Research Article

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Extraction, phytochemical characterization, and antifungal activity of *Salvia rosmarinus* extract

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Abstract: In the current study, we used high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) to investigate and analyze the methanolic extract of *Salvia rosmarinus* leaves. HPLC analysis showed that the extract revealed a diverse array of polyphenolic compounds, including apigenin, catechin, chlorogenic acid, cinnamic acid, caffeic acid, coumaric acid, daidzein, ellagic acid, ferulic acid, gallic acid, hesperetin, kaempferol, methyl gallate, naringenin, pyrocatechol, quercetin, rutin, syringic acid, and vanillin. Furthermore, three fungal isolates from symptomatic strawberry plants were obtained and identified as *Botrytis cinerea* (OR116486), *Fusarium oxysporum* (OR116505), and *Rhizoctonia solani* (OR116525). The extract's antifungal activity was evaluated at concentrations of 0, 50, 150, 200, and 300 µg/mL. At 200 µg/mL, the extract showed growth inhibition percentages of 74.56, 58.19, and 56.67% for *R. solani*, *F. oxysporum*, and *B. cinerea*, respectively, while at 300 µg/mL, all the tested fungi were completely suppressed. The GC–MS analysis revealed that the major compounds of the methanolic extract identified based on their retention times and relative peak areas (%) included β-caryophyllene (12.06%), germacrene d (13.55%), caryophyllene oxide (3.13%), methyl palmitate (5.26%), hexadecanoic acid (4.9%), and methyl stearate (6.02%). These results show rosemary extract's potential as a source of natural antifungal agents against plant photogenic fungi. As a result, it provides a safer alternative to the current protective approaches for plant disease management.

Keywords: *Salvia rosmarinus*, methanolic extract, HPLC, GC-MS, antifungal

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1 Introduction

Rosemary is an evergreen perennial plant that belongs to the family Lamiaceae, previously known as *Rosmarinus officinalis*. Recently, the genus *Rosmarinus* was combined with the genus *Salvia* in a phylogenetic study and became known as *Salvia rosmarinus* [1] It is an aromatic shrub that can grow up to 2 m high, and the leaves are the main part that is used for different purposes. Its leaves can be collected either dry or fresh and have been intended for culinary, medicinal, and cosmetic purposes since ancient times due to their human health benefits. Furthermore, the dried leaves, when cursed, emit a camphoraceous odor, which can be implemented in many dishes and recipes. Additionally, the oil derived from the leaves is used in the cosmetic industry [2–4]. However, it is today regarded as one of the most significant ornamental and medicinal plants in the world. Due to its numerous health benefits as

an antibacterial, anti-inflammatory, antioxidant, anti-apoptotic, and anti-tumorigenic herb, rosemary has been grown for many years and used in folk medicine, cosmetics, and phytocosmetics. In traditional medicine, rosemary extract is used to treat neurological illnesses, peripheral vascular issues, chronic weariness, urinary tract infections, and hair loss. Additionally, rosemary has a long history of usage as an emmenagogue, diaphoretic, choleretic, tonic, rubefacient, antispasmodic, anti-inflammatory, expectorant, emmenagogue, digestive aid, and diuretic. Rosemary plants are becoming more and more well-liked due to their antioxidant and health-promoting qualities [2].

Recently, rosemary extracts have been the focus of research for their effective antimicrobial properties against a variety of pathogens. Studies have shown major components in rosemary oil, including α-pinene, myrcene, 1,8-cineole, camphor, camphene, α-terpineol, and borneol, to have antimicrobial activity. Rosemary oil is effective against *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *E. coli* [3]. An additional study presented the higher efficiency of rosemary essential oil at low concentrations compared to two commonly used fungicides against ginseng root rot disease [5]. In a different study, rosemary extract was also effective in controlling *Macrophomina phaseolina* and charcoal rot in soybean [6]. Thus, these studies indicate the valuable antifungal properties of rosemary extract.

Strawberry (*Fragaria* × *ananassa*) is considered one of the most valuable fruit crops worldwide. The strawberry fruit contains vitamins, nutrients, and antioxidants in addition to its sweet taste and distinct aroma, making it a highly demanded crop in the market [7,8]. Thus, strawberry consumption has been rising quickly in recent years as this fruit production area has grown dramatically. However, the microclimate, high humidity, and temperatures in greenhouses make it easier for a variety of diseases to spread [9]. Several plant pathogens impair strawberry productivity, but recently, soilborne fungal pathogens are among the most restricting diseases in strawberry fields [8]. *Rhizoctonia solani*, *Fusarium oxysporum*, and *Botrytis cinerea* are some of the major pathogens that affect strawberry production worldwide. These pathogens can cause root rot and gray mold diseases. Which greatly affects the strawberry crop yield worldwide.

The spread of plant fungal infections around the world can be managed in a variety of ways. Using pesticides is the most popular and effective way to eradicate diseases and pests in agriculture [10]. Unfortunately, the increased use of pesticides in farming to address these problems before and throughout the fruit harvesting process can lead to dangerous outbreaks, and the pathogenic fungi can become resistant to the fungicides [11,12]. Therefore, current research focuses on developing novel strategies for lowering the levels of pesticides left in the soil using eco-friendly components. To establish an atmosphere free of chemicals, natural extracts are among the safest and most affordable substitutes for the widely used synthetic chemical antifungal agents [13].

In this study, we aimed to use an eco-friendly natural extract of *S. rosmarinus* as a novel method to manage strawberry pathogens (*R. solani*, *F. oxysporum*, and *B. cinerea*). Through isolating and molecularly characterizing the causative agents associated with strawberry root rot and grey mold disease. Then, evaluate the antifungal properties of a methanolic extract derived from *S. rosmarinus* leaves against the fungal pathogens responsible for the aforementioned diseases. Additionally, the research sought to identify the predominant phytochemical constituents present in the extract using a combination of high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) analysis.

2 Materials and methods

2.1 Phytopathogen isolation, purification, and preservation

Three types of fungi were isolated from the roots and fruits of strawberry plants from EL-Beheira, Egypt. To maintain their purity, the fungi were cultured on potato dextrose agar (PDA) medium, utilizing 9 cm Petri dishes. These dishes were then placed in a controlled incubator set at 27°C for 1 week, allowing the fungi to grow and develop. After the incubation period, the three fungal isolates were carefully transferred to slants and securely stored in a refrigerator at 4°C. This storage ensured their viability and provided a means for future investigation, analysis, and research purposes.

