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Biochemical Activity of Propolis Alcoholic Extracts against *Fusarium oxysporum* hm89

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PROPOLIS was responsible for *in vitro* growth suppression of some tested phytopathogenic fungi. Four tested species were partially inhibited by using propanolic or ethanolic extract associated with promising growth reduction of *Fusarium oxysporum* in a percent of growth recorded 38.2% or 58.9% during propanolic or ethanolic application, respectively, while *Helminthosporium* sp. and *Cladosporium* sp. showed unexpected activation of growth during propanolic or ethanolic extract applications. The antimicrobial products identified during GC-MS analysis of the propolis propanolic extract were Pyrazole, Quinic acid, D-lactic acid, Pentanoic acid, Erythritol and sulfonamide derivatives. The SDS gel electrophoresis of soluble proteins of *Fusarium oxysporum* treated with propanolic or ethanolic propolis extracts showed a specific protein band at 26.002kDa with the untreated pathogen, and several characterized bands at 21.160, 26.012, 28.666, 38.44, 102kDa related to propolis propanolic extract (2) and finally a two markedly visible bands at 18.871, 33.083kDa with propolis ethanolic extract (3). The decrease in enzyme activity of cellulase and pectinase of *Fusarium oxysporum* was recorded under treatment with either propanolic or ethanolic extracts. There was suppression in the degree of infectivity such as the number of rotted seeds, wilting, brown discoloration of *Phaseolus* seedlings presoaked in either of the two propolis extracts compared to infected plants with more reduction individually in case of propanolic extract over that of ethanolic one.

Keywords: Fungal hydrolytic enzymes, *Fusarium oxysporum,* GC-MS analysis, Kidney bean (*Phaseolus vulgaris*), Propolis extracts, SDS gel electrophoresis.

Introduction

Propolis is a hive product collected by bees from plant buds. It's characterized by its resinous nature and its color varies from green to dark brown depending on the plant source. It contains flavonoids, essential oils, wax, pollens, minerals, amino acids, and organic matter (Walker & Crane, 1987; Greenaway et al., 1990). It is a natural antimicrobial agent which is generally recognized as safe substances (GRAS) (Alencar et al., 2007). Studies showed that propolis had antifungal, antiviral, antibacterial, and antioxidant activities (Ghisalberti, 1979; La Torre et al., 1990; Yamauchi et al., 1992; Burdock, 1998) which is attributed to phenolic compounds particularly flavonoids that were distributed through the plant seeds, fruits, leaves, and other plant parts (Amoros et al., 1994; Packer & Luz, 2007; Simões et al., 2008).

Propolis has effective antimicrobial characteristics against *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus* sp., and *Candida* spp. (Sforcin, 2016; Zabaiou et al., 2017). Ethanolic extract of propolis had shown antiseptic, anesthetic, and antioxidant effects. The extract also inhibited vegetable germination as garlic and potato (Yamauchi et al.,1992; Hemeida & Abd Alfattah, 1993).

Hashem et al. (2012) studied the mechanism of the antifungal potential of propolis and its effect on the growth, aflatoxins production, and lipids metabolism of *Aspergillus parasiticus*. They reported a decrease in conidial behavior including

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production and germination as well as mycelial growth of *A. parasiticus*. It was also detected that aflatoxins production by *A. parasiticus* was largely suppressed at concentrations 0.2 and 0.4g/100mL of propolis, while the concentration 0.6g/100mL induced complete inhibition of all aflatoxins production. The biochemical assay of lipid cytotoxicity of *A. parasiticus* suggested clear cystic suppression of lipids metabolism by propolis. Gas chromatographic analysis of cellular fatty acids indicated that propolis enhanced the accumulation of saturated fatty acids suggesting a resistance mechanism of a fungal membrane via decreasing its fluidity and elasticity.

Ôzcan (1999) reported that water aqueous extracts at the concentrations of 0.5, 1, 2, 3 and 4%, of propolis, were assayed for inhibition of *Aspergillus parasiticus*, *Aspergillus niger*, *Botrytis cinérea*, *Fusarium oxysporum*, *Alternaría alternata* and *Penicillium digitatum* in culture media. The most potent concentration of propolis was 4%. This concentration showed more than 50% inhibition against all tested microorganisms. The most susceptible microorganisms among the tested fungi were *Alternaría alternata* and *Penicillium digitatum*.

The objective of the current work was to identify the antimicrobial potential of either the propanolic or the ethanolic extract of propolis toward some tested phytopathogenic fungi. The research also clarified the effect of these extracts on some physiological activities of *Fusarium oxysporum,* including fungal pathogenicity, the production level of hydrolytic enzymes, in addition to protein production, therefore providing a better understanding of the responses of the tested pathogen to the antimicrobial action of propolis as an alternative way to chemical control.

The current research also focused on the antimicrobial compounds associated with GC- MS analysis of propolis propanolic extract.

Materials and Methods

Propolis origin

We purchased propolis samples from an apiary beside tomato fields in El Badrashine, Giza, Egypt, during the winter season. Hand-collected propolis specimens were kept desiccated in a dark container till processing.

Extraction and sample preparation

One hundred grams of collected propolis samples were mixed and divided into two 50-gram portions. We extracted the active ingredients using 120mL of two extraction solvents separately; ethyl alcohol 95% and absolute propanol (Vechet, 1978). The two propolis extracts were filtered, and alcohol was evaporated under vacuum at 30°C using a rotary evaporator (Buchi model 011) until the complete removal of the solvents. Then the two different extracts underwent filtration using cellulose acetate (CA) membrane filters. The extracts were kept cool in the refrigerator (4°C) until use.

Isolation of tested microorganisms from Egyptian clay soil

We collected the soil samples from a garden inside Cairo University (Giza, Egypt). We isolated soil fungi using the serial dilution method (Waksman, 1994) on Czapek-Dox′s agar medium. Petri-dishes were incubated for 7 days at 25 ±2ºC. Then axenic cultures were recovered, purified, grown on agar slants and kept at 4ºC for further studies. The isolated fungal species were morphologically and microscopically examined for identification (Watanabe, 2002).

Molecular identification of Fusarium sp.

DNA extraction was done by using Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo research D6007). ITS1 (5′TCCGTAGGTGAACCTTGCGG 3′) and ITS4 (5/TCCTCCGCTTATTGATATGC 3/) primers were used in PCR for amplification of the 5.8S ribosomal RNA region of ITS. Sequencing of the PCR net product was carried at the GATC Company by using an ABI 3730xl DNA sequencer.

