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Antifungal Activity of Clove against Phytopathogenic Fungi and HPLC analysis of Extract

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Abstract: ***The objectives of this work were to evaluate the antifungal activity of clove flower buds extracts in management of three pathogenic fungus were isolated from strawberry, which molecularly identified through Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing, Alternaria alternata (accession no. PP737870), Botrytis cinerea (accession no. PP758474) and Fusarium oxysporum (accession no. PP737874). The results of study revealed weak to high level antifungal activities against tested phytopathogenic fungi at concentrations of 2000, 1000, 500 and 250 µl ml^{-1}. The highest antifungal activity was recorded at 2000 µLml^{-1}; on this concentration clove flower buds extract caused growth inhibition of A. alternata, B. cinerea, F. oxysporum were observed 50.00%, 60.37%, 76.30% respectively, while the weak antifungal activity against A. alternata, B. cinerea, F. oxysporum at 500 µLml^{-1} were observed 8.15%, 5.93%, 28.89% respectively. The inhibition rate of the growth of the three fungi with the pesticide (Topsin - M 70 WP) was 75.56 to 100% at 2 µl/ml concentration, compare to the inhibition rate with the control was 0.00%. Clove flower buds extract was analyzed using High Performance Liquid Chromatography (HPLC) for phenolic and flavonoid compounds, the highest amounts of polyphenolic compounds viz., Ferulic acid, P - Coumaric, Caffeic acid, Catechol, Syringenic acid, Gallic acid and Cinnamic with 13.95, 13.68, 13.52, 7.31, 6.82, 6.65and 2.20 µg/gm, respectively. Application of readily available aqueous plant extracts to controlling plant disease pathogens is an eco - friendly.***

Keywords: *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, HPLC, ITS, clove, Phytopathogenic fungi.

1. Introduction

Strawberries (*Fragaria* × *ananassa*) is affected by several pathogens including bacteria, nematodes, fungi and viruses. The most economically impactful pathogens of strawberry are fungi, which can cause severe damage or death by infecting all parts of the plant, viz., *Alternaria alternata* has been identified as the pathogen causing black spot diseases, when brown circular lesions and sometimes with a yellowish halo on the leaves, and causes significant economic losses to strawberry growers in both the growing and harvest seasons (Dingley 1970, Sun et al., 2023). Also, *Botrytis cinerea* is the causal agent of strawberry grey mould, this pathogen affects fruit in the field, transport, storage and market. The most frequent cause of fruit rejection by producers, shippers, and consumers is the presence of grey mould, which results in large financial losses (Gordon et al., 2016, Petrasch et al., 2019). In addition to, the fusarium wilt, a lethal disease caused by *Fusarium oxysporum*, which were the predominant pathogens responsible for the main economic losses of strawberry in different regions of the world (Henry et al., 2020). It colonizes the strawberry plant's xylem, causing obstruction and xylem breakdown, that results include wilting, yellowing, stunting, and eventual death of the plant (Castellanos et al., 2020).

Because phytopathogenic fungi are impact a wide range of economically valuable hosts, management of these diseases are necessary. Usually, crop diseases are effectively and efficiently managed with synthetic pesticides, the use of these harmful chemicals leads to environmental pollution. Methods must be sought alternative approaches to disease management are receiving a lot of attention. A range of methods, including biological and cultural ones, can be used to control plant diseases (Fu et al., 2020). Over the past three decades, the use of fresh plant extracts that include antifungal chemicals has become more significant for the treatment of plant diseases. The use of modern agrochemical research is influenced by the application of plant derived fungicides, which have a large potential to prevent microbial pathogen attacks because they contain secondary metabolites from plants (Mostafa et al., 2023).

