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Eco-friendly Approach for the Biocontrol of Two *Fusarium* spp. Using *Allium sativum* Extract

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ABSTRACT

Biological control strategies for overcoming the fungal pathogens infections are being great explored worldwide. Plant extracts are less toxic, economical, eco-friend and highly efficient as biocontrol as compared with chemical treatments. So, the present study aimed to determine the potential biocontrol activity of ethyl acetate and ethanolic *Allium sativum* (garlic) extracts against *Fusarium oxysporum* and *F. moniliforme in-vitro*. Results obtained clearly demonstrate that the ethyl acetate extract exhibit more anti-fungal activity as compared to the ethanolic extract. The diameter of inhibition zones for the ethanolic extract was 8.66 mm against *F. moniliforme* and had no effect on *F. oxysporum*. While the ethyl acetate extract were 39 mm and 30 mm on *F. oxysporum* and *F. moniliforme,* respectively. The MIC of the ethyl acetate extract was 28 mg/mL for *F. oxysporum* and 58 mg/mL for *F. moniliforme*. LC/MS analysis of garlic extract showed different peaks that correspond to important phytochemicals commonly present in garlic such as, Ally-Methyl-Sulfoxide, Pinocembrin, N-Y-Glutamyl phenylalanine, 3,4-Dihydroxy-benzoic acid, Alline, Ajoenes (Z-Ajoene, E-Ajoene), S-Ally-L-cysteine, and Allicin. The antifungal action of garlic is probably due to Allicin or Ajoene which disturb the performance of some important enzymes essential for fungal growth and propagation. It can be concluded from this study that ethyl acetate garlic extract showed potential antifungal activity against both *Fusarium* spp. suggesting its safe usage for biocontrol.

Keywords: Fusarium, garlic, antifungal, biocontrol

1. Introduction

Medicinal plants are widely used throughout the world to overcome many disorders and diseases. These plants produce numerous bioactive secondary metabolites involved in multi-biological functions, where it plays vital roles for plants to flourish in the natural environment, various protective roles related to abiotic stress which may resulted from lack of nutrient supply, water deficiency, ambient temperature, as well as insect pests. (Rajagopal *et al.,* 2022). The medicinal and antimicrobial activities of plants extract are gaining the attention of researchers worldwide. Modern medicine has its advantages and side effects, so, plant-based products are getting more popular, as they are safe to use, and comparatively easily available and cheap. Many extracts possess antifungal activity (Siripornvisal *et al.,* 2009). Its usage worldwide has a long history being an important food spice plant, it has a significant role in disease prevention and control (Kutawa *et al.,* 2018)

Garlic is one of the oldest cultivated plants used for both culinary and medicinal purposes, with history even predating the ancient Egyptian civilization (Russel and Mussa, 1977; Obagwu and Korsten, 2003). Many diseases can be cured with garlic (*Allium sativum* L.) (Yousuf *et al.,* 2010). It is cultivated worldwide intemperate regions and is used both for feed and for medical purposes. At a global level, 1.5 million hectares were occupied by this crop in 2018 (FAO-STAT, 2018). It is a biennial herb belonging to the family Alliaceae that consists of 30 genera and about 600 species. It is an important

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vegetable crop, which is grown in Egypt, Spain, Mexico, India, China, Italy, Korea, Thailand, Nigeria, Argentina, and the United States of America (Agi and Azike, 2019). *Allium sativum* is of interest for scientific research as it has anticoagulant, antihistaminic, antiparasitic, antifungal, antiprotozoal and antiviral properties (Adak and Teixeira, 2010). Also, the anticancer properties of garlic have been widely demonstrated, where increasing the dietary consumption of garlic bulbs was shown to decrease the risk of several cancer types like colon, pancreas, stomach cancer, and breast cancers (Mikaili *et al.,* 2013; Nouroz *et al.,* 2015; Isbilen and Volkan, 2020). Substantial studies have shown that garlic and its bioactive constituents exhibit antioxidant, anti-inflammatory, antibacterial, antifungal (Shang *et al.,* 2019). Some earlier works concerns the bioaction of garlic against pathogens. The garlic organic sulfur compounds inhibit the growth of many disease-causing bacteria and fungi. Researchers have found that Allicin blocks certain enzymes that infectious organisms use to damage or invade tissues (Levetin and McMahon, 2006). Garlic contains diverse bioactive compounds, such as Allicin, Alliin, diallyl sulfide, diallyl disulfide, diallyl trisulfide, ajoene, and S-allyl-cysteine. The use of garlic extract was found to be fungicidal against a broad range of soil-borne fungal organisms, but the concentration required to kill the organisms varied depending on the root substrate (Nene *et al.,* 2000; Tan *et al.,* 2017) such as *Fusarium spp.*