Antifungal activity of propolis

Sterilized Czapek- Dox′s medium was amended with either propanolic or ethanolic extract of propolis to yield a final concentration of 2mg/mL. The amended medium was then transferred to Petri dishes. Each petri dish was inoculated with a fungal disc. Three replicates were made for each test fungus. The plates were then incubated at 27°C for 7 days, then the final fungal radial growth was recorded (Quiroga et al., 2001).

The results were recorded as a percentage of control.

$$[(control - treatment) \div control] \times 100\ \%$$

Disc of fresh fungal culture (containing approximately 2×10^5 spores) was placed in 10mL of liquid Dox′s medium supplemented with different concentrations of either propolis propanolic extract (PPE) or propolis ethanolic extract (PEE). The concentrations used were 0.5, 1, 2, 3, 4 and 5mg/mL of each extract. Then incubation period was 7 days at 27ºC under shaking (180rpm). The culture broth was then filtered to obtain mycelia and filtrate using Whatman filter paper. The obtained fungal mycelia were oven-dried till constant weight at 80ºC. The dried fungal mycelial weight was obtained. The cultures were made in triplicate.

The results were recorded as a percentage of control as previously described.

Antifungal activity of fungicide

As the above-described method, the fungicide Eleven 10% FS (Fludioxonil 4% + Tebuconazole 6%) was added to Czapek- Dox′s medium inoculated with a fresh fungal disc with different concentrations of fungicide 5, 10, 15, 20µg/mL. Then it was incubated for 7 days at 27°C. After the incubation period, the dry weights of the fungal mat were measured. The cultures were made in triplicate.

SDS-PAGE analysis of soluble proteins in the growth medium of Fusarium oxysporum under propanolic or ethanolic treatments of propolis extracts.

The technique used for the separation of proteins was (SDS-PAGE) (Sodium dodecyle sulphate, polyacrylamide gel electrophoresis) (Laemmili, 1970) after modification (Hames, 1995) by reduction of TEMED to 25µL from 30µL and reduction of APS to 1.3mL from 1.5mL. Three samples of the tested fungal isolate were studied, 2 of them treated with different propolis extracts and one used as a control. Gel documentation Alphatec 2200 software for protein band analysis with protein marker Tris-Glycine 15%.

The protein content that is present in the supernatant was measured using albumin of bovine serum as standard protein (Bradford, 1976). The protein content was adjusted to a 2mg/mL sample. The gel was stained with Coomassie Brilliant Blue. The resulting bands were analyzed using a gel documentation system.

GC-MS analysis

The first step before GC-MS analysis is derivatization. 150µl of MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide) previously diluted with anhydrous pyridine in 1:1% percentage was added to the dried PPE. One hundred µL of PPE was placed in screw-cap vials and under the stream of nitrogen gas left to evaporate until complete dryness. Then it was incubated at 60ºC for about 45min. before analysis using GC-MS (using Rtx-5MS 0.25mm inner-diameter, 30m length and 0.25m film) (Ali et al., 2016).

Physiological assays

Cellulase assay

For cellulolytic enzyme production, 1disc from growing tips of 7 day old culture of *F. oxysporum* was grown up in 10mL Czapek-Dox´s broth medium supplemented by 1% carboxymethylcellulose (CMC) as the sole carbon source instead of sucrose. The cultures were treated with 0.5, 1, 2, 3, 4 or 5mg/mL of PPE or PEE and then incubated at 27°C for 7 days. The control and treated cultures were maintained in triplicates. After the incubation period, the fungal mat was separated from the liquid medium by Whatman No.1 double-layered filter paper using Buchner funnel under vacuum pump suction. Centrifugation of the filtrate was carried out at 5000rpm at high speed, cooling centrifuge for 10min. The supernatant was removed and used as the enzyme source.

Cellulase activity was measured using carboxymethylcellulose (CMC) as the substrate (Areekijseree et al., 2004; Mukesh et al., 2012 after Bernfield, 1955 modification). The reaction mixture consisted of 0.3mL of enzyme extract, 1.7mL of phosphate citrate buffer pH 6, and 1mL of 0.25% of CMC with a final volume of 2ml. The reaction was allowed to proceed for 15min and then stopped using 1ml of 3,5-dinitrosalicylicacid (DNS) solution. The mixture was placed in a 100°C water bath for 10 minutes then cooled to room temperature and the optical density was measured at 546 nm. The results were expressed with a microgram of liberated glucose/mg of the substrate.

The results were recorded as percentage of control.

$$[(control - treatment) \div control] \times 100\ \%$$

Pectinase assay

For pectinolytic enzyme production, the above-described method was used except for using 1%

citrus pectin as the sole carbon source instead of sucrose in the liquid medium.

Pathogenicity degree test

This method was carried out according to Kloepper (1991) with modification for the soil *in vivo* experiment. *Phaseolus vulgaris* seeds were obtained from the agricultural research Centre, Giza, Egypt. Surface sterilization of *Phaseolus vulgaris* seeds was made using sodium hypochlorite (2%) for 2min. After surface sterilization the seeds were soaked separately in sterilized distilled water (as control), PPE 3mg/mL, PEE 4mg/ml for 2h, then 5 seeds were placed in a Petri plate (9cm in diameter) with sterile wetted cotton (5 replicates for each treatment). Then incubation was at 30°C for 2 days until radical emergence then the germinating seeds were transferred to plates (5 seeds per plate) with fully grown cultures of *F. oxysporum* hm89 (7 days old, originally inoculated with a fungal disc of approximate 2×10^5 spores) with 5 replicates for each treatment. Over each plate, 40g of sterilized sieved fine particles of soil (sterilized for 2 hours at autoclave with 1.5 Bar and 121°C) were spread. Each plate was moistened with 15mL sterile distilled water. The plates were covered and incubated at 30°C until seedling growth and then the plate cover was removed (after approximately 3 days). The results were recorded after 7 days as a percentage of control.

$$[(control - treatment) \div control] \times 100\ \%$$

Where control: Healthy seeds without infection and without the addition of any propolis extract (non-infected untreated samples).

Statistical analysis

A randomized complete block design with two factors was used for the analysis of data with three replications for each parameter. The standard error (SE) was measured.

Results

Effect of different propolis extracts on radial growth of some phytopathogenic fungi

It is clear in Fig. 1 that either PPE or PEE caused the same pathway regarding growth inhibition or activation. The growth values recorded for PPE and PEE were 38.2% and 58.9% against *Fusarium* sp. while the growth values were (80% and 87%) against *Rhizoctonia* sp. (91.15% and 93.79%) against *Botrytis* sp. (93.47% and 97.64%) against *Sclerotium* sp., whereas PPE and PEE showed growth values (95.39% and 98.46%) against *Stemphylium* sp.

