44
Caffeic acid	C ₉ H ₈ O ₄	Hydroxycinnamic acids	5.872	385.7984	0.996	1492.04
Syringic acid	C ₉ H ₁₀ O ₅	Hydroxybenzoic acids	6.297	1.0434	26.952	33,583.13
Rutin	C ₂₇ H ₃₀ O ₁₆	Flavonol glycosides	7.059	83.622	0.216	661.67
Ellagic acid	C ₁₄ H ₆ O ₈	Phenolic acids	7.294	30.767	0.079	139.77
Coumaric acid	C ₉ H ₈ O ₃	Hydroxycinnamic acids	8.880	4080.939	10.541	6714.78
Vanillin	C ₈ H ₈ O ₃	Phenolic aldehydes	9.131	0.000	0.000	ND
Ferulic acid	C ₁₀ H ₁₀ O ₄	Hydroxycinnamic acids	9.671	115.942	0.299	316.89
Naringenin	C ₂₇ H ₃₂ O ₁₄	Flavanones	10.530	134.572	0.347	590.76
Rosmarinic acid	C ₁₈ H ₁₆ O ₈	Ester of phenolic acids	11.951	11.924	0.0308	58.33
Daidzein	C ₁₅ H ₁₀ O ₄	Isoflavones	16.035	8.479	0.0219	25.84
Quercetin	C ₁₅ H ₁₀ O ₇	Flavonols	17.440	64.646	0.167	201.44
Cinnamic acid	C ₉ H ₈ O ₂	Hydroxycinnamic acids	19.285	11.393	0.0294	9.77
Kaempferol	C ₁₅ H ₁₀ O ₆	Flavonols	20.586	34.0471	0.087	114.23
Hesperetin	C ₁₆ H ₁₄ O ₆	Flavonones	21.480	40.063	0.103	86.73

mAU milli-absorbance units, ND not detected, RT retention time (min)

Table 2 Phenolic profile of the methanolic extract in *Ceratonia siliqua* branches using HPLC–DAD

Compounds	Formula	Group	RT (min)	Area (mAU*s)	%Area	Concentration (µg/g extract)
Unknown	-	-	2.987	1913.552	22.641	-
Gallic acid	C ₇ H ₆ O ₅	Phenolic acids	3.592	2156.128	25.511	8461.39
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Hydroxycinnamic acids	4.170	33.755	0.399	210.05
Catechin	C ₁₅ H ₁₄ O ₆	Flavan-3-ols	4.429	34.988	0.414	387.56
Methyl gallate	C ₈ H ₈ O ₅	Methyl ester of gallic acid	5.510	984.486	11.648	2424.98
Caffeic acid	C ₉ H ₈ O ₄	Hydroxycinnamic acids	5.939	0.000	0.000	ND
Syringic acid	C ₉ H ₁₀ O ₅	Hydroxybenzoic acids	6.304	2417.218	28.600	7779.52
Rutin	C ₂₇ H ₃₀ O ₁₆	Flavonol glycosides	7.099	37.522	0.444	296.90
Ellagic acid	C ₁₄ H ₆ O ₈	Phenolic acids	7.268	0.000	0.000	ND
Coumaric acid	C ₉ H ₈ O ₃	Hydroxycinnamic acids	8.896	686.754	8.125	1129.99
Vanillin	C ₈ H ₈ O ₃	Phenolic aldehydes	9.299	58.479	0.691	105.29
Ferulic acid	C ₁₀ H ₁₀ O ₄	Hydroxycinnamic acids	9.657	17.229	0.203	47.09
Naringenin	C ₂₇ H ₃₂ O ₁₄	Flavanones	10.528	46.763	0.553	205.29
Rosmarinic acid	C ₁₈ H ₁₆ O ₈	Ester of phenolic acids	11.921	2.584	0.030	12.64
Daidzein	C ₁₅ H ₁₀ O ₄	Isoflavones	16.266	16.827	0.199	51.28
Quercetin	C ₁₅ H ₁₀ O ₇	Flavonols	17.729	25.340	0.299	78.96
Cinnamic acid	C ₉ H ₈ O ₂	Hydroxycinnamic acids	19.304	2.271	0.026	1.95
Kaempferol	C ₁₅ H ₁₀ O ₆	Flavonols	20.551	4.977	0.058	16.70
Hesperetin	C ₁₆ H ₁₄ O ₆	Flavonones	21.145	12.751	0.150	27.60

mAU milli-absorbance units, ND Not detected, RT retention time (min)

Table 3 Phenolic profile of the methanolic extract in *Ziziphus spina-christi* leaves using HPLC–DAD