Fusarium genus and its species are examples of phytopathogenic and toxin-producing fungi that have been reported to be widespread throughout the world. It can cause serious health problems. *Fusarium* is a soil born and plant pathogenic fungus and is responsible for destroying crops and dramatically reducing production yields (Agrios, 2005; Matos and Ricardo, 2006). *Fusarium* species are a large genus of hyaline filamentous mold fungi, responsible for fruit and vegetable crop rot. Numerous species of Fusarium contribute to yield loss and reduced quality to varying degrees by infection with some mycotoxins. The fruit rot caused by *Fusarium* incurs enormous yield losses and is often observed in fields and markets (Baria *et al.,* 2015). Many species of the genus *Fusarium* resemble each other morphologically. In many cases morphological differentiation is difficult, and molecular tools are used. Pathogenic as well as non-pathogenic isolates have the same habitat and colonize plant root systems with equal measure (Patil and Sriram, 2020). *F. oxysporum* displays high functional and genetic diversity (Steinberg *et al.,* 2016). Evidence of its diversity lies in its impressive plant host range, which includes dicots (e.g., bean, carnation, and tomato) and monocots (e.g. banana, orchids, and palms). *F. oxysporum* strains cause wilts or root and crown rots economically important field crops (banana, cotton, soybean), many markets garden crops (melon, onion, and tomato), as well as ornamental crops (cyclamen, gerbera, and orchids), and even on weeds or parasitic plants (broomrape and witchweed) (Edel-Hermann and Lecomte, 2019). *F. moniliforme* one of the facultative fungal endophytes, it causes the fumonisins in maize (Bacon *et al.,* 2001). *Fusarium moniliforme* results in diseases in Rice (*Oryza sativa*). The disease mostly infects the plants via the roots and crowns. Clear visible symptoms with abnormal elongation is observed more than symptoms on the healthy plants in the field (Kabilan *et al.,* 2021).

On the other hand, the application of fungicides has proven to be very effective in controlling the growth and development of Fusarium. The excessive use of fungicides not only pollutes the environment, but also results in fungicide-resistant pathogens and poses a serious threat to human, animal and plant health (Baibakova *et al.,* 2019). So, alternative strategies to overcome the fungal pathogens infections are being extensively explored worldwide. Plant- natural extracts have been shown to be less toxic, economical and highly efficient for such strategies (Chen *et al.,* 2019; Anaya Esparza and Montalvo-González, 2020; and Rizwana *et al.,* 2021). Due to the aforementioned reasons, the present study aims to determine potential *in-vitro* biocontrol of ethyl acetate and ethanolic garlic extracts against *F. oxysporum* and *F. moniliforme.*

2. Material and Methods

2.1. Plant materials

Garlic cloves were obtained from the farm of Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Sadat City, Menoufia Governorate. Garlic cloves were peeled washed under running tap water and then with sterile distilled water, air-dried in a well-ventilated area in the laboratory. Plant material was grinded into small pieces using an electric blender (Moulinex, 1000 watts).

2.2. Plant crude extraction

20 g grinded cloves were separately immersed in 200 mL of 70% ethanol or 99% ethyl acetate for 24 h at room temperature. Mixtures were incubated on a rotary shaker at 190-220 rpm for 48 h at room temperature to complete the extraction. After maceration, the suspensions were filtered using three layers of muslin cloth (Tan *et al.,* 2017). Then, another 200 mL of each solvent were separately added to filtrated plant materials, gently shaken and reincubated on a rotary shaker at 190-220 rpm at room temperature for 24 h. Suspensions were re-filtrated and added to the first filtrate. The final filtrates were concentrated in a rotary evaporator to remove the solvents (Fig. 1). The final crude extracts were sterilized by being passed through a sterilized membrane filter (0.22 µL) and collected separately in dark glass bottles and stored in a refrigerator at 5°C until needed (Orisajo *et al.,* 2007; Ghazalbash and Abdollahi, 2013).