Cloves (*Syzygium aromaticum* L.) are the aromatic dried flower buds, it is a source of natural ingredients which are reported to have various biological activities that have beneficial effect to human health. Clove is widely used by traditionally as a wound medicine, massage oil, body warmer, and spices for cooking (Cortés - Rojas et al., 2014), clove extract also used as antibacterial and antifungal (Aulifa. Et al., 2015, Hu et al., 2018). Clove extract has a bioactive compounds such as flavonoids, saponins, phenolics, tannins, steroids, terpenoids, and alkaloids (El Ghallab et al., 2020, Razafimamonjison et al., 2023). Because phytopathogenic fungi are impact a wide range of economically valuable hosts, management of these diseases are necessary. Usually, crop diseases are effectively and efficiently managed with synthetic pesticides, the use of these harmful chemicals leads to environmental pollution. Methods must be sought alternative approaches to disease management are receiving a lot of attention. A range of methods, including biological and cultural ones, can be used to control plant diseases (Mohamed et al., 2019 and 2020a).

High Performance Liquid Chromatography HPLC analysis indicated that polyphenolic compounds, such as flavonoids and phenolic acids are important constituents in alot of plants,

their quantification and identification can give vital information about the antifungal. Phenolic acids are hydroxylated derivatives of cinnamic and benzoic acid, the most common hydroxybenzoic acid are vanillic, benzoic, protocatechuic and gallic acids which are mainly present in the form of glucosides. The most common forms of hydroxycinnamic acid are caffeic, ferulic, and coumaric acids. Chlorogenic acid is the most familiar one (Haouala et al., 2008, Salih and Al Dabagh. 2021, Vesna et al., 2004).

The main objectives of the present work were evaluate the antifungal activity of clove flower buds extract against several pathogens such as, *A. alternata*, *B. cinerea*, *F. oxysporum*, which as molecularly identified through Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing and to screen phytochemical analysis of phenolic acids and flavonoids using HPLC.

2. Materials and Methods

Extraction of clove flower buds

Clove flower buds powder (100 g) were extracted by soaking it in 1000 ml of distilled water in a glass bottle at room temperature for 24 h. Then it was filtered through a muslin textile and dried in an oven at 50 °C for 72 h (Babar et al., 2019) . The final dried extract was stored at 4 °C in a refrigerator until used and analyzed by HPLC (Shadid et al., 2021) .

HPLC analysis of phenolic and flavonoid compounds

The phytochemicals identified from clove flower buds extract was analyzed using An Agilent 1260 Infinity HPLC Series (Agilent, Santa Clara, CA, USA), equipped with a Zorbax Eclipse and a Quaternary pump plus C18 column (100 mm × 4.6 mm i. d.) (Agilent Technologies, Santa Clara, CA, USA (Elbanoby et al., 2024). The injection volume was 20 µL, and the device was run at 30 °C. with the following ternary linear elution gradient; (1) HPLC grade water 0.2% H_3PO_4 (v/v), (2) methanol, and (3) acetonitrile. The detection was set at 284 nm to identify the existed compounds. Standard HPLC analysis of phenolic and flavonoid compounds including: ferulic, pyrogallol, quinol, syringenic, hydroxybenzoic, gallic acid, *P* - coumaric acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, benzoic acid, rutin, ellagic acid, catechol, cinnamic, o - coumaric acid, salicylic acid, resveratrol, myricetin, quercetin, rosmarinic acid, naringenin, and caffeine as well as kaempferol, were used for the HPLC analysis (Shadid *et al*., 2021, Elbanoby *et al*., 2024).

Isolation of the Pathogen

This research was conducted in seed pathology department, plant pathology research institute, agricultural research center (ARC), Alexandria, Egypt, under the supervision of the assistant professor Abeer Abozeed Mohamed. The leaves, Roots and fruits were collected from infected Strawberries plants showing the characteristic symptoms of black spot caused by *Alternaria* sp., grey mould caused by *Botrytis* sp. and wilt caused by *Fusarium* sp. . The infected part of plants interface were cut into small pieces, then surface sterilized with 0.1% Sodium hypochlorite (NaClO) for 30 seconds. Following three rounds of washing in sterile distilled water, the pieces were aseptically placed onto Potato Dextrose Agar (PDA) medium. The inoculated plates were incubated at 27 ± 2ºC (Hassan et al., 2021).