Compounds	Formula	Group	RT (min)	Area (mAU*s)	%Area	Concentration (µg/g extract)
Unknown	-	-	2.984	1150.111	33.853	-
Gallic acid	C ₇ H ₆ O ₅	Phenolic acids	3.590	631.796	18.597	2479.39
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Hydroxycinnamic acids	4.261	12.517	0.368	77.89
Catechin	C ₁₅ H ₁₄ O ₆	Flavan-3-ols	4.587	169.124	4.978	1873.36
Methyl gallate	C ₈ H ₈ O ₅	Methyl ester of gallic acid	5.720	19.718	0.580	48.57
Caffeic acid	C ₉ H ₈ O ₄	Hydroxycinnamic acids	5.939	0.000	0.000	ND
Syringic acid	C ₉ H ₁₀ O ₅	Hydroxybenzoic acids	6.306	74.703	2.198	243.39
Rutin	C ₂₇ H ₃₀ O ₁₆	Flavonol glycosides	6.943	262.413	7.724	2076.35
Ellagic acid	C ₁₄ H ₆ O ₈	Phenolic acids	7.536	698.624	20.564	3173.65
Coumaric acid	C ₉ H ₈ O ₃	Hydroxycinnamic acids	8.404	3.194	0.094	5.26
Vanillin	C ₈ H ₈ O ₃	Phenolic aldehydes	9.024	92.555	2.724	166.63
Ferulic acid	C ₁₀ H ₁₀ O ₄	Hydroxycinnamic acids	9.651	13.021	0.383	35.59
Naringenin	C ₂₇ H ₃₂ O ₁₄	Flavanones	10.349	199.059	5.859	873.84
Rosmarinic acid	C ₁₈ H ₁₆ O ₈	Ester of phenolic acids	12.205	4.461	0.131	21.82
Daidzein	C ₁₅ H ₁₀ O ₄	Isoflavones	16.467	6.645	0.195	20.25
Quercetin	C ₁₅ H ₁₀ O ₇	Flavonols	17.376	24.464	0.720	76.23
Cinnamic acid	C ₉ H ₈ O ₂	Hydroxycinnamic acids	19.324	6.530	0.192	5.60
Kaempferol	C ₁₅ H ₁₀ O ₆	Flavonols	20.651	7.691	0.226	25.80
Hesperetin	C ₁₆ H ₁₄ O ₆	Flavonones	21.216	20.657	0.608	44.72

mAU milli-absorbance units, ND not detected, RT retention time (min)

the presence of main compounds gallic acid (5999.97 µg/g extract), catechin (1507.08 µg/g extract), chlorogenic acid (460.64 µg/g extract), syringic acid (448.58 µg/g extract), naringenin (432.91 µg/g extract), and rutin (170.18 µg/g extract) (Table 4).

3.2 Identification of isolated fungi

The morphological identification of the fungal isolates provided preliminary insights into their species. *Alternaria alternata* colonies exhibited a velvety, dark brown appearance with distinctive concentric rings, typical of this species. Microscopic examination showed septate, darkly pigmented hyphae, and large conidia with transverse and longitudinal septa, confirming the presence of *A. alternata*. In contrast, colonies of *Fusarium oxysporum* were fast-growing and displayed a white to light pink, cottony texture. Microscopic analysis revealed the presence of fusiform macroconidia with 3–5 septa, alongside characteristic chlamydospores. This morphological profile aligned well with the known characteristics of *F. oxysporum*. The third fungal species, *Rhizoctonia solani*, formed pale brown colonies with a distinctive web-like growth pattern. Under the microscope, the hyphae were multinucleate and branched at right angles, a defining feature of *R. solani*.

The molecular identification via PCR amplification of the ITS region further confirmed the species identity of the

isolates. Successful amplification of the ITS region was achieved for all three fungal species, with the expected PCR product size being approximately 600 base pairs. Sequencing of these PCR products and subsequent BLAST analysis revealed high sequence homology with reference sequences in the GenBank database. The phylogenetic tree, constructed based on ITS sequences, provides a clear visualization of the genetic relationships among isolates of *A. alternata*, *F. oxysporum*, and *R. solani*. Each species is grouped into distinct clades, with the numbers on the tree representing bootstrap values, indicating confidence in these relationships (Fig. 3).

In the *Alternaria alternata* clade, the isolate PQ326444 *Alternaria alternata* (Zhal11) serves as the root, closely grouped with other *A. alternata* isolates such as MN706186, PQ286515, PP724415, MF102104, and KR912289. This grouping is strongly supported by a high bootstrap value of 100, demonstrating minimal genetic divergence among these isolates and strong confidence in their evolutionary relationship. Within this clade, the internal branches are delineated by lower bootstrap values of 14, 8, and 4, reflecting minor sequence variations among the isolates. While these values are lower, the overall grouping remains well-supported due to the robust terminal value of 100 (Fig. 3).