In contrast, the results of PPE and PEE showed an opposable line with *Helminthosporium* sp. and *Cladosporium* sp. with unexpected activation of growth.

Molecular identification of Fusarium oxysporum hm89.

Fusarium oxysporum was morphologically and microscopically identified and then further confirmed by ITS sequencing. The PCR product was sequenced and the result was deposited at the Genebank while given an accession number: MH485380, and a strain identifier: *Fusarium oxysporum* hm89.

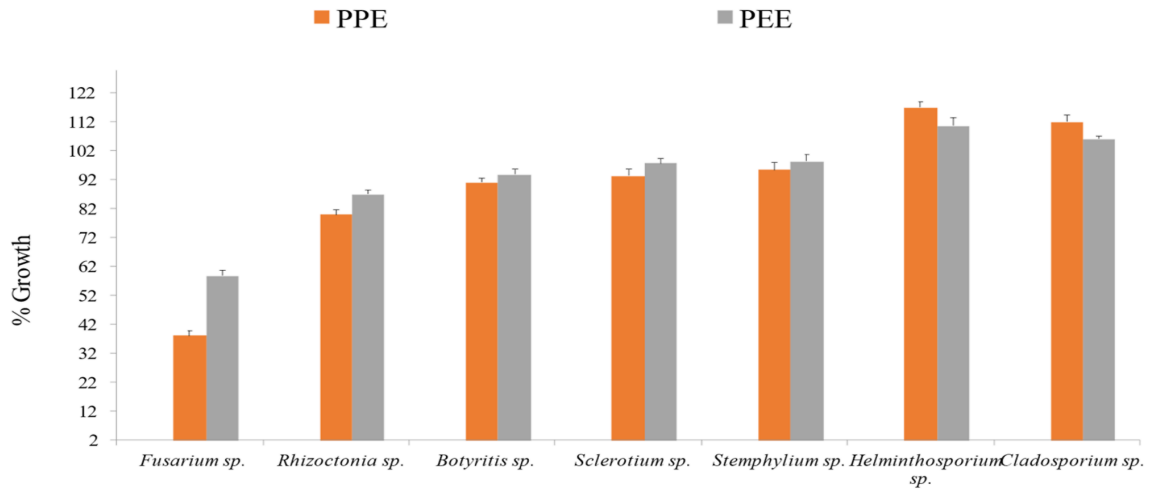

Fig. 1. Effect of PPE or PEE (2mg/mL) on radial growth of some phytopathogenic fungi [Bars show means. Error bars show mean + SE]

Effect of propolis extracts on percent growth of Fusarium oxysporum hm89

In this experiment, the gradual increase in the concentration of PPE or PEE was used, and the results were monitored (Fig. 2). The data showed high inhibitory activity of both propolis extracts on percent growth of *Fusarium oxysporum* hm89 at the concentration of 3mg/ml for PPE and 4mg/ml for PEE which recorded 2.1% and 2.5% in growth, respectively. The higher concentrations did not show a decrease in the growth of the two extracts.

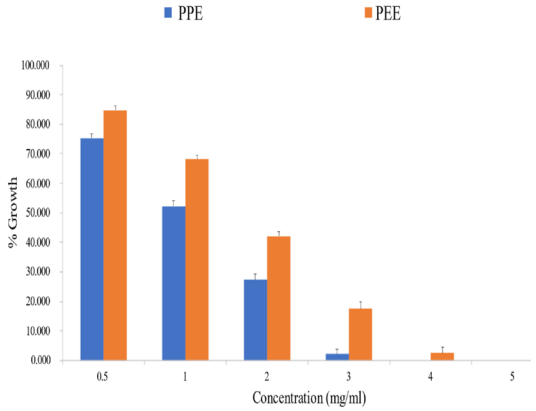

Fig. 2. Effect of PPE or PEE on percent growth of *Fusarium oxysporum* hm89 [Bars show means. Error bars show mean + SE]

Effect of fungicide on percent growth of Fusarium oxysporum hm89

The gradual increase in the concentration of the fungicide Eleven 10% (FS) was used, and the results were monitored (Fig. 3). The data showed high inhibition in growth at the concentration of 15µg/ml which recorded 1.5% growth. The higher concentrations did not show a significant decrease in growth.

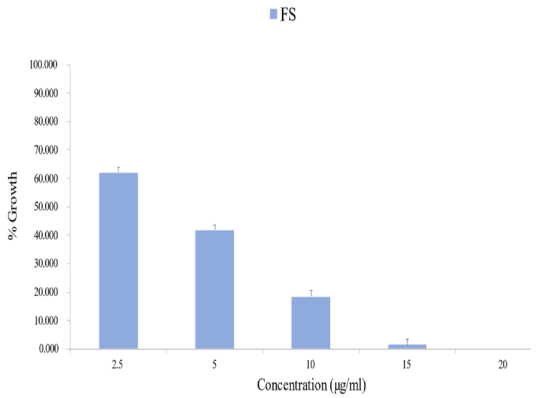

Fig. 3. Effect of fungicide (FS) on percent growth of *Fusarium oxysporum* hm89 [Bars show means. Error bars show mean + SE]

Protein pattern of Fusarium oxysporum hm89 treated with either PPE or PEE.

The relative molecular weights of soluble proteins in *Fusarium oxysporum* hm89 treated with PPE or PEE are shown in Fig. 4.

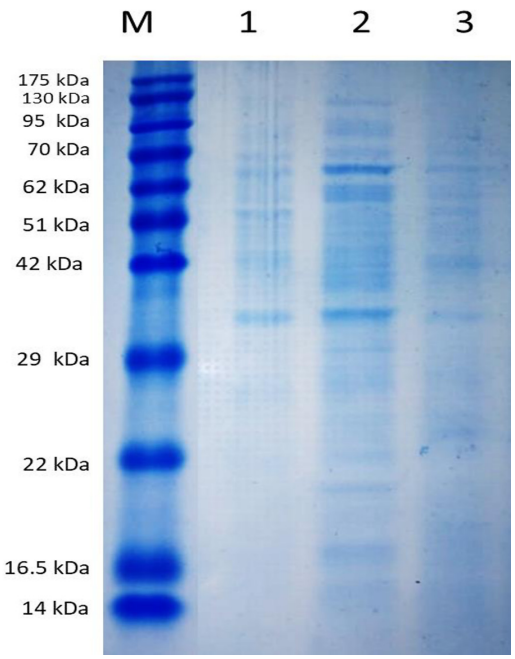

Fig. 4. SDS-PAGE analysis of soluble proteins in fungal growth medium amended with *Fusarium oxysporum* hm89 singly or amended with either PPE or PEE [M: Marker proteins, 1: *Fusarium oxysporum* hm89 soluble proteins profile (control), 2: *Fusarium oxysporum* hm89 soluble proteins profile after treatment with PPE 3mg/mL, 3: *Fusarium oxysporum* hm89 proteins profile after treatment with PEE 4mg/mL]