2.2 Morphological and molecular analysis

For morphological and molecular analysis, the three fungal isolates obtained were cultured on PDA at a temperature of 27°C for a week. The plates were observed for visible morphological characteristics colony appearance, color, texture, and growth pattern. Also, the properties of the mycelia and spores were determined *via* a light microscope following established identification protocols [14]. These morphological

features were documented and analyzed to differentiate the three fungal isolates. In addition to morphological analysis, molecular techniques were employed to further characterize the fungal isolates. The mycelia were collected to extract the DNA, and after that, using the CTAB procedure [15], the isolated DNA was used at a concentration of 100 ng/μL for PCR amplification in the future. For each of the fungal isolates, the whole rDNA-ITS (ITS) region was amplified using certain PCR primers (ITS1/ITS4) [16]. The amplified DNA fragments were then subjected to sequencing analysis, either through Sanger sequencing methods. The obtained DNA sequences were compared to existing databases and analyzed to determine the identity and genetic relatedness of the fungal isolates.

2.3 Plant sampling

The leaves of *S. rosmarinus* plants cultivated in Alexandria, Egypt, were collected for experimental use. For the investigation, only healthy leaves free of morphological abnormalities were used. The leaves were first carefully washed with running water to discard the surface impurities. After that, they were allowed to dry for a further 10 days at room temperature. The sample was then turned into a powder by being finely pulverized before using them in the trials.

2.4 Plant leaf extracts' preparation

The preparation of methanolic extract involved several steps. Initially, we mixed 50 g of the plant powder with 500 mL of 99% methanol in separate Erlenmeyer flasks. We agitated the mixture on a rotary shaker at a speed of 100 rpm and left them at room temperature for one night. This allowed the solvents to extract the desired compounds from the plant material. After the extraction phase, we used Whatman No. 1 filter paper to remove any contaminants or solid residues from the leaf extract. We used vacuum-assisted drying to get rid of any leftover methanol. The leftover solvent was made to evaporate by exposing the extract to a temperature of 30°C. The obtained dried extract was then kept for subsequent use in a refrigerator at 4°C.

2.5 *S. rosmarinus* extract antifungal activity

The poisoned food technique was used to evaluate the antifungal activity of *S. rosmarinus* extract [17]. A negative control (NC) comprised of PDA plates that had been treated with dimethyl sulfoxide was used in place of various extract concentrations (50, 100, 150, 200, and 300 μg/mL). Round fungal discs (0.5 cm in diameter) were positioned on the PDA plates and cultured for 5 days at 25°C. Two pesticides were used to compare extract results (positive control [PC] 1, Copper formate, and PC2, Ridomil gold SL, at concentrations of 200 μg/mL). Three duplicates of the experiment were run. The growth inhibition percentage (%) was calculated by comparing the impact of the extract on the mycelial expansion of the fungi relative to the control, utilizing the equation $[(C - T)/C] \times 100$. In this formula, C denotes the length of fungal growth observed in the control, while T signifies the length of mycelial growth observed in the treated sample [18]. Also, the 50% inhibition concentration (IC50) was calculated according to the formula IC50 = [(tested concentration × 50)/inhibition percentage (%)].

2.6 HPLC conditions used

The assessment of polyphenolic constituents in the *S. rosmarinus* methanolic extract was performed using a selection of reference compounds, including apigenin, catechin, chlorogenic acid, cinnamic acid, caffeic acid, coumaric acid, daidzein, ellagic acid, ferulic acid, gallic acid, hesperetin, kaempferol, methyl gallate, naringenin, pyrocatechol, quercetin, rutin, syringic acid, and vanillin. To identify these phenolic compounds, an Agilent 1260 Infinity HPLC Series was employed, featuring a Quaternary pump and a Zorbax Eclipse Plus C18 column with dimensions 100 mm × 4.6 mm i.d. The separation process was executed at 30°C using a gradient elution composed of (A) HPLC grade water with 0.2% H_3PO_4 (v/v), (B) methanol, and (C) acetonitrile, maintaining a flow rate of 1 mL/min. A volume of 25 μL of the sample extract was injected. A variable-wavelength detector set to 284 nm was used for compound detection. The resulting data were integrated using ClarityChrom® Version 7.2.0, a software developed by Knauer Wissenschaftliche Geräte GmbH, located in Berlin, Germany [19,20].

2.7 GC–MS analysis

In the analysis, we examined the chemical composition of the methanolic extract of *S. rosmarinus* using GC–MS. The analysis was carried out with a Thermo Scientific Trace GC Ultra ISQ mass spectrometer, which originates from Waltham, MA, USA. For the analysis, we utilized the TraceGOLD TG-5MS

direct capillary column with dimensions of 30 m × 0.25 mm × 0.25 m film thickness. To dissolve the methanolic extract, we used methanol of the highest caliber suitable for spectroscopy. The column oven temperature was initially set to 50°C and then increased by 5°C/min until it reached 230°C, where it was held for 2 min. Subsequently, the temperature was further increased to a maximum of 290°C and maintained at that level for an additional 2 min. For the injector and mass spectrometer transfer lines, temperatures of 250 and 260°C, respectively, were maintained. Helium was chosen as the carrier gas, and a sample volume of 1 L was injected at 250°C using a 1:30 split ratio. The mass spectrometer operated in the electron ionization mode at 200°C with an energy of 70 eV. The study of mass spectra was conducted within the scan range of 40 to 1,000 *m*/*z*. The 40–1,000 *m*/*z* scan range was chosen for the study of mass spectra. By comparing the mass spectra and retention periods to information found in the Wiley and NIST MS library databases, components were identified [21,22].

2.8 Statical analysis

A one-way analysis of variance was used to statistically analyze the antifungal efficacy of various extract concentrations. A post hoc analysis utilizing the least significant difference test was carried out with a significance level of 0.05 to compare the means.

3 Results

3.1 Isolation and identification of fungal isolates

All the fungal isolates derived from the strawberry plants were subjected to initial morphological identification. The fungal strain obtained from diseased strawberry fruits exhibited distinct characteristics of *B. cinerea* through cultural, morphological, and microscopic analyses. Conidiophores carrying conidia with typical features of *B. cinerea* were observed. The conidiophores were tall, stout, and dichotomously branched, with short, dark, and septate sporogenous branches near each conidiophore's apex. Terminal ampullae were present on each branch, containing clusters of conidia on short, fine denticles. The conidia were ovate, consistent with the characteristics of most species within the *Botrytis* genus.