The *Fusarium oxysporum* clade also shows clear genetic relationships. OR116513 *Fusarium oxysporum* (FO-101) anchors this group, with isolates MT560380

Table 4 Phenolic profile of the methanolic extract in *Ziziphus spina-christi* branches using HPLC–DAD

Compounds	Formula	Group	RT (min)	Area (mAU*s)	%Area	Concentration (µg/g extract)
Unknown	-	-	2.984	2557.770	52.889	-
Gallic acid	C ₇ H ₆ O ₅	Phenolic acids	3.593	1528.909	31.614	5999.97
Chlorogenic acid	C ₁₆ H ₁₈ O ₉	Hydroxycinnamic acids	4.264	74.026	1.530	460.64
Catechin	C ₁₅ H ₁₄ O ₆	Flavan-3-ols	4.581	136.056	2.813	1507.08
Methyl gallate	C ₈ H ₈ O ₅	Methyl ester of gallic acid	5.492	6.842	0.141	16.85
Caffeic acid	C ₉ H ₈ O ₄	Hydroxycinnamic acids	5.939	0.000	0.000	ND
Syringic acid	C ₉ H ₁₀ O ₅	Hydroxybenzoic acids	6.408	139.380	2.882	448.58
Unknown	-	-	6.585	97.11859	2.008	-
Rutin	C ₂₇ H ₃₀ O ₁₆	Flavonol glycosides	6.935	21.508	0.444	170.18
Ellagic acid	C ₁₄ H ₆ O ₈	Phenolic acids	7.268	0.000	0.000	ND
Coumaric acid	C ₉ H ₈ O ₃	Hydroxycinnamic acids	8.915	58.155	1.202	95.69
Vanillin	C ₈ H ₈ O ₃	Phenolic aldehydes	9.322	42.876	0.886	77.19
Ferulic acid	C ₁₀ H ₁₀ O ₄	Hydroxycinnamic acids	9.923	3.075	0.063	8.41
Naringenin	C ₂₇ H ₃₂ O ₁₄	Flavanones	10.539	98.615	2.039	432.91
Rosmarinic acid	C ₁₈ H ₁₆ O ₈	Ester of phenolic acids	11.875	1.230	0.025	6.02
Daidzein	C ₁₅ H ₁₀ O ₄	Isoflavones	15.401	3.610	0.074	11.00
Quercetin	C ₁₅ H ₁₀ O ₇	Flavonols	17.376	12.385	0.256	38.59
Cinnamic acid	C ₉ H ₈ O ₂	Hydroxycinnamic acids	19.382	11.618	0.240	9.96
Kaempferol	C ₁₅ H ₁₀ O ₆	Flavonols	20.533	10.519	0.217	35.29
Hesperetin	C ₁₆ H ₁₄ O ₆	Flavonones	21.338	32.341	0.668	70.01

mAU milli-absorbance units, ND not detected, RT retention time (min)

and MT560342 clustering closely, supported by a high bootstrap value of 100. Additional isolates, including MN216218, MK268134, and MK226163, are part of the *F. oxysporum* group. The branching points within this clade display varying bootstrap values, such as 12, 8, 6, and 16, indicating moderate confidence in the internal relationships. However, the overall grouping remains highly reliable due to the consistent 100 bootstrap value for the main clade, suggesting strong evolutionary conservation within these isolates (Fig. 3).

The *Rhizoctonia solani* clade is similarly well-supported. The isolate OR116533 *Rhizoctonia solani* (RHS-302) groups with others, including MT484259, MT108198, MN872378, and MN398398, all part of the *R. solani* group. This clade also shows high genetic coherence, as reflected by a bootstrap value of 100, confirming the reliability of the genetic relationships. However, lower bootstrap values, such as 2 and 12, suggest less confidence in certain finer branches, indicating slight variability within the sequences. Despite this, the overall clade remains genetically coherent, with the high terminal bootstrap value providing strong support for the grouping (Fig. 3).

In summary, both morphological and molecular techniques provided consistent results, confirming the identities of the three fungal pathogens *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizoctonia solani*.