We compared the weights were to those in the untreated sample (Table 1). The three samples contained seven common proteins of molecular weights, 14.155, 54.730, 60.034, 66.164, 78.315, 85.287, 92.013kDa. The control sample (test pathogen only; 1) had 13 protein bands (the lowest value in all treatments) with a unique band at 26.002kDa. Meanwhile, the sample containing test pathogen amended with 3mg/ml PPE (2) had 18 protein bands (the highest value in all treatments) with specific protein bands at molecular weights, 21.160, 26.012 28.666, 38.44, 102.64kDa. Finally, the extract including test pathogen amended with 4mg/mL PEE (3) had 13 protein bands with particular protein bands at 18.871, 33.083kDa.

TABLE 1. Comparative analysis of different *Fusarium oxysporum* hm89 protein bands treated with PPE or PEE

Marker molecular weight (kDa)	Treatment					
	f_1		f_2		f_3	
	Band	RF	Band	RF	Band	RF
102.12	-	-	+	0.075	-	-
92.013	+	0.121	+	0.121	+	0.121
85.287	+	0.151	+	0.151	+	0.151
78.315	+	0.186	+	0.186	+	0.186
72.087	-	-	+	0.220	+	0.220
69.840	-	-	+	0.233	-	-
66.164	+	0.255	+	0.255	+	0.255
62.082	+	0.280	-	-	-	-
60.034	+	0.291	+	0.291	+	0.291
55.272	+	0.329	+	0.329	-	-
54.730	+	0.338	+	0.338	+	0.338
52.005	-	-	+	0.354	+	0.354
50.013	+	0.363	+	0.363	-	-
47.290	+	0.393	-	-	-	-
43.213	+	0.430	-	-	+	0.430
38.44	-	-	+	0.492	-	-
33.083	-	-	-	-	+	0.525
31.711	+	0.557	+	0.557	-	-
26.012	-	-	+	0.631	-	-
21.160	-	-	+	0.723	-	-
18.871	-	-	-	-	+	0.770
16.304	-	-	+	0.830	+	0.830
14.155	+	0.880	+	0.880	+	0.880

GC-MS analysis of PPE

We examined the Propolis Propanolic Extract (PPE) for its components through GC-MS analysis (Fig. 5).

Twenty compounds were identified in propolis propanolic extract (PPE) through GC-MS analysis. The compounds with their corresponding relative concentration (Area), retention time (RT) and their previously recorded biological activities are given in Table 2. In GC-MS analysis of the PPE extract, the spectrum of the unknown compound was compared with spectra of the NIST library stored known compounds.

Effect of PPE or PEE on percent cellulolytic activity of Fusarium oxysporum hm89.

Figure 6 shows the effect of either PPE or PEE on percent cellulolytic activity of *Fusarium oxysporum* hm89. The data indicated there was a significant reduction in the cellulolytic activity of *Fusarium oxysporum* hm89 till 3mg/ml concentration in the case of PPE that recorded 2.8% cellulase activity. The most effective reduction of cellulolytic activity in the case of PEE was at a concentration of 4mg/mL with a corresponding percent cellulolytic activity of 3.1%. At the same concentration, PPE was more inhibitory to percent cellulolytic activity than PEE.

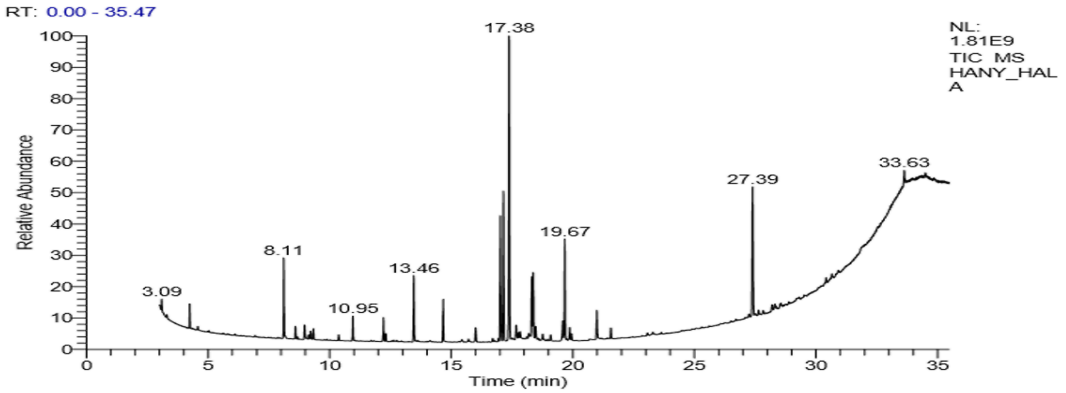

Fig. 5. GC-MS chromatogram of PPE

TABLE 2. List of components and their biological activity of PPE through GC-MS analysis

RT	Area%	Compound name	Reported activities
4.24	1.36	D-Lactic acid-DITMS	Antimicrobial (Neal-McKinney et al., 2012)
8.11	4.69	3,7-Dioxa-2,8-disilanonane	-
8.59	0.93	Pentanoic acid	Antibacterial (Freter et al., 1983)
8.97	1.10	Cis-2,5-di(2-cyanoethyl)-3-(thiophen-2-y l)-4,5-dihydropyrazole	Antibacterial (Elguero et al., 2002)
10.37	0.37	Propanoic acid	Antimicrobial
12.31	0.51	Erythritol PER-TMS	Inhibit growth of oral streptococci (Soderling & Hietala-Lenkkeri, 2010)
13.46	4.29	Methyl 2,2-dimethyl-3-[(4-chlorophenyl)amino]-3-(2-pyridyl)propanoate	-
14.67	2.83	(1S,1'R)-N-(2-Hydroxy-1-isopropylethyl)-o-(1'-hydroxy-1'-phenylmethyl)-Nmethylbenzenesulfonamide	Antifungal (El-Gaby et al., 2002)
17.02	8.81	D-(-)-Fructofuranose	-
17.15	10.83	D-(-)-Fructopyranose	-
17.38	20.95	S-[(E)-S-Phenyl-N-(p-tolylsulfonyl)-1-t rimethylsilyl-3-methylbut-1-enyl]sulfoxime	Insecticide
17.83	0.60	Quinic acid-PENTATMS	Anti-inflammatory, antioxidant, and antimuta-genic (Bai et al., 2018)
18.31	3.94	D-Psicose	Hypoglycemic, Hypolipidemic and Antioxidant (Matsuo et al., 2001)
18.37	4.07	á-D-(+)-Mannopyranose	Antitumor activity
19.59	1.57	Calystegine B2 tris (Trimethylsilyl) Derivative	Potent competitive inhibitor of β-glucosidases and α-galactosidases (Molyneux et al., 1993)
19.67	6.27	á-D-Glucopyranose	-
20.99	2.32	Myo-Inositol	Anticancer activity (Bizzarri et al., 2016)
27.39	10.35	Octakis(trimethylsilyl) ether	-
27.64	0.43	Tristrimethylsilyl ether derivative of 1,25-Dihydroxyvitamin D	-
28.19	0.65	1-Monolinoleoylglycerol trimethylsilyl ether	Antimicrobial, antioxidant, antiinflammatory, antiarthritic, antiasthma, diuretic (Senthil et al., 2016)