Fig. 1: Steps for preparing the garlic extract, A: grinding the garlic cloves, B: mixing the crude extract with solvent after gently shaking for 48 h, C&D: filtration, and E: solvent removal by rotary evaporator.

2.3. Cultivation of fungi

2.3.1. Potato Dextrose Agar Medium (PDA)

The fungal medium was prepared according to the manufacturer's specifications. Medium contains: 200 g/L potatoes, 20 g/L Dextrose (Glucose), and 15 g/L Agar. All components were dissolved into a conical flask completed to one liter of sterile distilled water. It was then shaken to mix up, the final pH (at 25 °C) was 5.6±0.2. It was further sterilized by autoclaving at 121 °C for 20 min and allowed to cool down. The medium was dispensed into sterile Petri-dishes and allowed to solidify (Neela *et al.,* 2014).

Fusarium oxysporum and *F. moniliforme* were obtained from Industrial Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Sadat City. Spores of mycelium were swabbed on the PDA medium using a sterilized inoculating loop. The plates were incubated at a temperature of 28°C until fungal growth was visible. Developed fungi were stored in a refrigerator at 4°C and were maintained by periodic sub-culturing on PDA slants every 15 days (Srivastava *et al.,* 2015).

2.3.2. Antifungal activity using disc diffusion assay

The tested fungi *(F. oxysporum* and *F. moniliforme)* were cultivated on PDA medium for 5-7 days at 28 °C. The spore suspension was prepared by scraping the pathogen with sterile distilled water

to give a final spore concentration (1×10^6) and was used on the same day. Spore suspension (150 µL) was added to 20 mL cooled PDA medium, then distributed into a sterilized petri dish. At the same time, crude extracts were dissolved in a solvent to a final concentration of 500 mg/mL. Then, the sterilized discs (6 mm in diameter) were loaded with 40 µl of prepared extracts solution and placed on the inoculated PDA media. The inoculated plates were incubated at 28 °C for 48 h. The diameter of the clear zones of inhibition (mm) around each disc were taken as measure of the antifungal activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zones was calculated and expressed as its antifungal activity (Korukluoglu *et al.,* 2008).

2.4. Determination of antifungal inhibition percentage

Plant extracts were tested for their efficiency against the pathogen by using an agar dilution technique. 20 mL of PDA media was supplemented with 150 µL of plant extract (500 mg/mL). Then, the mixture of medium and extract were poured into the Petri-dishes. After medium solidification, mycelia stock (50 µL) from a 5-7-day-old *F. oxysporum* or *F. moniliforme* was separately added to the wells of each plate and incubated at 28 ˚C. The colony diameter of *F. oxysporum* and *F. moniliforme* were measured after 2 days of incubation. The media without plant extracts amended with mycelia block was considered as a positive control. The efficacy of plant extracts was observed and the percentage of radial mycelial growth over the control was calculated by using the following formula (Neela *et al.,* 2014).

$$\text{Inhibition \%} = [(C-T)/C\times100$$

where, C and T represent the diameter of control and treated colony, respectively.

2.5. Determination of minimum inhibitory concentration (MIC)

The diluted broth method was used to determine the MIC of the plant extract against the tested pathogens as previously described (Bahrulolum *et al.,* 2021). Serial concentrations of plant extracts (500–30 mg/mL), were prepared by dissolving crude extracts into the solvents. Sterile filter paper discs (Hi media) were saturated with 40 µL of each concentration, then inoculated on 20 mL PDA medium containing 150 µL of pathogens and incubated at 28°C for 48h. Clear area around the disc was measured. After determining the range of concentrations that affect pathogens, serial dilutions of crude extract including 25–30 mg/mL for *F. oxysporum* and 55-60 mg/mL for *F. moniliforme*, were mixed with Potato Dextrose Broth medium (PDB) and inoculated with 150 µL of each fungus separately. The media were incubated at 28°C for 48 h. The turbidity of each concentration was determined (Tayel *et al.,* 2016).

2.6. Determination of the minimum fungicidal concentration (MFC)

One mL of each crud concentrations was spread onto fresh PDA (20mL) plates and incubated at 28 °C until observation the fungi growth on positive control medium. The appearance of any fungi growth on PDA medium plates compared with positive control refer to the minimum fungicide concentration (MFC) of the crude extract (Rahmouni *et al.,* 2019).