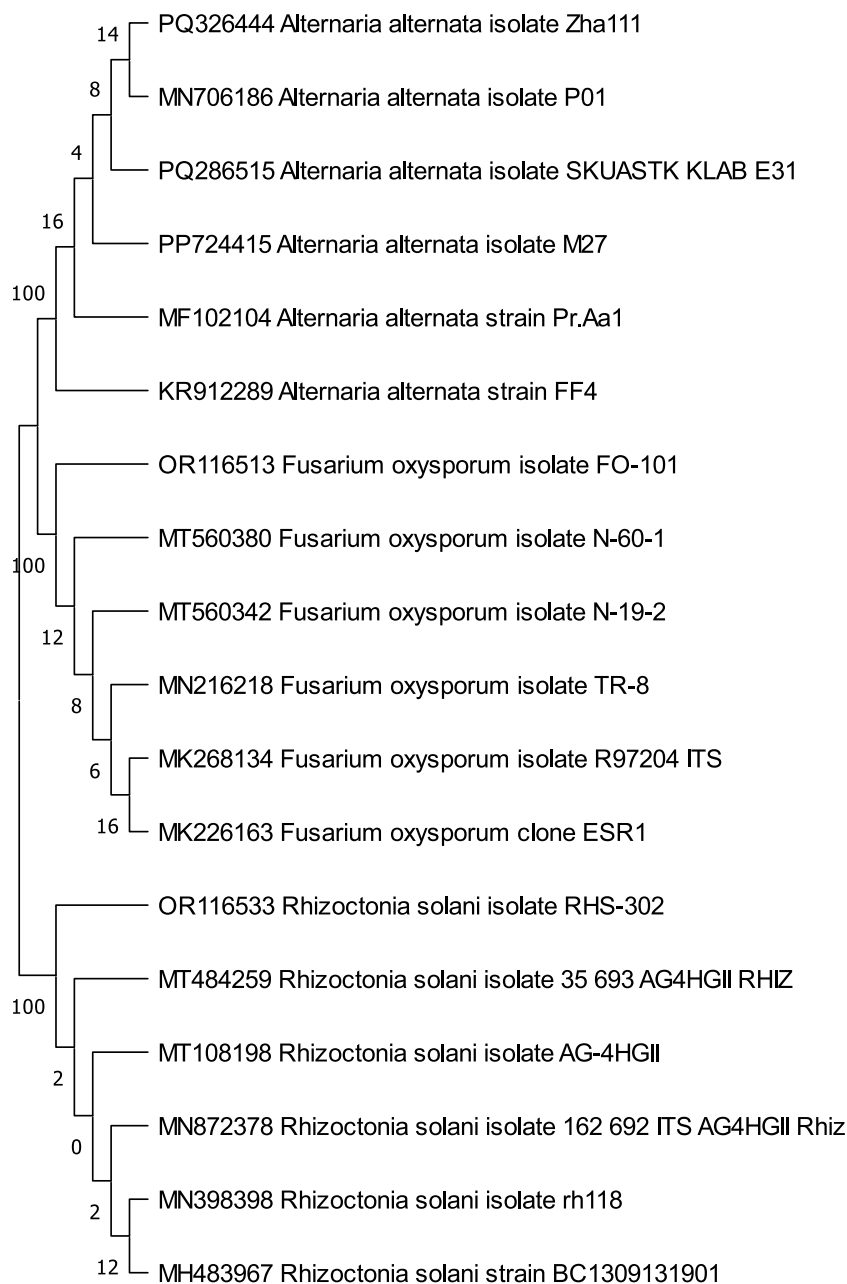
3.3 Antifungal activity of wood treated with leaf and branch extracts

After being treated with 150 µL of the concentrated ME, each wood sample was placed over the substrate that became infested with fungi. Therefore, the leaching or spreading of dissolved extracts may be the reason behind the suppression of fungal development. The antifungal activity of *P. sylvestris* wood blocks treated with the MEs from *C. siliqua* and *Z. spina-christi* leaves and branches against the growth of three mold species: *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizoctonia solani* compared with the positive control are shown in Figs. 4, 5, and 6, respectively.

Figure 4 illustrates the overall impact of the ME concentration and tree part on *A. solani* growth. Compared with the positive control, the ME at 4% was observed the highest effect. Additionally, the MEs from leaves and branches of *C. siliqua* were more effective than those from *Z. spina-christi*. The same trend was observed against the growth of *F. oxysporum* (Fig. 5). For the overall effects against *R. solani*, the ME at 4%, and the MEs from *Z. spina-christi* (leaves and branches) had the highest effects (Fig. 6).

A. alternata, *F. oxysporum*, and *R. solani* development can be inhibited to diverse degrees in wood samples treated with MEs of *C. siliqua* and *Z. spina-christi* leaves and branches at different concentrations (1%, 2%, and 4%), as

Fig. 3 The phylogenetic relationships among *Alternaria alternata* (PQ326444), *Fusarium oxysporum* (OR116513), and *Rhizoctonia solani* (OR116533) isolates were inferred using ITS sequence data. The tree was constructed using the maximum likelihood method, with bootstrap values (1000 replicates) indicated at the branch points. The scale bar represents the number of nucleotide substitutions per site. High bootstrap values at major nodes indicate strong support for the grouping of isolates within each species clade



shown in Table 5. These were compared to the control treatment (wood samples treated with 10% DMSO) and positive control Carbendazim 50 WP (250 µg/mL). The wood blocks exposed to positive control Carbendazim 50 WP (250 µg/mL) exhibited the highest percentage of inhibition against the growth of *A. solani* and *R. solani*.

The MEs at 4% from *C. siliqua* leaves and branches showed the highest fungal growth inhibition (FGI %) values of 72.22 and 71.48%, respectively, when compared to the positive control (74.81%) against the growth of *A. alternata*. This was followed by the ME of the leaves from *Z. spina-christi* at 4%, which showed a FGI % value of 70% against the growth of *A. alternata*. The extracts' MEs followed

these at 2% from *C. siliqua* (leaves and branches) and *Z. spina-christi* leaves with FGI % values of 61.85, 61.11, and 61.48%, respectively, against the growth of *A. solani*.

The highest FGI % values observed by the MEs against the growth of *F. oxysporum* were 77.77, 66.66, 61.11, and 61.11% by the application of the MEs on wood were from *C. siliqua* branches and leaves and *Z. spina-christi* leaves and branches, respectively, when compared to the positive control (75.92%). Also, the ME at 2% from *C. siliqua* branches observed moderate activity against the growth of *F. oxysporum* (FGI, 61.11%).