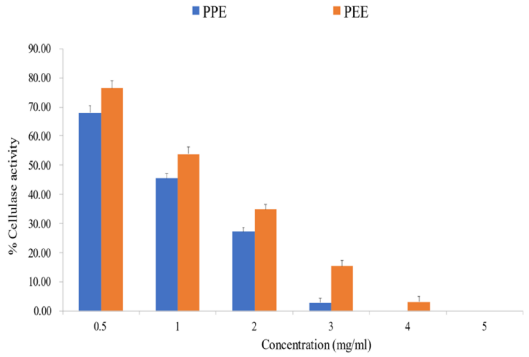

Fig. 6. Effect of PPE or PEE on percent cellulolytic activity of *Fusarium oxysporum* hm89 [Bars show means. Error bars show mean + SE]

Effect of PPE or PEE on percent pectinolytic activity of Fusarium oxysporum hm89.

The effect of either PPE or PEE on the percent pectinolytic activity of *Fusarium oxysporum* hm89 is elucidated in Fig 7. The data indicate there was a significant reduction in the pectinolytic activity of *Fusarium oxysporum* hm89 till 3mg/mL concentration in the case of PPE that recorded 2.9% pectinase activity. The most effective reduction of pectinolytic activity in the case of PEE was at a concentration of 4mg/mL with a corresponding percent pectinolytic activity of 3.3%. At the same concentration, PPE was more inhibitory to percent pectinolytic activity than PEE.

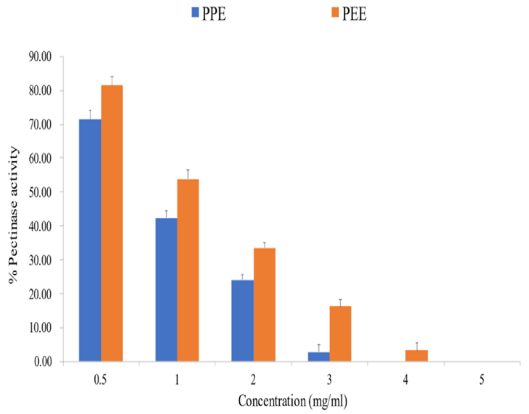

Fig. 7. Effect of PPE or PEE on percent pectinolytic activity of *Fusarium oxysporum* hm89 [Bars show means. Error bars show mean + SE]

Effect of PPE or PEE applications on the degree of pathogenicity of Fusarium oxysporum hm89 infecting Phaseolus vulgaris

There is suppression in the degree of infectivity in *Phaseolus* seedlings presoaked in either of the two propolis extracts compared to infected plants (Fig. 8). Also shown is the higher growth-enhancing and disease-suppressing effect of PPE over PEE (Table 3).

It is obvious from data in Table 3 that the degree of infectivity decreased in both propolis extract applications. The best result was obtained when PPE was applied at the concentration of 3mg/mL as compared to the corresponding value of 4mg/mL of PEE application. The suppression of infectivity in both propolis applications was partially compared with the degree of infectivity that occurred in infected untreated *Phaseolus vulgaris* seedlings.

Data recorded in Fig. 9 clearly shows that the application of propolis extracts increased the length of epicotyl and the length of the hypocotyl of *Phaseolus vulgaris* seedlings as compared with untreated infected control. Seedling growth parameters were higher in the case of PPE over that of PEE.

Seed presoaking in PPE gave the best result concerning the length of epicotyl, which recorded 2.6cm compared to healthy ones that measured 3.5cm. The length of hypocotyl recorded 12.3cm as compared to healthy ones that measured 13.2cm. The lowest value of epicotyl and hypocotyl lengths was recorded with the infected untreated seedlings and measured 0.7cm and 7.4cm, respectively (Fig. 9 a).

It is clear (Fig. 9 b) that the two propolis extract treatments (PPE and PEE) significantly increased total fresh (0.045g and 0.039g) and dry weights (0.009g and 0.008g) of *Phaseolus vulgaris* seedling leaves, respectively, compared with the infected untreated seedlings (0.036g and 0.007g).

The maximum increase was obtained when PPE was used which resulted in 1.162g and 0.232g in either fresh or dry weight of seedlings, respectively. The seedling fresh and dry weight of infected non treated *Phaseolus vulgaris* recorded 0.58g and 0.116g, respectively (Fig. 9 c).

The data recorded in Fig. 9 d clearly show that the application of propolis extracts (PPE and PEE) increased the leaf area of *Phaseolus* vulgaris seedling leaves (1.4cm² and 1.2cm²) as compared with untreated infected control (0.95cm²).