2.7. LC/MS analysis of garlic extract

LC/MS was performed according to Zhu *et al.* (2016) method using Agilent 1260 Infinity LC system (Faculty of pharmacy, Ain Shams University, Egypt), coupled to an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, USA). The separation was conducted on a SUPELCO Discovery HS F5 column (3 × 150 mm*3 µm) using a binary mobile phase composed of A (water+0.1% formic acid) and B (acetonitrile). The mobile phase was programmed as follows: 0–15 min, B 0–100 %; 15–25 min, B 100 %. 5 µL sample was injected and the mobile flow rate was 0.4 mL/min, column oven was maintained at 30 C. The mass spectrometer was operated using an Agilent Jet Stream electrospray ionization (ESI) source in positive and negative ion modes. The optimal MS parameters were as follows: gas pressure, 50 psi; capillary voltage, 3.5 kV; dry gas temperature, 280 °C; sheath gas temperature, 350 °C; dry gas flow rate, 10 L/min; and sheath gas flow rate, 12 L/min. Quantitative analysis was performed in SRM mode.

2.8. Statistical analysis

The data shown in results are means of triplicate values and expressed as ± SD (standard deviation) for antifungal activity assay, percentage of inhibition of mycelial growth.

3. Results and Discussion

Results obtained (Table 1) indicated that ethanolic and ethyl acetate extracts of garlic gloves inhibited the tested fungi. The ethanolic extracts (at concentration 500 mg/mL) showed inhibition zone of 8.6 mm against *F. moniliforme*, while the ethanolic extracts did not inhibit *F. oxysporum*. On the contrary, garlic ethyl acetate extract showed the highest effect on both tested pathogens, where the inhibition zones were 39 ± 1.15 and 30±0.00 mm for *F. oxysporum* and *F. moniliforme,* respectively. Similarly, Kutawa *et al.,* (2018) reported that 20 mg/mL of the ethanolic garlic extract was very effective on *Fusarium sp.* and *Rhizopus sp*. with inhibition zone diameter of 14.3 mm and 12.2 mm, respectively. In a very recent study, Cinar *et al.,* (2022) reported that 50 mg/mL of black garlic extract has antimicrobial activity against Gram-positive bacteria such as *Bacillus cereus*, Gram-negative bacteria *such as Listeria monocytogenes*, and yeasts.

In our study, the percentage of both tested fungi inhibition growth was affected by the two garlic extract solvents (Fig. 2).

Table 1: Garlic extracts against two fungi using agar disc diffusion and percentage of inhibition

Tested organisms	***F. oxysporum***		***F. moniliforme***	
	Inhibition			
Solvent extract	**Zone (mm)**	**Percentage (%)**	**Zone (mm)**	**Percentage (%)**
Ethanol extract	0 ± 0	7.69 ± 1.92	8.66 ±.75	4 ± 0
Ethyl acetate extract	39 ± 1.15	100 ± 0	30 ± 0	100 ± 0

Data are averages of 3 replicates ±STD.