For *Rhizoctonia solani*, the highest FGI % values were observed by the MEs at 2% from *Z. spina-christi* branches

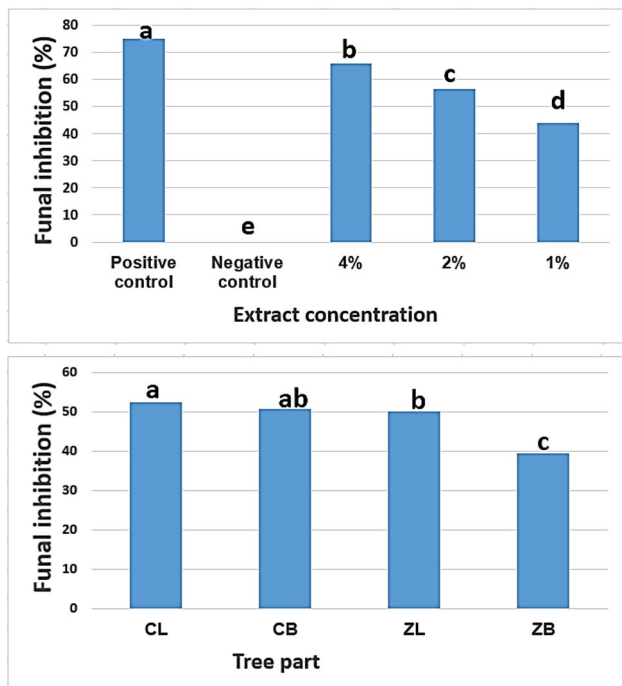


Fig. 4 The main effects of extract concentration and tree part compared with positive and negative controls against the growth of *Alternaria solani*. *C. siliqua* leaves (CL), *C. siliqua* branches (CB), *Z. spina-christi* leaves (ZL), and *Z. spina-christi* branches (ZB). Means with the same letters for each figure are not significantly different according to LSD at 0.05 probability level

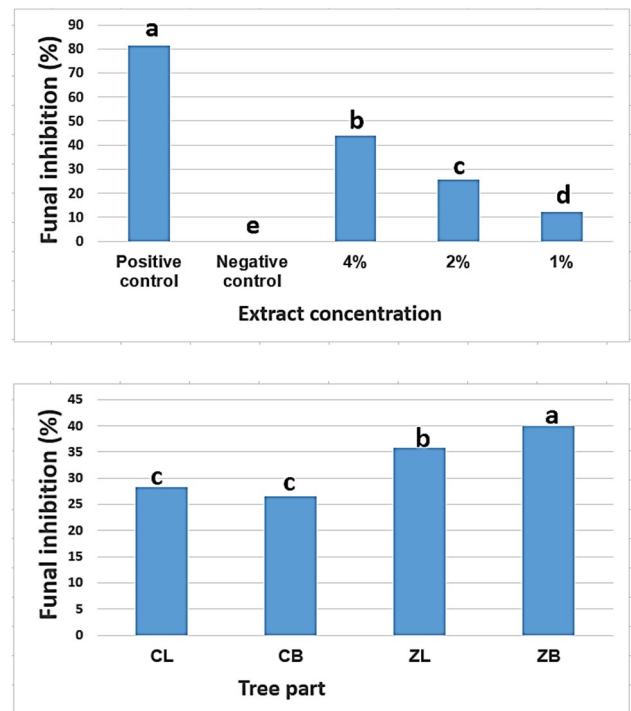


Fig. 6 The main effects of extract concentration and tree part compared with positive and negative controls against the growth of *Rhizoctonia solani*. *C. siliqua* leaves (CL), *C. siliqua* branches (CB), *Z. spina-christi* leaves (ZL), and *Z. spina-christi* branches (ZB). Means with the same letter for each figure are not significantly different according to LSD at 0.05 probability level

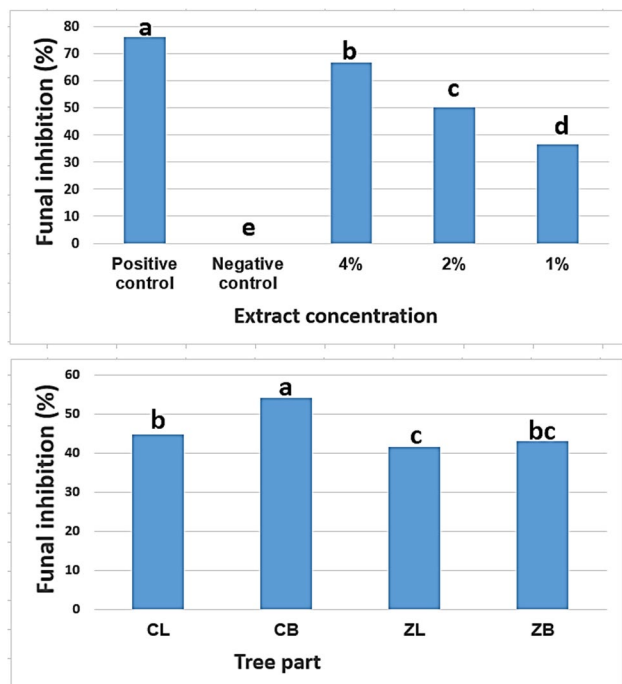


Fig. 5 The main effects of extract concentration and tree part compared with positive and negative controls against the growth of *Fusarium oxysporium*. *C. siliqua* leaves (CL), *C. siliqua* branches (CB), *Z. spina-christi* leaves (ZL), and *Z. spina-christi* branches (ZB). Means with the same letters for each figure are not significantly different according to LSD at 0.05 probability level

and leaves (59.26 and 42.22%, respectively), compared to the positive control (81.48%). This was followed by the MEs from *C. siliqua* branches at 4% (FGI 40.74%) and *Z. spina-christi* at 2% (FGI 40.74%).