Molecular identification of fungal isolates

Alternaria sp., *Botrytis* sp. and *Fusarium* sp. isolates were grown on potato dextrose agar (PDA) medium to identify according to the molecular identification through Internal Transcribed Spacer (ITS) ribosomal RNA (rRNA) sequencing, a quick micro preparation process was used (Shakam et al., 2022, Khatab and Mohamed 2024). The ITS DNA region of this isolates were amplified via PCR using universal primers, the PCR products were sent for sequencing by Macrogen, Scientific Services Company, Korea (Kumar et al., 2016). The sequences were contrasted with GenBank sequences (http: //www.ncbi. nlm. nih. gov) using a BLAST search on the National Center for Biotechnology Information (NCBI). The sequences of the three isolates used in the present study were submitted to GenBank.

Antifungal activity of clove flower buds extract

Clove flower buds extract was tested for their anti - fungal activity against *Alternaria* sp., *Botrytis* sp. and *Fusarium* sp. isolates. The extract was prepared a series of two fold dilutions ranging from 250 to 2000 µl ml - 1 (Mohamed et al.2020a, 2020b, Shakam et al., 2022, Khatab and Mohamed 2024) were compared to reference fungicide (Thiophanate methyl) was also tested at the recommended dosage (2 µl/ml) for antifungal activity using the poisoned food technique (Gupta et al., 2015). The anti - fungal activity of the Clove flower buds extract was assessed against *Alternaria* sp., *Botrytis* sp. and *Fusarium* sp., isolates by linear growth. After the isolates were grown on PDA medium, a 6 - mm culture disc was taken out of the actively growing cultures using a corkborer and put in the middle of Petri dishes with varying concentrations. The Petri dishes were left in a 28°C incubator for one week (El - Hefny et al.2023, Khatab and Mohamed 2024). The plates without Clove flower buds extract served as control. The radial mycelial growth (mm) was measured when the pathogen in the control plates completely covered the surface. All experiments were carried out in triplicates. Fungal growth inhibition (FGI %) of the tested fungal isolates were calculated with the following formula according to Mohamed et al., 2020a and Hassan et al., 2021: Fungal growth inhibition % (FGI %) = [(Average increase in mycelial growth in control plate - Average increase in mycelial growth in treatment plate) / Average increase in mycelial growth in control plate] × 100.

Statistical analysis

According to Snedecor and Cochran (1989), mean comparisons were performed using the least significance difference (LSD) at the 5% level of significance after the collected data had been statistically examined using a statistical program. Using Excel version 2016, Pearson's correlation coefficient was calculated.

3. Results

Molecular identification of fungal isolates

Alternaria sp., *Botrytis* sp. and *Fusarium* sp. isolates were also identified as *Alternaria alternata*, *Botrytis cinerea* and *Fusarium oxysporum* by ITS sequence analysis, and the nucleotide sequence was submitted to GenBank under the

accession number PP758474, PP737874 and PP737870. The sequence analysis revealed a 97 to 100 % identity with *A. alternata*, *B. cinerea*, and *F. oxysporum* isolates, confirming the fungal pathogen.

Phytochemical compounds in clove flower buds extract by HPLC

The phytochemical compounds identified in clove flower buds extract is shown in Table 1 with the HPLC chromatographic charts Fig.1. The results showed that the most abundant phenolic compounds were catechol (7.31 µg/gm), syringic acid (6.82 µg/gm), *P* - coumaric (13.68 µg/gm), cinnamic acid (2.20 µg/gm), caffeic acid (13.52 µg/gm), gallic acid (6.65 µg/gm) and ferulic acid (13.95 µg/gm).