The fungal strain *R. solani* was isolated from infected strawberry root plants. This isolated strain displayed slightly melanized hyphae with asymmetrical forms and brownish sclerotia. Microscopic examination revealed hyphae with 90° branching, followed immediately by hyphal constriction and septa development. Notably, no conidia, clamp connections, or rhizomorphs were observed during the investigation. The *F. oxysporum* strain, isolated from infected strawberry roots, showed a varying range of white, creamy mycelium, from scarce to profuse growth. The macroconidia of this strain typically had an average of 3–4 septa and exhibited a sickle-shaped morphology with a slightly flattened apical end. Additionally, numerous oval to kidney-shaped microconidia were observed. These microconidia were primarily formed through elongated monophialides that produced false heads.

To verify the morphological characterization of the isolates, DNA extraction was carried out, followed by PCR amplification of the ITS region, which produced 600 bp products. The nucleotide bases derived from the PCR products were then subjected to sequencing. A BLAST search

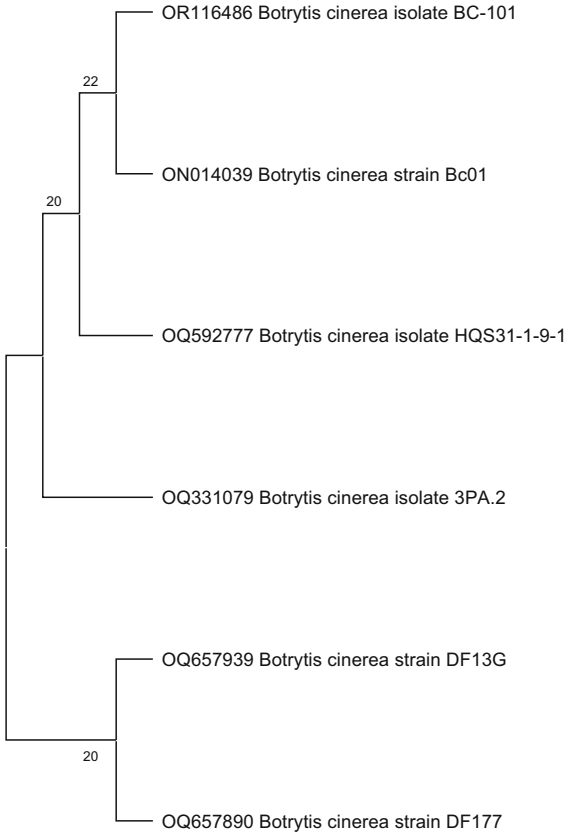


Figure 1: Utilizing the maximum likelihood method, a phylogenetic tree was created to demonstrate the genetic relatedness between our isolated fungus *B. cinerea* isolate BC-101 (accession number, OR116486) and the other identified *B. cinerea* isolates retrieved from the NCBI-GenBank portal relied on internal transcribed spacer gene sequences (ITS region). The tree bootstrapped from 1,000 tested repetitions.

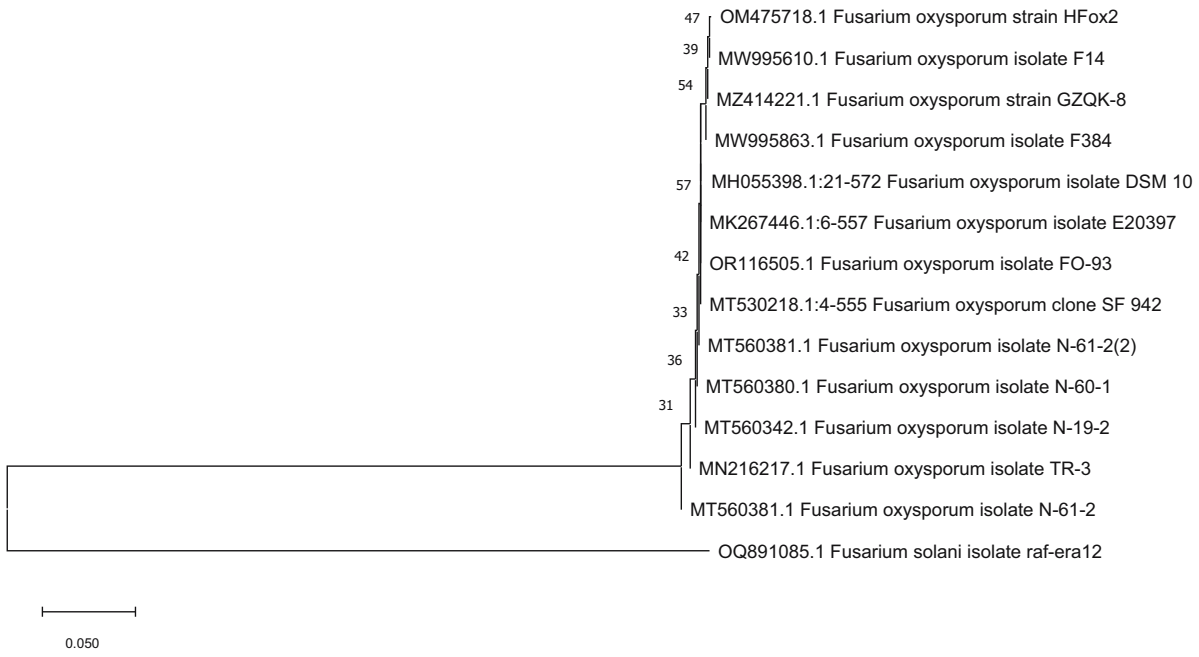


Figure 2: Utilizing the maximum likelihood method, a phylogenetic tree was created to demonstrate the genetic relatedness between our isolated fungus *F. oxysporum* isolate FO-93 (accession number, OR116505) and the other identified *F. oxysporum* isolates retrieved from the NCBI-GenBank portal relied on the internal transcribed spacer gene sequences (ITS region). The *F. solani* isolate raf-era 12 was designated as an outgroup fungus. The tree bootstrapped from 1,000 tested repetitions.