4 Discussion

In recent years, there has been a rise in the use of agro-industrial wastes, resulting from tree pruning or wood manufacturing, such as tree leaves, bark, fruits, flowers, and branches, for producing sustainable bioactive chemicals [41–43]. Furthermore, the importance of bioactive substances obtained from waste having natural properties with added value as opposed to synthetic ones. The circular economy flow, residue reduction, and development of high-end uses would all benefit from the use of biomass residues from *Ceratonia siliqua* and *Ziziphus spina-christi*.

In this context, the methanol extracts (MEs) from leaves and branches of *C. siliqua* and *Z. spina-christi* grown in Egypt were analyzed for their phytochemicals, particularly phenolic and flavonoid compounds. The HPLC analysis showed the presence of several bioactive compounds with various concentrations according to the part used from each tree.

Table 5 Antifungal activity of extracts from *C. siliqua* and *Z. spina-christi* leaves and branches against *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizoctonia solani*

Treatment	Conc (%)	Fungal inhibition (%)		
		<i>Alternaria alternata</i>	<i>Fusarium oxysporum</i>	<i>Rhizoctonia solani</i>
<i>Ceratonia siliqua</i> leaves	1	53.71 ± 3.21 ^{d*}	32.96 ± 0.64 ^g	8.29 ± 2.77 ^g
	2	61.85 ± 1.28 ^c	48.15 ± 3.21 ^e	18.15 ± 3.57 ^{ef}
	4	72.22 ± 5.55 ^{ab}	66.66 ± 0.00 ^b	33.33 ± 5.55 ^d
<i>Ceratonia siliqua</i> branches	1	46.29 ± 3.21 ^{ef}	55.55 ± 0.00 ^d	0.00 ^h
	2	61.11 ± 5.55 ^c	61.11 ± 5.55 ^c	10.74 ± 6.12 ^{fg}
	4	71.48 ± 3.39 ^{ab}	77.77 ± 0.00 ^a	40.74 ± 8.48 ^{cd}
<i>Ziziphus spina-christi</i> leaves	1	44.44 ± 0.00 ^{fg}	27.77 ± 5.55 ^h	22.22 ± 0.00 ^e
	2	61.48 ± 5.59 ^c	42.59 ± 3.21 ^f	33.33 ± 0.00 ^d
	4	70.00 ± 2.93 ^b	61.11 ± 5.55 ^c	42.22 ± 3.84 ^c
<i>Ziziphus spina-christi</i> branches	1	31.48 ± 3.21 ^h	30.00 ± 2.94 ^{gh}	18.52 ± 6.41 ^{ef}
	2	40.74 ± 3.21 ^g	48.15 ± 3.21 ^e	40.74 ± 6.41 ^{cd}
	4	50.00 ± 5.55 ^{de}	61.11 ± 5.55 ^c	59.26 ± 6.41 ^b
Negative control ^a	0	0.00 ⁱ	0.00 ⁱ	0.00 ^h
Positive control ^b	250 µg/mL	74.81 ± 2.79 ^a	75.92 ± 3.21 ^a	81.48 ± 6.41 ^a
<i>p</i> -value		< 0.0001	< 0.0001	< 0.0001
LSD 0.05		4.742	4.693	8.118

*Values are means ± standard deviations (SD); means with the same letters within the same column are not significantly different according to LSD at 0.05 probability level

^aDMSO 10%; ^bthe fungicide, Carbendazim 50 WP (250 µg/mL)

By HPLC, the abundant compounds identified in the MEs from leaves and branches from *Ceratonia siliqua* were gallic acid, chlorogenic acid, catechin, methyl gallate, caffeic acid, syringic acid, rutin, coumaric acid, and naringenin. Numerous studies have determined the biological activities associated with the bioactive chemicals found in *C. siliqua* leaves [44]. Furthermore, gallic acid is a plentiful phenolic substance found in carob leaves, pulps, and seeds [45, 46].

Mostly flavonols—quercetin-3-rhamnoside and myricetin, which are usually the most abundant flavonoids in carob—made up the polyphenols present in carob leaves [47–49]. Gallic acid constituted almost 90% of the phenolic acids, the second most significant group of polyphenols. This is consistent with the literature, as gallic acid has been demonstrated to be the primary representative of phenolic acids in carob leaves [11, 47]. Leaves extract from carob showed the presence of the following flavonoids (mg/kg extract) myricetin (1486.0), kaempferol (476.4), quercetin (406.2), genistein (76.8), naringenin (52.7), and luteolin (31.8) [49]. Gallic acid, (–)-epigallocatechin-3-gallate, myricitrin, isoquercitrin, catechin, chlorogenic acid, and malic acid were isolated from the methanol extract from leaves with potential antimicrobial activity [50]. Vanillin, fraxidin, and 2,4-bis(dimethylbenzyl)–6-butylphenol were isolated from leaves [44, 51]. 4-Hydroxy-coumaric acid was isolated from leaves [52, 53]. Myricitrin, daidzein, flavonol, and morin were isolated from leaves [44, 50, 52–54].