Fig. 8. *Phaseolus vulgaris* seedlings; a: Control, b: Infected with *Fusarium oxysporum* hm89, c: Treated with PEE and infected with *F. oxysporum* hm89, d: Treated with PPE and infected with *F. oxysporum* hm89

TABLE 3. Effect of PPE or PEE applications on growth parameters and degree of infectivity in healthy and *Fusarium oxysporum* hm89 - infected *Phaseolus vulgaris* seedlings

Treatments	Growth parameters of *Phaseolus vulgaris* for 25 germinated seeds			
	% of completely rotted seeds	% of soft watery rotted seeds	% of seedlings with brown discoloration	% of wilted seedlings
Fusarium oxysporum hm89 infected seeds	80	64	48	80
PPE + infected seeds	16	20	16	32
PEE + infected seeds	48	44	32	52

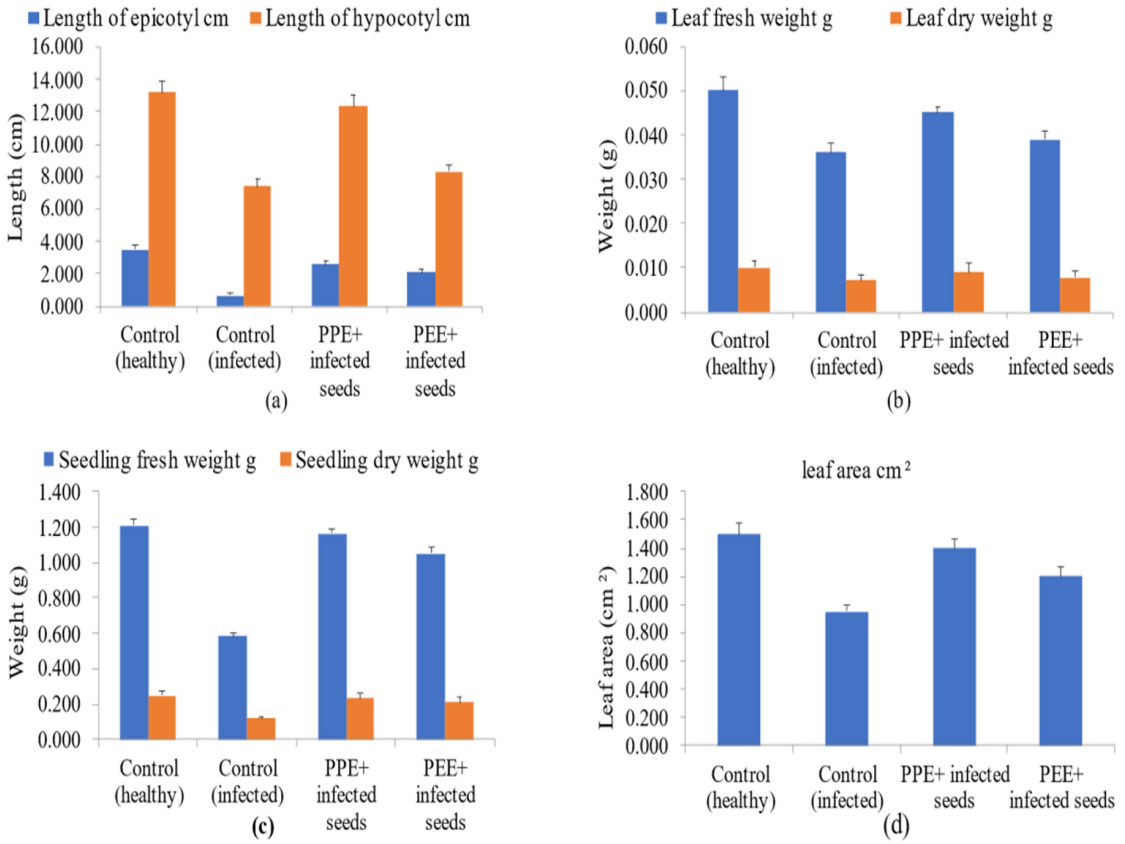

Fig. 9. Effect of PPE and PEE applications on *Fusarium oxysporum* hm89 infected *Phaseolus vulgaris* seedlings after 10 days; (a) Length of epicotyl and hypocotyl, (b) Leaf fresh and dry weight, (c) Seedling fresh and dry weight, (d) Leaf area [Bars show means. Error bars show mean + SE]

Discussion

Fusarium oxysporum is a fungal strain that causes wilt disease on some plants e.g., *Phaseolus vulgaris* L. and *Lycopersicon* sp. (Alves-Santos et al., 2002; Alfiky & Eldenary, 2019). The pathogen penetrates healthy tissue of the root usually through wounds and/or natural openings (Abawi, 1989).

In this study, there was a trial to apply ethanolic or propanolic extract of propolis to reduce the pathogenicity of seven fungal species (*Fusarium* sp., *Rhizoctonia* sp., *Botrytis* sp., *Sclerotium* sp., *Stemphylium* sp., *Cladosporium* sp. and *Helminthosporium* sp). The most susceptible isolate was *Fusarium oxysporum* with a percent growth of 38.2% and 58.9% in the case of PPE or PEE application, respectively, and hence it was selected for further work during this study. There were unpromising nonsignificant growth suppressions that occurred with *Rhizoctonia* sp., *Botrytis* sp., *Sclerotium* sp. and *Stemphylium* sp. whereas unexpected activation was observed with *Cladosporium* sp. and *Helminthosporium* sp. when either of the two extracts was applied.

Ôzcan (1999) found that propolis was able to induce inhibition of aflatoxigenic fungi and also decrease conidial growth in *Aspergillus flavus*. Propolis samples collected from different areas showed activity against *Candida albicans, C. guilliermondii* and *C. krusei*. Out of 26 or more constituents of propolis, pinocembrin, pinobanksin-3-acetate, 3-acetylpinobanksin, caffeic acid and p-coumaric were reported to have antifungal activity. Caffeic acid showed antifungal activity against *Helminthosporium carbonum*, and this result is practically inconsistent with our data. This may be due to the presence of amino acids together with tryptophan among the parts of propolis extract (Walker & Crane, 1987). Therefore, the utilization of such a product may activate the tested microorganism compared with untreated control. This activation may be also due to the greater tolerance of the spore (multicellular with thick cell wall conidia) against propolis extract or may be due to the absence of caffeic acid in tested propolis extract. The same reasons may also explain the motivation of fungal growth in *Cladosporium* sp. when treated with our crude propolis extracts.

Evaluation of the antifungal activity of propolis ethanolic extract (PEE) against *C. albicans*, *C. tropicalis, C. parapsilosis* and *C. guilliermondii* showed that 98% of the fungal samples are sensitive to concentrations less than 5% (Fernandes Jr. et al., 1994). In another study *in vitro*, it was observed that a concentration of 5% or 10% of propolis ethanolic extract prevented the growth of *Trichophyton verrucosum* (Lori, 1990). Propolis was also observed by La Torre et al. (1990) to have *in vitro* antifungal activity against some phytopathogenic fungi (La Torre et al., 1990).