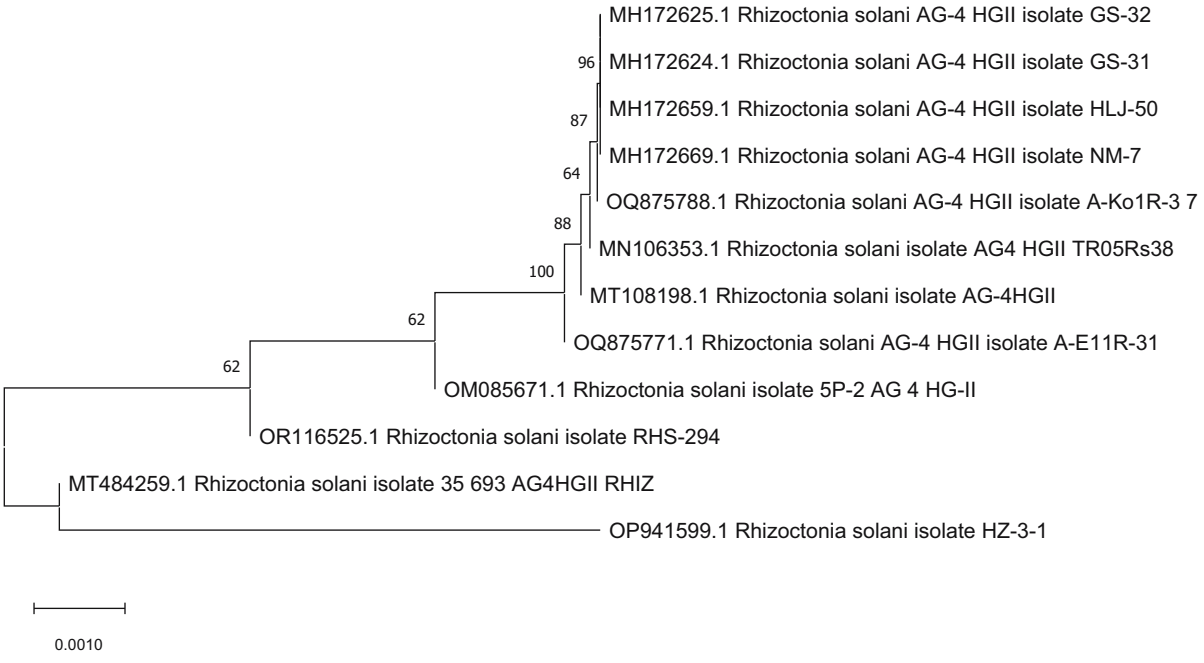


Figure 3: A phylogenetic tree was assembled employing the maximum likelihood method, to demonstrate the genetic relatedness between the *R. solani* isolate (RHS-294) and additional *R. solani* isolates available in GenBank.

Table 1: Percentage of *in vitro* growth suppression of plant pathogenic fungi subjected to *S. rosmarinus* leaf extract treatments

Concentrations (µg/mL)	Inhibition percentage (%) and IC50					
	Rhizoctonia solani	IC50	*Fusarium oxysporum*	IC50	*Botrytis cinerea*	IC50
50	52.59d	47.54	38.52e	64.90	37.41e	66.83
100	55.93c	89.40	40.09d	124.72	38.80e	128.87
150	56.30c	133.21	43.04d	174.26	40.37d	185.78
200	74.56b	134.12	58.19b	171.85	56.67b	176.46
300	100.00a	150.00	100.00a	150.00	100.00a	150.00
NC	0.00e	0.00	0.00f	0.00	0.00f	0.00
PC1 (Copper formate) 200	73.7b	135.69	53.33c	187.51	42.12c	237.42
PC2 (Ridomil Gold SL) 200	97.57a	102.49	100.00a	100.00	100.00a	100.00

In each column, varying letters denote that the data exhibit significant differences as determined by the LSD test at a probability level of 0.05.

was conducted to analyze the resulting sequences. The molecular identification findings from the BLAST search corroborated and reinforced the morphological identification of the examined isolates. The three fungal strains were identified as *B. cinerea*, *F. oxysporum*, and *R. solani*, and each was archived in the GenBank database under accession numbers OR116486, OR116505, and OR116525, respectively.

Phylogenetic analysis of the *B. cinerea* nucleotide sequence with the other known retrieved isolates from GenBank revealed that the *B. cinerea* isolate investigated in this study exhibited notable genetic similarity (as depicted in Figure 1). In a relative phylogenetic analysis of the *B. cinerea*-ITS region, it was observed that our fungal isolate *B. cinerea* BC-101 (OR116486) displayed the highest genetic similarity (100%) to the *B. cinerea* isolate from China (ON014039), as illustrated in Figure 1.

By performing a relative phylogenetic analysis of the *Fusarium*-ITS region, it was noted that our fungal isolate *F. oxysporum* isolate FO-93 (OR116505) shared the highest genetic similarity (100%) with *F. oxysporum* isolate from USA (MK267446), as shown in Figure 2. Additionally, the *F. oxysporum* isolate demonstrated a disclosed phylogenetic relationship with *F. solani* from Egypt (OQ891085) which was used to root the phylogenetic tree. Furthermore, when comparing the *R. solani* isolate from this study to other *R. solani* isolates in the NCBI database (as depicted in Figure 3), noticeable genetic differences were observed. Our *R. solani* isolate when compared with existing isolates in the GenBank database, it became evident that the maximum genetic similarity of 100% was noted with *R. solani* isolate reported in Turkey (accession number OM085671). In contrast, the minimum nucleotide sequence similarity, at 99.69%, was established between our isolate and the isolate identified in China (accession number OP941599). The identification of this *R. solani* isolate raises concerns about its potential increased virulence and the significant threat it may pose to economically important crops.