In this work, the leaves and branches from *C. siliqua* showed good antifungal activity against the growth *Alternaria alternata* and *Fusarium oxysporum* and moderate activity against the growth of *Rhizoctonia solani*. Strong microbial inhibitors are present in the phenolics, tannins, and propyl gallate found in Turkish *C. siliqua* leaves [5].

Because carob leaves contain significant amounts of tannins, flavonoids, and phenolic substances, the carob tree's leaf extracts demonstrated strong antibacterial activity against *Pectobacterium atrosepticum* [19]. Another investigation verified that the biologically active compounds found in *C. siliqua* leaves, including gallic acid, quercetin-glucoside, kaempferol-rhamnoside, quercetin-rhamnoside, 1,2,6 tri-O-galloyl-glucopyranose, (e)-epigallocatechin-3-O-gallate, kaempferol, and quercetin, could account for the observed activity [55]. The presence of flavonoids and tannins in the acetone extract of *C. siliqua* leaf explained its potent antibacterial activity and synergistic impact when combined with infected potato tuber extract. Three carob flavonoids—rutin, apigenin, and naringin—have been shown to have antibacterial properties [56]. *Geotrichum candidum* mycelium growth was reduced (100%) by the extract from *C. siliqua* leaves [57].

The following phenolic and flavonoid compounds were identified in the carob leaves including myricetin, gallic acid, 3,4-dihydrobenzoic acid hexoside, apigenin-6-C(O-deoxyhexosyl)-hexoside, syringic acid, protocatechuic

acid, rosmarinic acid, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, catechin, epicatechin, epigallocatechin gallate, procyanidin trimer, luteolin-6-C glucoside, p-coumaric acid, rutin, ferulic acid, quercetin-3-glucoside, apigenin, kaempferol-3-rutinoside, kaempferol-3-O-hexoside, quercetin-3-pentoside, isorhamnetin-3-hexoside, quercetin-3-rhamnoside, kaempferol-3-O-pentoside, kaempferol-3-O-deoxyhexoside, and luteolin [58].

In many plant-pathogen interactions, phenolics are significant as induced phytoalexins and inherent defensive metabolites [59]. P-glycoprotein, salicylic acid, quercetin, naringin, chlorogenic acid, catechin, 4-hydroxybenzoic acid, orcinol, and 3-hydroxybenzoic acid were detected in leaf extract of carob [60]. Furthermore, numerous phenolic substances were found in leaves, including polydatin, kaempferol, tannic acid, and catechin hydrate [2]. *C. siliqua* extract exhibits strong biological activity against plant diseases including *Fusarium oxysporum* f. sp. *cucumerium* and *Monillia fructigena* [61]. Higher levels of antifungal action were demonstrated by quercetin + rutin, quercetin + morin, and quercetin + quercitrin together than by any of these flavonoids alone. It was also mentioned that despite rutin's lack of action on its own, rutin's presence increased the antifungal properties of quercetin + morin [62].

The methanol extracts from *Ziziphus spina-christi* leaves and branches observed moderate activity against *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizoctonia solani*. These could be related to the presence of phenolic compounds identified by HPLC, such as gallic acid, chlorogenic acid, catechin, syringic acid, rutin, and naringenin. The total phenolic content of extracts from *Z. spina-christi* ranged between 11.8 and 52.5 mg/g expressed in terms of gallic acid equivalent (mg of GAE/g extract) [63].

Compounds including apigenin-7-O-glucoside, quercitrin, quercetin, isovitexin, rutin, and quercetin-3-O-glucoside-7-O-rhamnoside were detected in *Z. spina-christi* leaf extract. The *Z. spina-christi* extract influenced the spore formation and germination of the cellulolytic and pectolytic activity of *Fusarium solani* [64]. Extract from *Z. spina-christi* showed antifungal activity *A. alternata*, *F. oxysporum*, *F. solani*, and *R. solani* using agar dilution bioassay [23]. Because *Z. spina-christi* extract contains several phenolic and antioxidant compounds, such as rutin, naringin, myricetin, quercetin, kaempferol, hesperidin, syringic acid, eugenol, pyrogallol, gallic acid, and ferulic acid, which stimulate the biochemical immunity of plants, foliar spraying inhibits the harmful effects of *F. oxysporum* [65]. Gallic acid, tyrosol, hydroxytyrosol, p-hydroxybenzoic acid, vanillic acid, caffeic acid, quercetin, apigenin, chlorogenic acid, ferulic acid, cinnamic acid, p-coumaric acid, and tannic acid were among the 13 phenolic chemicals found in Sidr juice extracted from leaves [66]. The main constituents of the leaf extract were gallic and ellagic acids. The methanolic extracts of *Z.*

spina-christi leaves showed notable antifungal activity, with varying inhibition percentages at different concentrations, against *Alternaria alternata*, *A. citri*, and *A. radicina* [67].