In a correlated study, propolis activity was investigated using some strains of Enterobacteriaceae, *Pseudomonas* spp., *Lactobacillus Plantarum*, *Fusarium oxysporum* and yeasts (*Debaryomyces hansenii* and *Saccharomyces cerevisiae*). By using microdilution methods after modification and viable count, these microorganisms were inoculated at low and high levels inoculum. Propolis antimicrobial effects depend on many factors such as the type of microorganisms, the concentration of microbial inoculum and partial inhibition is the main mode of action of propolis than complete inhibition (Petruzzi et al., 2020).

The isolated *Fusarium oxysporum* strain in this work was molecularly identified and the gene sequence of the isolated strain was similar to that of many species of the genus *Fusarium* spp., according to a BLAST search of the GenBank database.

It seemed more worthy in the current work to study, *in vitro*, the potentiality of the two propolis extracts or Eleven 10% (FS) as a standard fungicide against *Fusarium oxysporum* hm89. The data showed high inhibitory activity in growth percentage at the concentration of 3mg/ml for PPE and 4mg/mL for PEE which recorded 2.1% and 2.5% respectively. The higher concentrations did not show a significant increase in the inhibitory effect of the two extracts. So, we optimized 3mg/ml as a proper for PPE and 4mg/ml as a proper for PEE during the further survey in our recent study. Ôzcan (1999) reported that the concentration of 4% of propolis extract could reduce the infectivity of *F. oxysporum* f. sp. *melonis* by 50% compared with the wild type.

Although the chemical fungicide, Eleven 10% (FS), is used in very low concentrations

(micrograms), it must be noted its damages, especially as it accumulates in the soil as a result of frequent use. Eleven 10% (FS) is a commercial chemical fungicide that is recommended by the Agricultural Research Center in Giza, Egypt, to be used for controlling *Fusarium* species. Although Eleven 10% (FS) exhibited an MIC of 15µg/mL which is much lower when compared with the 3mg/ml or 4mg/mL MIC values of PPE or PEE, respectively, it should be taken into consideration that Eleven 10% (FS) is a mixture of Fludioxonil and Tebuconazole which are known to be toxic. Fludioxonil is a broad-spectrum fungicide (Gullino et al.,2000) that belongs to phenylpyrrole fungicides which inhibit germination of spores, elongation of germ tube and mycelial growth and also have been reported to induce germ tube distortions and cell bursting (Rosslenbroich & Stuebler, 2000). Tebuconazole is an effective multifunctional fungicide that belongs to triazoles fungicides that penetrate the plant through roots and vegetative organs and are known to be phytotoxic (Ahemad & Khan, 2012a, b). On the other hand, propolis is natural and safe (Alencar et al., 2007). Meanwhile, in the El-Yazal (2019) study, it was demonstrated that increasing the concentration of propolis extract as seed presoaking application increased growth parameters of spinach. The best result was obtained by the middle concentration of 7000mg/L.

Studies showed that many different compounds like flavonoids and phenolics may be responsible for the antifungal activity of the ethanolic extract of propolis. Their mode of action depends on changing the cell membrane permeability that leads to cell death by causing complete leakage of the cell constituents such as proteins, nucleic acids, and inorganic ions like potassium and phosphate (Shehu et al., 2016). It was recorded that 5mg/mL (5000ppm) of ethanolic extract of propolis completely inhibited *F. oxysporum* radial growth (Ahmed et al., 2008).

The chemical compositions of PPE were analyzed by GC-MS to identify the major and minor constituents that are present in our extract sample. GC-MS profile of the PPE was composed of aliphatic hydrocarbons, aliphatic acids and their related esters, aromatic acids and their related esters, sugars, and miscellaneous compounds. Antifungal products like Pyrazole, Quinic acid, D-lactic acid, Pentanoic acid, N-methylbenzenesulfonamide and Erythritol could have a critical role in the inhibitory action of PPE on the tested pathogen *Fusarium oxysporum* hm89.

Pyrazole possesses widespread pharmacological activities. These properties include analgesic, anti-hyperglycemic, anti-inflammatory, anti-bacterial, antipyretic, sedative-hypnotic, and hypoglycemic activity (Elguero et al., 2002).

Quinic acid (QA), which is a natural phenolic acid, was reported by Bai et al. (2018) to have antioxidant, anti-inflammatory, and antimutagenic activities, and show transition metal-chelating activity. QA also showed broad-spectrum antibacterial activities. Studying the effect of QA on the cellular physiology of *Staphylococcus aureus* showed a decrease in intracellular pH that reduces the activity of succinate dehydrogenase causing a decrease in the concentration of intracellular ATP. It also reduced *S. aureus* DNA content. Additionally, it interacted directly with genomic DNA.

Lactic acid bacteria produce lactic acid, an organic acid showing antimicrobial activity (Neal-McKinney et al., 2012). The other various compounds, produced by lactic acid bacteria, include diacetyl, organic acids, hydrogen peroxide, and bacteriocins (proteins that have bactericidal activity produced during the fermentation of lactic acid). Some bacteriocins also showed inhibitory activity against food-borne pathogens and/or food spoilage (Cleveland et al., 2001). The inhibitory mechanism of lactic acid against microorganisms includes membrane destabilization (Sjögren et al., 1990), interference with proton gradient (Gänzle & Vogel, 2003), reactive oxygen species creation (Lindgren & Dobrogosz, 1990; Schaefer et al., 2010) and enzyme inhibition (Lindgren & Dobrogosz, 1990).

N-methylbenzenesulfonamide was demonstrated to have antifungal, antibacterial and antitumor activity (El-Gaby et al., 2002).

Pentanoic acid or valeric acid is one of the short-chain fatty acids that have antibacterial activity (Freter et al., 1983).

An *in vitro* study was conducted (Si et al., 2019) on the antifungal activity of several

novel niacinamide and pyrazole carboxamide derivatives against four fungi using the inhibition of mycelium growth method. These fungi (*Rhizoctonia solani*, *Botrytis cinerea*, *Alternaria solani* and *Fusarium graminearum*) cause severe diseases to plants. The results showed that some of such novel compounds have higher antifungal activity at a concentration of 100µg/mL toward *B. cinerea* more than toward *Rhizoctonia solani, Alternaria solani*, and *Fusarium graminearum*.