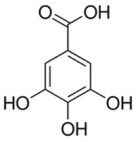
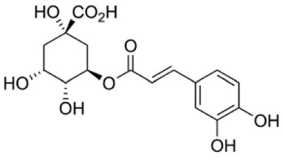
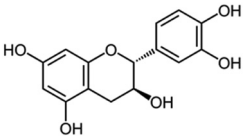
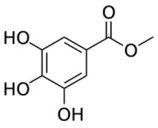
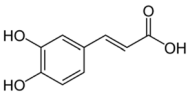
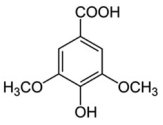
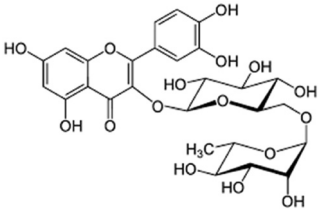
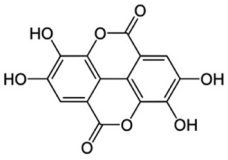
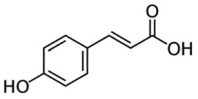
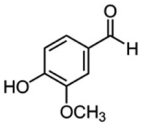
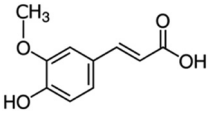
3.2 Effect of the rosemary extracts on the fungal pathogens

The provided Table 1 presents the growth inhibition percentages (%) and IC50 values of three plant pathogenic fungi, *R. solani*, *F. oxysporum*, and *B. cinerea*, in response to different concentrations (µg/mL) of *S. rosmarinus* leaf extracts.

At a concentration of 50 µg/mL, the methanolic extract showed growth inhibition percentages of 52.59% and the IC50 is 47.54 µg/mL for *R. solani*, 38.52%, and the IC50 is 64.90 µg/mL for *F. oxysporum*, and 37.41% and the IC50 is 66.83 µg/mL for *B. cinerea*. Increasing the concentration to 100 µg/mL, the methanolic extract displayed growth inhibition percentages of 55.93% for *R. solani* (with an IC50 of 89.40 µg/mL), 40.09% for *F. oxysporum* (with an IC50 of 124.72 µg/mL), and 38.8% for *B. cinerea* (with an IC50 of 128.87 µg/mL). At 150 µg/mL, the methanolic extract resulted in growth inhibition percentages of 56.3% for *R. solani* (with an IC50 of 133.21 µg/mL), 43.04% for *F. oxysporum* (with an IC50 of 174.26 µg/mL), and 40.37% for *B. cinerea* (with an IC50 of 185.78 µg/mL). The concentration of 200 µg/mL demonstrated significant growth inhibition for the extract. The methanolic extract displayed growth inhibition percentages of 74.56% for *R. solani* (with an IC50 of 134.12 µg/mL), 58.33% for *F. oxysporum* (with an IC50 of 171.85 µg/mL), and 56.67% for *B. cinerea* (with an IC50 of 176.46 µg/mL). The inhibition percentage increases with higher concentrations, reaching 100% inhibition at the highest concentration of 300 µg/mL against all tested fungi.

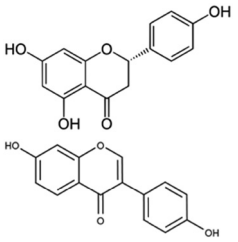
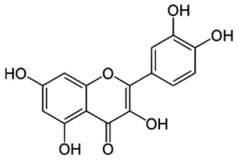
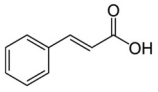
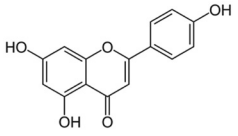
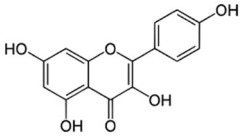
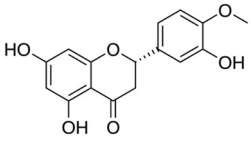
The NC had no growth inhibition for any of the fungi, with 0% inhibition. The PC1 using copper formate at a concentration of 200 µg/mL showed a growth inhibition percentage of 73.7% for *R. solani,* while the PC2 using Ridomil Gold SL at the same concentration inhibited the growth of *R. solani* by 97.49% and totally suppressed *F. oxysporum* and *B. cinerea*. Statistical analysis ($p \leq 0.05$)

Table 2: Polyphenolic compounds revealed from the HPLC analysis of the methanolic extract of *S. rosmarinus*

Compounds	Concentration (µg/g)	Chemical structure
Gallic acid	564.98	
Chlorogenic acid	144.27	
Catechin	1094.63	
Methyl gallate	100.05	
Coffeic acid	868.92	
Syringic acid	310.83	
Pyro-catechol	*ND	
Rutin	226.78	
Ellagic acid	72.58	
Coumaric acid	8.32	
Vanillin	274.46	
Ferulic acid	2017.27	
Naringenin	2038.44	

(Continued)

Table 2: *Continued*

Compounds	Concentration (µg/g)	Chemical structure
Daidzein	236.26	
Querectin	266.32	
Cinnamic acid	10.79	
Apigenin	360.14	
Kaempferol	320.57	
Hesperetin	172.35	

*ND = not detected.

indicated significant differences in growth inhibition percentages compared to the NC.

3.3 *S. rosmarinus* extract's polyphenolic content

Table 2 provides the concentrations (µg/g) of various polyphenolic compounds present in the methanolic extract. In the methanolic extract, the predominant compounds were gallic acid (564.98 µg/g), catechin (1094.63 µg/g), caffeic acid (868.92 µg/g), and ferulic acid (2017.27 µg/g). Other notable compounds included chlorogenic acid (144.27 µg/g), syringic acid (310.83 µg/g), rutin (226.78 µg/g), and ellagic acid (72.58 µg/g). Lower concentrations were observed for compounds such as coumaric acid (8.32 µg/g), cinnamic acid (10.79 µg/g), and daidzein (236.26 µg/g) Figure 4. These results indicate that both the methanolic extract contains a range of polyphenolic compounds with varying concentrations. The presence of these compounds suggests that the extracts may possess potential antioxidant and bioactive properties, which can contribute to the medicinal and therapeutic properties associated with *S. rosmarinus*.

