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Full Length Research Paper

Antifungal effect of some plant extracts on *Alternaria alternata* and *Fusarium oxysporum*

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***In vitro* studies were carried out to test the antifungal activity of 5 plant extracts; *Cinnamomum zeylanicum* (Cinnamon), *Cymbopogon proximus* (Halfa barr), *Laurus nobilis* (Laurel), *Persea americana* (Avocado) and *Zingiber officinale* (Ginger) performed with either cold distilled water (CDW) or boiling (BDW) on two pathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. The results revealed that plants extracts especially those performed with CDW had a strong antifungal activity with significant inhibition on the growth of the 2 tested fungi and their hydrolytic enzymes, β-glucosidase, pectin lyase and protease. Extracts of Halfa barr and ginger were the most effective to inhibit the growth of the tested fungi followed by avocado, cinnamon and laurel. Findings from this study confirmed that plant extracts can be used as natural fungicides to control pathogenic fungi, thus reducing the dependence on the synthetic fungicides. Halfa barr, which was found to be the most efficient extract (75% inhibition), might be a promising material for controlling these fungi.**

Key words: Antifungal agents, *Alternaria alternate*, *Fusarium oxysporum*, plant extracts, pectin lyase, β-glucosidase, protease.

INTRODUCTION

The inappropriate use of agrochemicals especially fungicides were found to possess adverse effects on ecosystems and a possible carcinogenic risk than insecticides and herbicides together (Cameron and Julian 1984; Research Council Board of Agriculture 1987; Osman and Al-Rehiayam 2003; Masuduzzaman et al., 2008; Siva et al., 2008). Moreover, resistance by pathogens to fungicides has rendered certain fungicides ineffective (Zhonghua and Michailides, 2005). Due to the aforementioned considerations, there may be a need to develop new management systems to reduce the dependence on the synthetic agrochemicals. Recent trends favour the use of alternative substances derived from natural plant extracts to control pests (Lale and Abdulrahman, 1999; Xan et al., 2003; Islam et al., 2004). Plant extracts and their essential oils show antifungal activity against a wide rang of fungi (Cowan, 1999; Kurita et al., 1981; Grane and Ahmed, 1988; Abd-Alla et al., 2001; Wilson et al., 1997). Several authors studied the effect of different plant extracts on the growth of fungi: *Cymbopogon proximus* against the toxigenic fungi *Fusarium verticillioides* and *Aspergillus flavus* (El-Assiuty et al., 2006); garlic (*Allium sativa*) against *Aspergillus niger* (Yoshida et al., 1987); *Allium sativum, Cymogopogon proximus, Carum carvi, Azadirchia indica* (neem) and *Eugenia caryophyllus* against *Fusarium oxysporum* f. sp. *lycopersici, Botrytis cinerea* and *Rhizoctonia solani* (Aba AlKhail, 2005); and *Aristea ecklonnii* and *Agapathus inapertus* against *Botrytis cinerea, Fusarium oxysporum, Rhizoctonia solani* (Pretorius et al., 2002). Also, garlic and neem have been recommended against *Pesteloxia palmarum* (Islam et al., 2004; Pandey et al., 2002).

Fusariosis induced by *Fusarium oxysporum* is one of the most difficult to control and it is a severe disease of several crops, greenhouse plants and trees. It causes significant losses in crop production and has been reported in at least 32 countries (Jones et al., 1991). Also, *Alternaria alternata* can have a certain pathogenic effect; it has been recorded as a saprophytic or a weak pathogen causing so-called "indefinite or opportunistic disease" on a number of crops (Nishimura 1980; Ibraheem et al., 1987; Abbas et al., 1995). Filtrates of pathogenic fungi containing their respective toxins caused necrosis within 48 h and eventually mortality on susceptible cultivars.

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The spores of these fungi caused cellular leakage and reduced chlorophyll content in susceptible cultivars. The current chemical control is either not efficient or difficult to apply. Therefore, the use of alternative substances derived from natural plant extracts can be a promising alternative to prevent the diseases caused by the aforementioned fungi and seems to be one of the possible solu-tions (Alabouvette et al., 1984).

Cellulolytic enzymes often have different modes of action. They are known as the cellulase complex (exoglucanase E.C. 3.2.1.91, endoglucanase E.C.3.2.1.4 and β-glucosidase (Cellobiase) E.C. 3.2.1.21) (Enzyme Nomenclature Database, 2001). β-Glucosidase, which plays an important role in complete enzymatic saccharifications of cellulose is the object of this study. Pectin lyase [poly (methoxygalacturonide) lyase, PMGL, PNL or PL; E.C.4.2.2.10] seems to be the only pectic enzyme capable of breaking down pectin with high degree of esterification (like those found in fruits) into smaller molecules (Fogarty and Ward, 1974; Fogarty and Kelly, 1983; Wijesundera et al., 1984; Alana et al., 1990; Delgado et al., 1992; Serra et al., 1992). Proteolytic enzymes are by far the most important group of enzymes produced commercially and are used in many areas of application, such as in detergents, brewing, photographic, leather and dairy industries (Yang et al., 2000).