The proteomic analysis for *Fusarium oxysporum* f. sp. cubense as the cause of *Fusarium* wilts in the root cells of banana showed Pathogenesis-related protein 1, NADP-dependent malic enzyme, Protein IN2-1 homolog B, Protein disulfide-isomerase, L-ascorbate peroxidase, Caffeoyl-CoA O-methyltransferase, Superoxide dismutase (Mn) 3.1, Probable glutathione S-transferase GSTF1, and Isoflavone reductase homolog, with molecular weights: 92, 60, 66, 67, 58, 61, 57kDa respectively (Li et al., 2013). In our study, the *Fusarium oxysporum* hm89 protein profile exhibited seven common proteins of molecular weights,14.155 54.730, 60.034, 66.164, 78.315, 85.287, and 92.013kDa in the control and the two propolis extracts. There are five common protein bands in our work that may be correlated to *Fusarium oxysporum* hm89 and they were commonly matched with the corresponding ones found during a running protein SDS page by Li et al. (2013). They were identified as Pathogenesis-related protein 1, Protein IN2-1 homolog B, NADP-dependent malic enzyme, Protein disulfide-isomerase, Caffeoyl-CoA O-methyltransferase, L-ascorbate peroxidase.

It was recorded that proteins with 33 kDa molecular weight called Thaumatin-like proteins (TLP) was induced in bioagent-stressed tomato plant root tissues which are infected with *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy et al., 2002). Results showed that defense enzyme induction is involved in the pathway of phenylpropanoid and phenolics accumulation and PR-proteins may be responsible for invasion restriction of the pathogen in roots of tomato. In the current, work the corresponding protein band with molecular weight 33.083kDa in *Fusarium oxysporum* hm89 stressed with 4mg/ml PEE may be related to Thaumatin-like proteins that limited the fungal growth in this treatment.

It seemed beneficial in the current work to study *in vitro* the activity of hydrolytic enzymes including cellulase and pectinase of *Fusarium oxysporum* hm89 in response to different concentrations of tested propolis extracts (from 0.5mg/mL to 5mg/ml). The results clarified a progressive reduction in cellulolytic and pectinolytic activities of the test pathogen with an increase in propolis concentration. Lindgren & Dobrogosz (1990) assumed that the reduction in enzymatic activity of the tested pathogen could be attributed to the inhibitory activity of lactic acid identified as a constituent of propolis propanolic extract during GC-MS technique in our work. Hence, the chemical constituents of propolis extracts played an important function in reducing the activity of enzymes that play the main role in host-pathogen interaction and may induce host infection.

Wanjiru et al. (2002) demonstrated that hydrolytic enzymes of *Fusarium compactum* play a critical role in the invasion of broomrape tubercles macerating parenchymatous tissue. The mechanism of tubercle invasion by *F. compactum* was by degrading cell wall components followed by invading host tissues. The activity of pectinolytic and cellulolytic enzymes results in the loss of the structural integrity of the host and characteristic damage of the root. The pathogen's enzyme activity was also responsible for the expansion of tubercle cells and hence symptom development. The level of fungal inoculum and the consequent degree of pathogenicity of *F. compactum* was due to enzyme production. The role of cellulase and pectinase in cell wall components lysis and plant-host interactions was also reported by other authors (Wanjiru et al., 2002; Chanliaud, 2004; El-Sharkawy & Alshora, 2020).

In our *in vivo* soil study, presoaking of *Phaseolus vulgaris* seedlings in either the two propolis extracts decreased the degree of infectivity induced by *Fusarium oxysporum* hm89. There was also enhancement of qualitative and quantitative growth parameters of seedlings which were concomitant with the results of the El-Yazal (2019) study.

Conclusion

Propolis is a natural and safe antimicrobial compound. In an *in vivo* soil study, the strategy of applying propolis extracts (ethanolic or propanolic)

to *Phaseolus vulgaris* decreased *Fusarium oxysporum* hm89 growth and pathogenicity. However, future work could be carried out under greenhouse conditions or inland areas.

Conflict of interest: The authors declare no conflict of interest.

Author contributions: Neveen M. Khalil contributed in conceiving the ideas, designing and supervising the research and contributed in data analysis, discussion, writing and editing of the manuscript. Hala M. Ali performed the experiments and contributed in data analysis, discussion, writing and editing of the manuscript. Ahmed E. Ibrahim contributed in conceiving the ideas, designing and supervising the research and contributed in data analysis, discussion, writing and editing of the manuscript.

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النشاط الكيميائى الحيوى لمستخلص البروبوليس الكحولى المضاد لفطر الفيوزاريوم اوكسيسبورم

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يقلل البروبوليس نمو بعض الفطريات الممرضة للنبات المختبره معمليا. تم تثبيط أربعة أنواع تم اختبارها جزئيًا باستخدام المستخلص البروبانولى والايثانولى للبروبوليس بإستخدام كل من البروبانول والايثانول وتم تسجيل نشاط ضد ميكروبى واعد منهما تجاه فطره الفيوزاريوم أوكسيسبورام بدرجة تثبيط سجلت ٪38.2 و ٪58.9 أثناء تطبيق مستخلصى البروبوليس بالبروبانول والإيثانول على التوالي بينما تطبيق نوعى المستخلص على فطرى الهيلمنثوسبوراوم والكلادوسبوريوم أظهر نشاطا غير متوقع للنمو. هذه المنتجات ذات النشاط الضد ميكروبى التي تم تسجيلها خلال التحليلى الطيفى الكتلى (جى سى - أم أس) هي البيرازول، وحمض الكينيك، وحمض اللاكتيك، وحمض البينتانويك، والاريثريتول، وحمض البروبيونك، ومشتقات السلفونامايد. وقد اتضح من خلال التحليل الكهربائى للبروتينات خاصه لكل معامله عند اوزان جزيئيه مختلفه فمثلا بروتين وحيد متفرد عند وزن جزيئى 26.002 كيلو دالتون مع المعامله المتعلقه بوجود الفطر الممرض فقط، وبروتينات متنوعه مميزه عند 21.160، 26.012، 28.666، 38.44، 102 كيلو دالتون لحاله الفطر المعامل بمستخلص البروبوليس البروبانولى (f2) وأخيراً بروتينان واضحان ومميزان عند اوزان جزيئيه 18.871 33.083 كيلو دالتون فى المعامله الخاصه بالفطر المعامل بمستخلص البروبوليس الإيثانولي (f3). تم دراسة تثبيط نشاط إنزيمى السيلولاز والبكتيناز لفطر الفيوزاريوم اوكسيسبورم الذى تم معاملته بمستخلصى البروبوليس البروبانولى والإيثانولى. كان هناك تثبيط في درجات و مظاهر العدوى بالنسبه لفطره الفيوزاريوم اوكسيسبورام والمتمثله فى عدد البذور المتعفنة والذبول وتغير اللون البني لبذور الفاصوليا المنقوعة مسبقًا في أي من مستخلصي البروبوليس مقارنة بالنباتات المصابة مع درجة تثبيط أكبر بشكل فردي في حالة المستخلص البروبانولى على المستخلص الإيثانولي.