3.4 *S. rosmarinus* extract's GC–MS content

The GC–MS chromatogram of the methanolic extract exhibited a total of 27 peaks (Figure 5). These peaks corresponded to the bioactive compounds that were identified by comparing their peak retention time (RT), relative abundance area (%), and compound class with those of known compounds cataloged in various mass spectral libraries. The major compounds of methanolic extract identified based

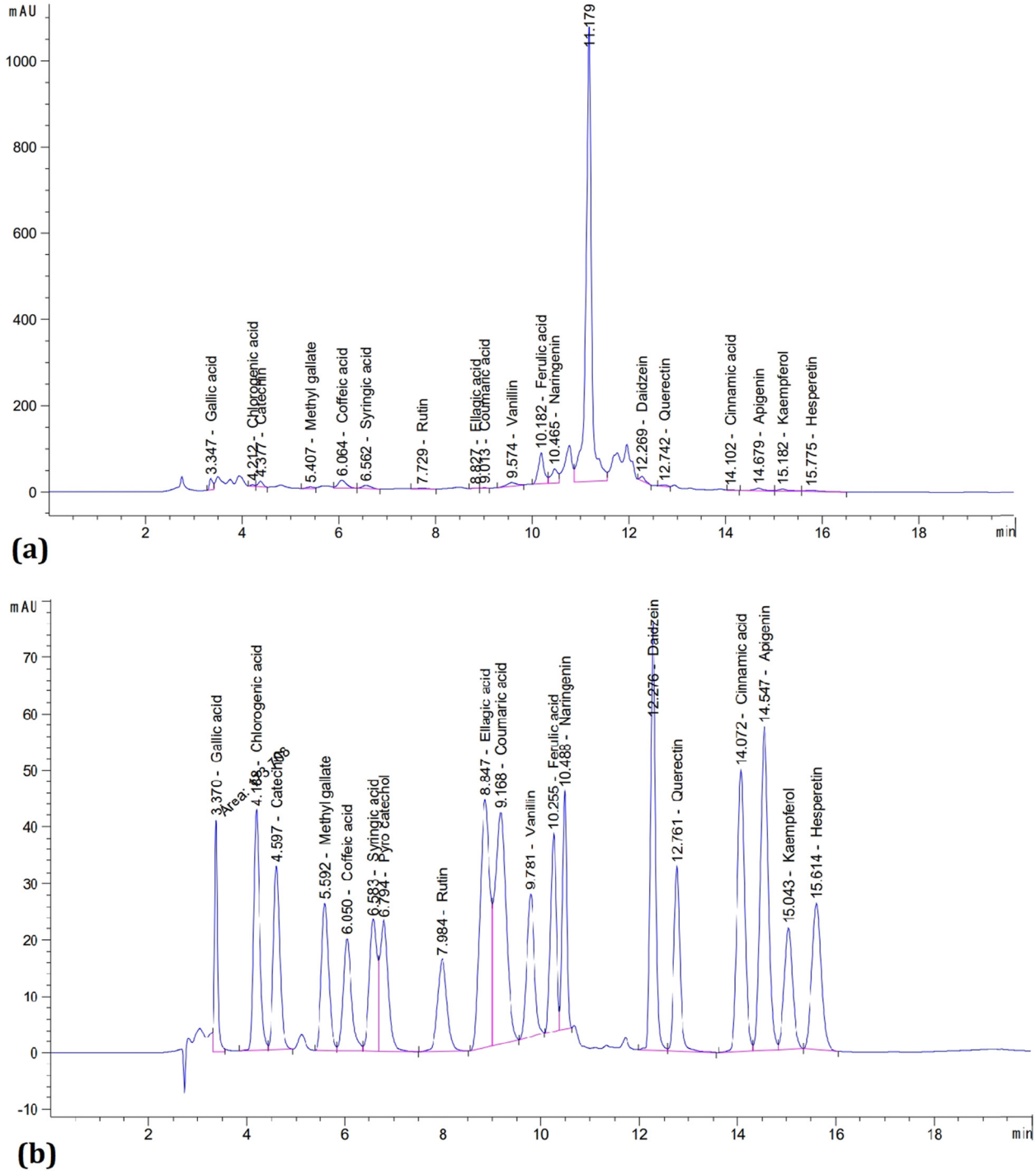


Figure 4: A chromatograph of the polyphenolic compounds observed in (a) the methanolic extract of *S. rosmarinus* and (b) the polyphenolic compound standards.

on their RT and relative peak areas (%) and their hit spectrums included β-caryophyllene (12.06%), germacrene d (13.55%), caryophyllene oxide (3.13%), methyl palmitate (5.26%), hexadecanoic acid (4.9%), and methyl stearate (6.02%). These compounds belong to various classes, including terpenoids, sesquiterpenes, fatty acid esters, fatty acids, and flavonoids (Table 3 and Figure 6).

Several terpenoids were detected, such as camphor (0.87%), (−)-borneol (3.48%), and α-terpineol (1.74%). Sesquiterpenes were also abundant, with compounds like γ-elemene (0.59%),

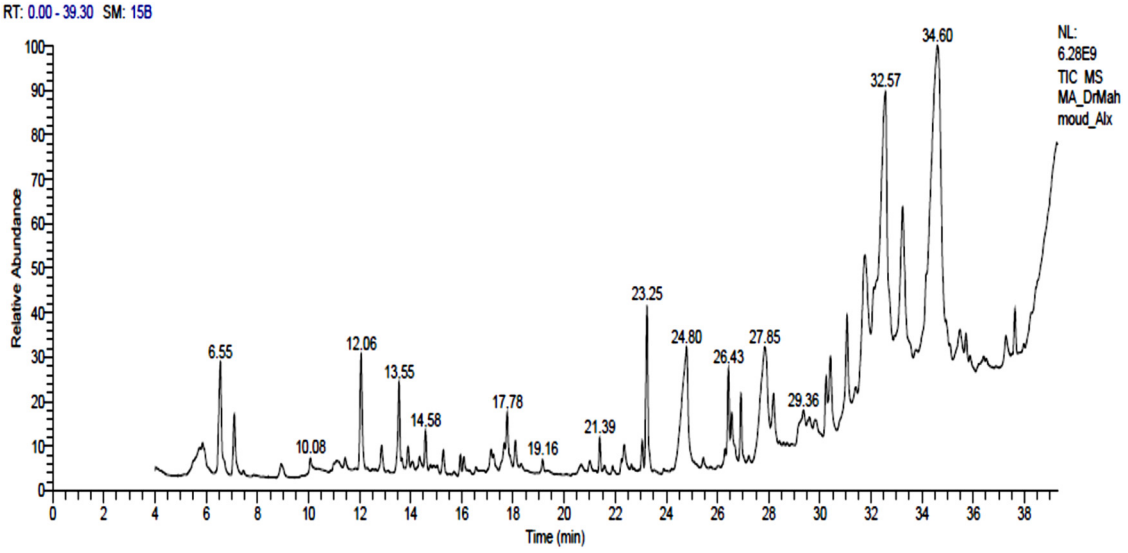


Figure 5: A chromatograph of phytochemicals detected in the methanolic leaf extract of *S. rosmarinus* using GC–MS.