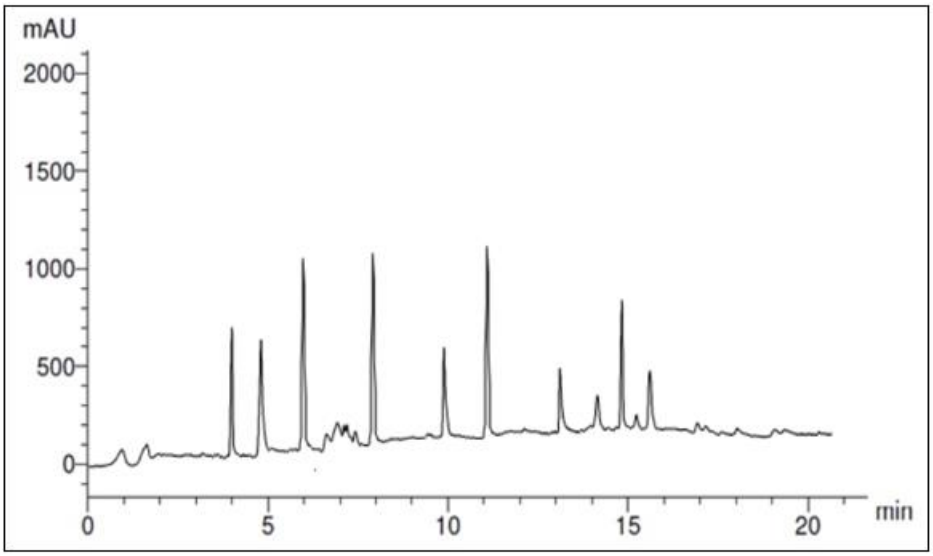

Figure 1: The HPLC analysis of the phenolic compounds in clove flower buds extract

Table 1: The phenolic compounds in clove flower buds extract.

RT *	Phenolic Compound	Concentration µg/gm
4.0	Catechol	7.31
5.0	Syringenic	6.82
6.0	*P* - Coumaric	13.68
7.0	Cinnamic	2.20
8.0	Caffeic	13.52
10.0	Gallic	6.65
11.0	Ferulic	13.95

*RT: Retention time (min)

Antifungal activities

The results of study revealed weak to high level antifungal activities against tested phytopathogenic fungi at concentrations of 2000, 1000, 500 and 250 µl ml $^{-1}$. The highest antifungal activity was recorded at 2000 µLml $^{-1}$; on this concentration clove flower buds extract caused growth inhibition of *A. alternata*, *B. cinerea* and *F. oxysporum* were observed 50.00%, 60.37%, 76.30% respectively, while the weak antifungal activity against *B. cinerea*, *F. oxysporum*, *A. alternata* at 500 µLml - 1 were observed 8.15%, 5.93%, 28.89% respectively. The inhibition rate of the growth of the three fungi with the pesticide (Topsin - M 70 WP) was 75.56 to 100% at 2 µl/ml concentration, Compare to the inhibition rate with the control was 0.00%. (Table and Fig.2).

Table 2: Antifungal activity of clove flower buds extract against *A. alternata*, *B. cinerea* and *F. oxysporum*

Concentration (µl ml $^{-1}$) Positive control Negative control	Fungal growth inhibition %			
	A. alternata	*B. cinerea*	*F. oxysporum*	Overall mean
2000	76.30 B	50.00 B	60.37 B	62.22
1000	32.96 C	32.96 C	31.85 C	32.59
500	28.89 D	08.15 D	05.93 D	14.32
250	00.00 E	00.00 E	00.00 E	0.00
Negative control	00.00 E	00.00 E	00.00 E	0.00
Positive control	100.0 A	100.0 A	100.0 A	100
Overall mean	39.69	31.85	33.02	
LSD. at 5%	1.1474	1.1671	1.1474	

Values are average of 9 amended PDA plates /treatment, values in every single column are significantly different with 0.05 probability if it is followed by a different letter or letters