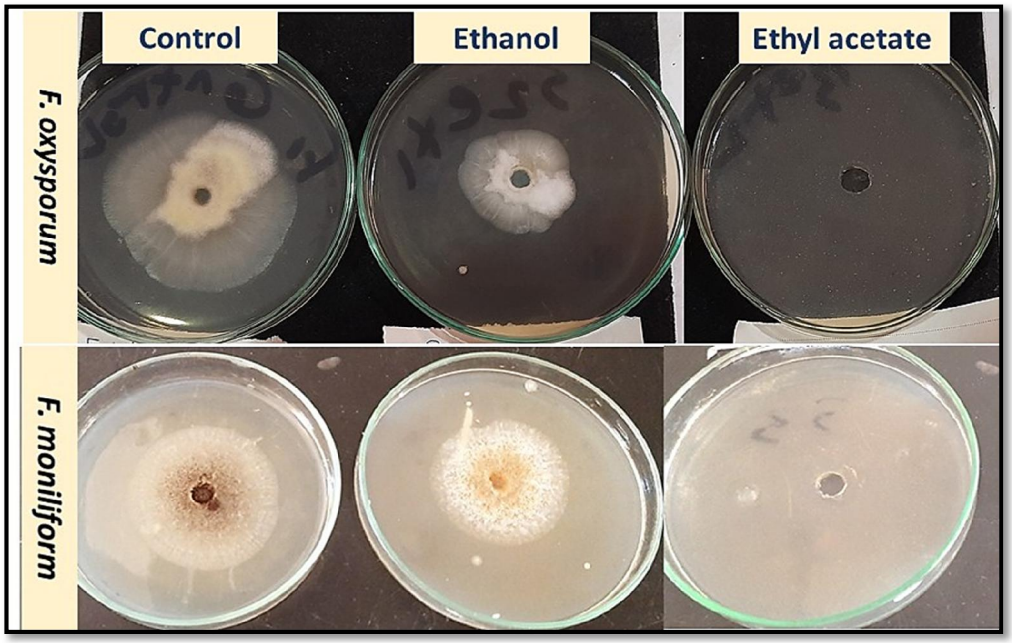

Fig. 2: The effect of ethanolic and ethyl acetate extracts of garlic cloves on the growth of *F. oxysporum* (A). and *F. moniliforme* (B).

However, ethyl acetate extract of garlic possessed the highest percentage of fungi inhibition (100%). The ethanolic extract of garlic gloves affected the inhibition growth percentage of the *F. moniliforme*, but it did not show any effect on the percentage of *F. oxysporum* inhibition. It seems that inhibition zones and percentage may be affected by pathogen and solvent types used for the extraction. Similarly, Neela *et al.,* (2014) reported 100% inhibition against *F. oxysporum* using the ethanolic and acetone

leaves extract of *Adhatoda vasica*. In addition, Shrestha and Tiwari (2009) used garlic extract against *Fusarium solani* which showed 47.7-100% inhibition. Thus, it can be concluded that microorganisms differ in their inhibition response to different plant extracts, depending on the extract and the solvent used.

3.1. Determination of MIC

Results obtained (Table 2) indicate that the ethanolic and ethyl acetate garlic extract inhibit the growth of *F. oxysporum* and *F. moniliforme*, but the ethyl acetate extract possessed the highest fungicidal effect on both fungi. Different concentrations of ethyl acetate garlic extract (25-500 mg/mL) were loaded into disc and inoculated on PDA medium and incubated at 28 °C for 48 h to examine its fungicide effect against *F. oxysporum* and *F. moniliforme* (Table 2). *F. oxysporum* was more sensitive to ethyl acetate garlic extract than *F. moniliforme*, where the *F. oxysporum* could not grow on concentrations from 60 to 500 mg/mL of garlic extract and the clear zone around the disc was 35 mm using concentration 30 mg/mL of ethyl acetate garlic extract. While *F. moniliforme* showed clear zone around the disc at the concentrations from 60 to 500 mg/mL with clear zone diameter that ranged from 6 to 30 mm. So, the MIC can be determined to be between 25-30 mg/mL for *F. oxysporum* and between 55-60 mg/mL for *F. moniliforme*. Results (Table 3) showed MIC of ethyl acetate garlic extract was found to be 28 mg/mL for *F. oxysporum* and 58 mg/mL for *F. moniliforme*, suggesting that the MIC was probably affected by the concentration of the extract and the fungi spp. Results are supported by the findings of Kutawa *et al.,* (2018) who reported that garlic is a spice with global recognition as inhibition agent against fungal growth. By varying the concentrations used, the MIC of ethanolic garlic extract is 2.5 mg/mL against *Fusarium spp.* (Kutawa *et al.,* 2018). In another study, Neela *et al.,* (2014) reported that the extracts of *Datura innoxia, Spilanthe acmella, Wedelia chinensis* and *Tagetes patula* have inhibitory effect on *F. moniliform* with 63.97%, 55.48%, 50.36%, 40.00% inhibition, respectively.

Table 2: Fungicidal effects of ethyl acetate garlic extract concentrations using disc diffusion method

Ethyl acetate extract Conc. (mg/mL)	**Inhibition zone (mm)**	
	F. oxysporum	***F. moniliforme***
500	-	30
400	-	21
300	-	20
200	-	18
150	-	18
100	-	12.5
80	-	13.5
70	-	11.5
60	-	6
50	40	+
40	37.5	+
30	35	+
25	+	+

Key: (+) means growth of fungi. (-) means no growth of fungi.

Table 3: Minimum inhibitory concentration (MIC) of ethyl acetate garlic extract against *F. oxysporum* and *F. moniliforme*

Extract Conc. (mg/mL)	**25**	**26**	**27**	**28**	**29**	**30**	**MIC**
F. oxysporum	+	+	+	-	-	-	28
Extract *Conc. (mg/mL)*	**55**	**56**	**57**	**58**	**59**	**60**	**MIC**
F. moniliforme	+	+	+	-	-	-	58

Key: (+) means growth of fungi. (-) means no growth of fungi.