An additional investigation revealed that *Z. spina-christi* contains tannins, flavonoids, terpenoids, saponin glycosides, and alkaloids in its bark extract, where the ethyl acetate and its alkaline extracts observed antifungal effects against *Aspergillus fumigatus*, *Syncephalastrum racemosum*, and *Geotricum candidum* [68]. Methanolic extracts of *Z. spina-christi* leaves and fruits exhibited promising antifungal activity, effectively inhibiting the growth of *Alternaria alternata*, *A. citri*, and *A. radicina* at varying levels depending on the concentration.

The purified compound gallic acid exhibited strong, dose-dependent antifungal activity against *Fusarium solani*, causing significant hyphal collapse and shrinkage at 500 ppm after 24 h of exposure [69]. Additionally, gallic acid and its derivatives showed enhanced antifungal effects and effective control of *F. graminearum* [70], and *Alternaria solani* in vitro [71] highlighted their potential as environmentally friendly alternatives for managing plant pathogenic fungi.

Water, methanol, and ethanol are examples of polar solvents that facilitate the extraction of phenolic compounds, which are secondary metabolites found in plants that have at least one phenol group (an aromatic ring connected to a -OH group) [72]. The following independent variables were also used to assess other cutting-edge techniques for improving the conditions of stirring extraction and ultrasonic-assisted extraction of phenolic compounds using response surface methodology: ratio of ethanol to water and duration of extraction from bio-waste from the pistachio industry [73].

A class of 15-carbon phenolic chemicals called flavonoids, or polyphenols, are distinguished by their biological activity. There have been reports of quercitrin, quercetin, isovitexin, rutin, epicatechin, vanillic acid, caffeic acid, quercetin, apigenin, chlorogenic acid, ferulic acid, and cinnamic acid in polar extracts of various medicinal plants [74–76]. Flavonoids' biological activity appears to be caused by their amphipathic properties, which enhance the antimicrobial activities of the chemical structure, particularly the hydrophobic substituents (prenyl groups, alkylamino, and alkyl chains, and heterocyclic fractions with N or O) [77, 78]. While methylation of the active hydroxyl groups reduces the activity, flavonoids with hydroxyls at specific locations on aromatic rings increase their activity [78].

Flavonoids have a significant antifungal activity effect; their exact mechanisms of action are still being investigated [79, 80]. By destroying the plasma membrane, causing mitochondrial malfunction, and preventing the production of cell walls, cell division, RNA/protein synthesis, and efflux-mediated pumping mechanism, flavonoids prevent the growth of fungi [81]. A study has shown that the structure of flavonoids and their biological activity are related. Flavones,

flavonols, and natural flavonones are among the flavonoid classes that have potent antifungal effects [82].

Flavonoids have been anticipated to be effective in the creation of antifungal drugs for human use due to their inhibitory effect on the spore germination of infections in plants. Numerous investigations on the antifungal activity, mode of action, structural change, and structure–activity relationships (SARs) of flavonoids have been conducted in recent years [83, 84].

In conclusion, the methanol extracts of both *C. siliqua* and *Z. spina-christi* exhibited significant antifungal activity against a range of pathogenic fungi, including *Alternaria alternata*, *Fusarium oxysporum*, and *Rhizoctonia solani*. Due to their rich phytochemical profiles, both plant species hold considerable promise as natural, eco-friendly alternatives for managing plant fungal diseases. The variations in antifungal potency between different plant parts and concentrations suggest that further exploration of these extracts could lead to more effective formulations for agricultural applications. Additionally, their potential to enhance plant immunity and mitigate disease progression highlights the value of *C. siliqua* and *Z. spina-christi* as valuable resources for sustainable crop protection.

5 Conclusion

The polyphenols from the leaf and branch extracts of *Ceratonia siliqua* and *Ziziphus spina-christi* are identified in this study by an HPLC. The extracts were evaluated for their antimicrobial activity against the molecularly identified fungal isolates *Alternaria alternata* (PQ326444), *Rhizoctonia solani* (OR116533), and *Fusarium oxysporum* (OR116513). *A. alternata* and *F. oxysporum* were effectively combated by the leaves and branches extract from *C. siliqua*, while *R. solani* was effectively inhibited by the leaves and branches extract from *Z. spina-christi* when applied to wood samples. These activities could be attributed to the presence of several polyphenolic compounds such as gallic acid, ellagic acid, catechin, chlorogenic acid, syringic acid, coumaric acid, methyl gallate, and rutin. According to the study, plant extracts may be utilized to eliminate fungal infestations on wood samples caused by *A. alternata*, *F. oxysporum*, and *R. solani*.

Author contribution All authors have equal contributions in conception of idea and experiment planning, laboratory analysis, data interpretation, experiment execution, literature search, proof reading, write up of manuscript, and overall formatting and technical proof reading of manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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