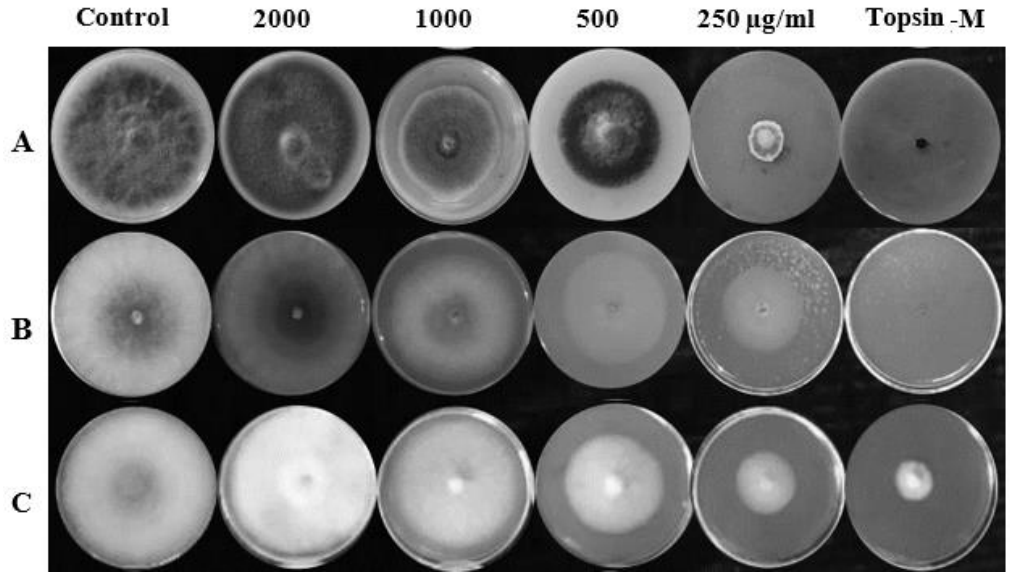

Figure 2: Inhibition effect of of clove flower buds extract on mycelium growth of *A. alternata (*A), *B. cinerea F. oxysporum* (B), (C).

4. Discussion

In this study, *Alternaria* sp., *Fusarium* sp., and *Botrytis* sp. were identified as causal agents of strawberry black spot, fusarium wilt, and grey mould diseases, respectively, in the main strawberry - producing regions. These results were consistent with a report by Petrasch et al. (2019), Henry et al. (2020), Fu et al. (2020), Mostafa et al. (2023), who confirmed that *A. alternata*, *B. cinerea* and *F. oxysporum* were the pathogens causing strawberry black spot, fusarium wilt, and grey mould respectively, diseases.

The species identified from *Alternaria* sp., *Fusarium* sp. and *Botrytis* sp. were based on ITS rDNA sequence analysis و were identified as, *B. cinerea*, *F. oxysporum*, *A. alternata*, which found to have similar loci as were found in previous observations (Bessadat et al., 2021, Shennan et al., 2018, Mohammed 2023).

The effect of clove buds extract is significant against the fungal isolates of *A. alternata, B. cinerea* and *F. oxysporum* according to El - Samawaty et al. (2013). These strains are very sensitive to essential oils of clove according to the interpretation of Belaich et al, (1979). The clove extracts confirmed the documented antifungal activity against *Fusarium* sp. (Boulenouar et al., 2012). Furthermore, results of the study are in agreement with the study conducted by Rena et al. (2011), who evaluated the antifungal activity of clove against *F. moniliforme*, *F. oxysporum*, *Aspergillus* sp. and *Mucor* sp., they reported that all fungal species were inhibited by the oil when tested and highest sensitivity was reported against *F. oxysporum*.

HPLC analysis was employed to define qualitative and quantitative content of flavonoids and phenols in investigated clove flower buds extract. The sample compounds were identified by comparing their online ultraviolet (UV) spectra and relative retention times (Rt) with those of the chromatogram of the standard. According to Arif et al. (2009) phenolic compounds are one of the main groups of secondary metabolites found in plants. There are roughly 10, 000 distinct polyphenols, and each one is crucial for plants' ability to resistance to pathogenic microorganisms. Ferulic acid was the predominantly identified phenol in clove with 13.95 µg/gm, *P* - Coumaric 13.68 µg/gm and Caffeic 13.52 µg/gm followed by Catechol 7.31 µg/gm, Syringenic 6.82 µg/gm, Gallic 6.65 µg/gm and Cinnamic 2.20 µg/gm, these results were in agreement with Koksal et al. (2016).

5. Conclusion

Plant extracts have been used as natural microbial agents for a long time because these extracts effectively inhibit the growth of a wide range of microorganisms. Clove extract could be promising as a source of natural eco - friendly phytofungicidal compounds for *in vivo* applications. These results demonstrate that HPLC analysis of clove buds extract (*Syzygium aromaticum*) identified several phenolic acids and flavonoids particularly of catechol, syringic acid, *P* - Coumaric, cinnamic acid, caffeic acid, ferulic acid and gallic acids in clove. Functional extracts could provide an alternative method of using hazardous chemical fungicides for treatment of strawberry fruits to reduce infections caused *B. cinerea*, *F. oxysporum* and *A. alternata*.

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