Table 3: GC–MS analysis of the methanolic extract of *S. rosmarinus*

RT	Area (%)	Name	Class
5.86	0.87	Camphor	Terpenoid
6.56	3.48	(−)-Borneol	Terpenoid
7.1	1.74	alpha-Terpineol	Terpenoid
12.06	4.47	β-Caryophyllene	Sesquiterpene
13.55	2.3	Germacrene D	Sesquiterpene
13.9	0.59	γ-Elemene	Sesquiterpene
14.58	1.07	δ-Amorphene	Sesquiterpene
15.28	0.73	α-Acorenol	Sesquiterpene
16.08	0.4	Guaiene	Sesquiterpene
17.15	0.63	(+)-Longifolene	Sesquiterpene
17.66	0.54	Methyl jasmonate	Methyl ester
17.78	3.13	Caryophyllene oxide	Sesquiterpene oxide
21.39	0.85	Neophytadiene	Diterpene
22.35	0.85	Clovanediol	Sesquiterpene alcohol
23.06	0.79	Cembrene	Sesquiterpene
23.25	5.26	Methyl palmitate	Fatty acid ester
24.80	4.9	Hexadecanoic acid	Fatty acid
26.43	6.02	Methyl stearate	Fatty acid ester
28.19	1.29	Stearic acid	Fatty acid
30.41	1.84	*cis*-Ferruginol	Diterpene alcohol
31.06	2.61	Podocarpa-1,8,11,13-tetraen-3-one,	Diterpene
31.75	4.51	Carnosol	Diterpene
32.58	10.04	5-Hydroxy-3′,4′,7-trimethoxyflavone	Flavonoid
33.24	4.74	Gonzalitosin I	Flavonoid
35.48	0.84	DL-α-Tocopherol	Vitamin E
35.72	0.72	Isochiapin b	Flavonoid
37.27	0.78	Isochiapin b	Flavonoid
37.63	1.24	1-Heptatriacotanol	Fatty alcohol

δ-amorphene (1.07%), and β-caryophyllene (4.47%) being identified. Additionally, fatty acid esters like methyl palmitate (5.26%) and methyl stearate (6.02%) were present in significant amounts (Table 3). Flavonoids, which are known for their antioxidant properties, were also detected in the extract. Compounds such as 5-hydroxy-3′,4′,7-trimethoxyflavone (10.04%) and gonzalitosin i (4.74%) were identified. Furthermore, diterpenes like podocarpa-1,8,11,13-tetraen-3-one (2.61% area) and carnosol (4.51% area) were found in the extract. The GC–MS analysis also revealed the presence of fatty acids, including hexadecanoic acid (4.9%) and stearic acid (1.29%). Additionally, DL-α-tocopherol (0.84%), a form of vitamin E, was identified.

4 Discussion

Plant fungal diseases became more devastating due to major climate changes leading to increasing crop yield losses worldwide. Efficient and green fungicides are in high demand to secure food required by a growing population [23]. The major steps for controlling any pathogen include pathogen identification and identifying the efficient antifungal element with the optimum concentration. The results demonstrated the antifungal activity of rosemary extract against three major fungal pathogens that were isolated from the strawberry plant. Our results showed the highest inhibition at 300 µg/mL of rosemary extract against the three pathogenic fungi with a 100% inhibition percentage. Interestingly, different concentrations of rosemary methanolic extract showed different inhibition percentages