3.2. Determination of the minimum fungicide concentration (MFC)

The garlic ethyl acetate extract was able to inhibit the growth of both *F. oxysporum* and *F. moniliforme* as compared with ethanolic extract of garlic cloves. All used concentrations of garlic ethyl acetate extract showed no visible fungal growth after 3 days of incubation at 28 °C. However, after 7 days of incubation the concentration of 25-26 mg/mL showed visible growth of *F. oxysporum*, and 55-56 mg/mL showed visible growth of *F. moniliforme* as compared with the positive control.

3.3. LC/MS analysis

LC/MS analysis (Table 4) of *A. sativum* extract showed different peaks that correspond to important phytochemicals commonly present in garlic such as, Ally-Methyl-Sulfoxide, Pinocembrin, N-Y-Glutamyl phenylalanine, 3,4-Dihydroxy-benzoic acid, 4-Hydroxy-benzoic acid, Alline, Ajoenes (Z-Ajoene, E-Ajoene), S-Ally-L-cysteine, Y-Glutamyl-S-Ally-Cysteine, C_6 H_6O phenol 2-elhyltiacy, $C_7H_{14}S$ Clohexane, $C_{10}H_{18}O_{22}$-3 Pentyl-2,4 Pentadien-1-ol, and Allicin (Table 4.). Similarly, Zhu *et al.,* (2016) and El-Saber Batiha *et al.,* (2020) reported the same phytochemicals.

A. sativum is reported to contain hundreds of phytochemicals, including sulfur-containing compounds (Wang *et al.,* 2016). Remarkably, Alliin, the main cysteine sulfoxide is transformed into allicin by the alliinase enzyme after cutting off the garlic. Several organosulfur compounds, such as S-allyl-cysteine is derived from Alliin (El-Saber Batiha *et al.,* 2020). Allicin is extremely unstable due to the presence of a thiol group which is rapidly metabolized into diallyl sulfide, diallyl disulfide, ajoene, etc. (Zhu *et al.,* 2016). The antifungal action of garlic is probably due to Allicin. It has strong antimicrobial and antifungal activities. Thus, the inhibition of fungi may be related to Allicin or Ajoene which curbs the performance of some important enzymes essential for the fungal growth and propagation.

Table 4: LC/MS analysis of garlic extract showing different bioactive compounds

Base peak (m/z)	Ret.Time (Min)	A/H	Area %	Compound
163.1	7.23	26.052	4.983	S-Ally-L-cysteine
293.2	12.14	24.13	1.85	Y-Glutamyl-S-Ally-Cysteine
163.1	7.23	26.052	4.983	Allicin
104	0.73	15.18	0.844	Ally-Methyl-Sulfoxide
100	7.61	7.752	0.166	E-Ajoene
541.1	1.22	7.836	1.752	C_6 H_6O phenol 2-elhyltiacy
145.1	12.97	17.262	1.682	C_7H_{14}S2-ethylthiacyclohexane
145.1	10.12	21.373	16.43	Z-Ajoene
339.3	20.9	30.578	2.368	$C_{10}H_{18}O_2$Z-3 Pentyl-2,4 Pentadien-1-ol
225.1	11.86	12.73	2.682	Pinocembrin
293.2	12.14	24.13	1.85	N-Y-Glutamyl phenylamine
152.1	1.24	7.384	2.172	3,4-Dihydroxy-benzoic acid
137.05	1.51	5.278	1.447	4-Hydroxy-benzoic acid
177.20	1.62	16.174	4.856	Alline

Conclusion

Alternative strategies to overcome fungal pathogens infections are being extensively explored worldwide. In this respect, medicinal plant extracts have been shown to be non-toxic, economical and highly efficient for biocontrol. The garlic extracts (ethanolic and ethyl acetate) have antifungal activity against two tested organisms (*F. oxysporum* and *F. moniliforme*). Ethyl acetate garlic extract had the highest inhibitory effect against both fungi. However, *F. oxysporum* was more sensitive to acetyl acetate garlic extract than *F. moniliforme.* The antifungal action of garlic is probably due to the compound Allicin and Ajoene which have been confirmed by LC/MS analysis, and affects the performance of some important enzymes essential for the fungal growth. Overall, biocontrol could be affected by plant materials, solvent type and different microorganisms.

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