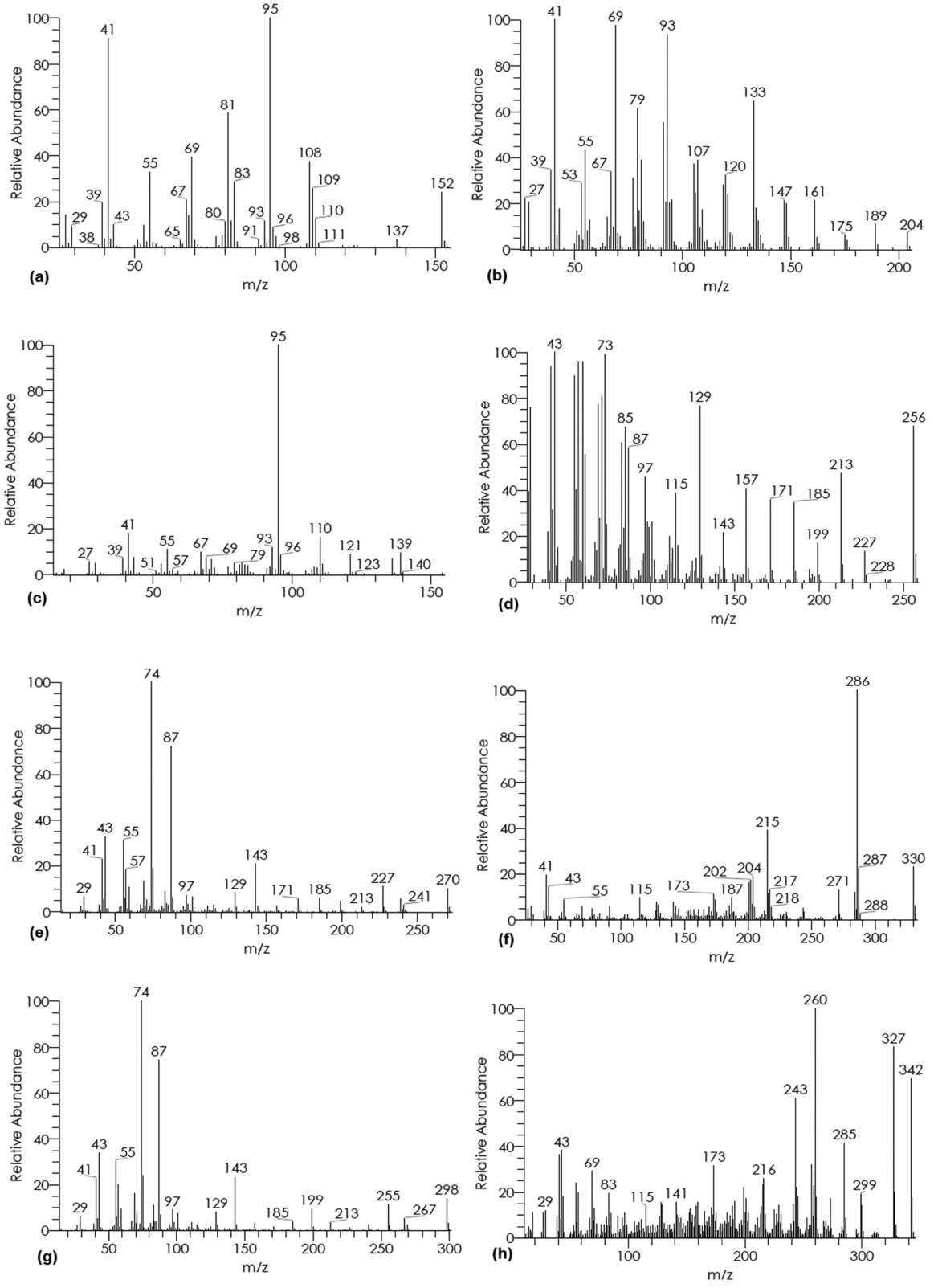


Figure 6: Hit spectrums of some GC–MS compounds detected in the methanolic leaf extract of *S. rosmarinus*, (a) camphor, (b) caryophyllene, (c) borneol, (d) hexadecanoic acid, (e) methyl palmitate, (f) carnosol, (g) methyl stearate, and (h) podocarpa-1,8,11,13-tetraen3-one.

having all treatments significantly higher inhibition when compared to the NC. This finding was revealed when analyzing the rosemary extract using HPLC and GC–MS.

For HPLC analysis, phenolic and flavonoid compounds have been known for their antimicrobial properties against plant pathogens as plants produce them naturally to defend against biotic stresses [24]. Our results showed high levels in the methanolic extract of gallic acid, catechin, caffeic acid, ferulic acid, chlorogenic acid, syringic acid, rutin, and ellagic acid. All these compounds were represented in recent studies having antimicrobial activities; however, most researchers focus on the separate effect of each compound. Other studies highly suggest that combining these compounds in the correct percentage will lead to enhanced antifungal effects at low concentrations [25–29].

For GC–MS, the major compounds of methanolic extract identified based on their RT and relative peak areas (%) included β-caryophyllene (4.47%), germacrene D (2.3%), caryophyllene oxide (3.13%), methyl palmitate (5.26% area), hexadecanoic acid (4.9%), and methyl stearate (6.02%). These compounds belong to various classes, including terpenoids, sesquiterpenes, fatty acid esters, fatty acids, and flavonoids. Camphor oil was found to be highly antifungal against a wide range of pathogenic fungus species as previously published studies showed higher camphor percentage in their GC–MS analysis related to the high antifungal activity [5,30].

In a different study, rosemary's inhibitory impact is due to the activity of several compounds, such as rosmarinic acid, carnosic acid, rosmanol, and isorosmanol. These substances interact with cell membranes, causing alterations in genetic material, nutrient modifications, disruptions in electron transport, leakage of cellular components, and changes in fatty acid production. Additionally, they interact with membrane proteins, leading to a loss of cell membrane function and structure [31].

Carnosic acid is more effective against pathogenic bacteria than other major extract constituents, including rosmarinic acid. Nevertheless, there are conflicting opinions in the literature regarding the possible associations between the composition of polyphenolic extracts and their antimicrobial properties [32]. In the same way, researchers have shown that rosemary's effectiveness is associated with a potential synergy between its contents of phenolic acids and diterpenes [33,34]. On the other hand, Bernardes et al. [35] claim that a strong correlation exists between the concentration levels of diterpenes and the bioactivities of rosemary extract.

There were investigators have found that the antimicrobial activity against *S. aureus* is enhanced when α-pinene is present as a major component. This outcome may be related to the ability of terpenes to disrupt cell membranes and promote lysis [36,37]. The effectiveness of rosemary essential oil against *E. coli* is linked to the combined actions of several minor components within its volatile fraction, rather than any individual component, which aligns with the findings of Zaouali et al. [36]. The literature contains reports of sesquiterpenoids exhibiting significant antifungal activities [38]. On the contrary, the ethanolic rosemary extract did not exhibit antifungal activity against *Aspergillus niger* and *Penicillium roquefortii* fungi [39]. In another study, the water and ethanolic extract extracted from rosemary were not effective against *Aspergillus flavus* or *A. versicolor* but exhibited good antimicrobial activity against *Penicillium* sp. (21 and 20% inhibition rate, respectively) and *P. purpurogenum* (12 and 14%, respectively) [40]. Other investigators proved that the aqueous and the ethanolic leaf extracts could suppress *Candida albicans* fungus [41]. Though their exact mechanism of action is not yet fully comprehended, it is hypothesized that their lipophilic nature may contribute to membrane disruption [42]. Many researchers contend that highly resistant bacteria and underscore the importance of the chemical composition and the balance of rosemary oil components of several terpenes in influencing their antimicrobial effectiveness [43].

Our study paved the way demonstrating rosemary extract using a methanolic solvent having effective antifungal properties against *R. solani*, *F. oxysporum*, and *B. cinerea*. However, further studies are required to investigate natural phenolic, essential oils, and flavonoid compounds' interaction along with their antimicrobial specificity effect against different fungus species in different plant species.

5 Conclusions

In conclusion, the current study utilized HPLC and GC–MS techniques to comprehensively analyze the methanolic extract of *S. rosmarinus* leaves, revealing a diverse array of polyphenolic compounds and major constituents. The antifungal activity of the extract was evaluated against three fungal isolates, *B. cinerea*, *F. oxysporum*, and *R. solani*, obtained from symptomatic strawberry plants. The results demonstrated significant growth inhibition percentages at a concentration of 300 µg/mL, indicating the potential of rosemary extract as an effective natural antifungal agent against plant pathogenic fungi. By harnessing the power of these bioactive compounds, rosemary extract presents a promising, eco-friendly alternative for managing plant diseases compared to conventional synthetic fungicides.

Future research could focus on elucidating the specific mechanisms of action and optimizing the application techniques for these natural antifungal agents in agricultural settings. Ultimately, the findings of this study contribute to the development of sustainable and environmentally responsible approaches for protecting crops and maintaining agricultural productivity.

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Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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