The present study was undertaken to evaluate the effectiveness of 5 plant extracts namely *Cymbopogon proximus* (Hochst. ex A. Rich.) Stapf (Halfa barr), *Cinnamomum verum* J. Presl., *Cinnamomum zeylanicum* Breyne (Cinnamon), *Laurus nobilis* L. (Laurel), *Persea americana* Mill. (Avocado) and *Zingiber officinale* Roscoe (Ginger) extracted with either cold distilled water (CDW) or boiling (BDW) on the growth of 2 phathogenic fungi: *Alternaria alternata* f. sp. *lycopersici* (Fr.) Keissl and *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen. Also, the effect of the 5 plant extracts with CDW on the production of hydrolytic enzymes; β-glucosidase, pectin lyase and protease from the 2 tested fungi was investigated.

MATERIALS AND METHODS

Micro-organisms and culture medium

Alternaria alternata f. sp. *lycopersici* (Fr.) Keissl NRRL 18822 and *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen NRRL 26037 were obtained from NRRL (Agricultural Research Service Culture Collection). They were kindly provided by united state department of agriculture (USDA), New Orleans, Louisiana 70179. The cultures were grown on Waksman's agar medium at 28˚C for 7 days before being used.

Preparation of plant extracts

The rhizome of ginger, stems and leaves of half-barr, leaves of cinnamon, avocado and laurel were obtained from the botanical garden of the faculty of education Ain Shams university. The tested fresh plants were ground to fine powder and then extracted by macerating 20 g of each plant in 100 ml of either sterilized cold distilled water (CDW) for 24 h, or boiling distilled water (BDW) for 1 h. The extracts were filtered [the extract with CDW sterilized by passing it through bacterial filter 0.25 µm (Seitz)]. These extracts were set as original concentrations (20%). Dilutions of 0.5, 1 and 2% of the tested extracts were prepared by adding 1.25, 2.5 and 5 ml of the original concentration to Erlenmeyer conical flasks of 250 ml capacity, contained 50 ml of warm Waksman's medium, respectively. Each treatment was replicated 3 times. The plant parts extracted by CDW were thoroughly mixed with the medium after autoclaving while those extracted by BDW were mixed with the medium before autoclaving. The medium without extracts served as control. Mycelial discs were prepared using a cork borer (5 mm diameter) from the peripheral of 7 days old culture of the tested fungi. The flasks were incubated in an incubator at 28°C for 7 days after inoculation.

Growth measurement

Measuring the fungal growth was made according to the method described by Kane and Mullins (1973). The flasks of each triplicate were filtered off using Whatman filter paper after the end of the incubation period and the mats were washed carefully and dried up to a constant dry weight at 70 - 80˚C for 24 h.

Determination of enzymes production

The previous procedure was repeatedly done with some modification to determine the effect of plant extracts (CDW only because the extracts with BDW showed no significant effect against the tested fungi) on the hydrolytic enzymes (β-glucosidase, pectin lyase and protease) of the tested fungi. Waksman's medium was prepared by replacing glucose content of the original medium with either cellobiose or citrus pectin (2% of each) to measure the production of β-glucosidase and pectin lyase respectively. The peptone content of the original medium was also replaced by casein to measure the production of protease.

Beta-glucosidase assay

β-glucosidase [(Cellobiase) E.C. 3.2.1.21] activity was assayed by using cellobiose as a substrate according to the method of Berghem and Petterson (1974). The assay mixture contained 1 ml of 1% cellobiose in 0.05 M citrate phosphate buffer pH 5.0 and 1 ml of enzyme solution. After incubation at 50˚C for 30 min, reducing sugars were determined using the 3,5 dinitro salicylic acid method (DNS) according to Miller (1959), using glucose as a standard. The absorbency was read at 540 nm using a spectrocolorimeter (Spekol). One unit of beta-glucosidase was defined as the amount of enzyme which liberates 0.0926 mg glucose min^{-1} ml^{-1} (Ghose, 1987).

Pectin lyase assay

Pectin lyase activity [poly (methoxygalacturonide) lyase, PMGL, PNL or PL; E.C.4.2.2.10] was evaluated by the method of Albersheim and Killias (1962) as follows: 1.1% (wt/vol) solution of citrus pectin (Sigma Chemical Co., St Louis, Mo) was dissolved in 0.05 M citrate-phosphate buffer under magnetic stirring at 30˚C, then 3.0 ml of this solution was added to 2.0 ml of crude enzyme that had been previously adjusted to pH 7.5 with concentrated NaOH. After 60 min, the reaction was stopped by adding 3.5 ml of 0.5 M H_2S0_4. For the blank test, the order of the reagent was reversed (that is, the acid was first added to the enzyme, followed by the pectin solu-

Table 1. The effect of plant extracts on mycelial growth of *Alternaria alternate* (g/50 ml medium).

Treatment	Aqueous extract (CDW)			Aqueous extract (BDW)		
	0.5%	1%	2%	0.5%	1%	2%
Control	0.43 ± 0.00	0.43 ± 0.00	0.43 ± 0.00	0.43 ± 0.00	0.43 ± 0.00	0.43 ± 0.00
Cinnamomum zeylanicum (Cinnamon)	0.35 ± 0.00 (H.S)	0.34 ± 0.01 (H.S)	0.23 ± 0.0 (S)	0.43 ± 0.05 (N.S)	0.43 ± 0.05 (N.S)	0.43 ± 0.05 (N.S)
Cymbopogon proximus (Halfa barr)	0.29 ± 0.02 (H.S)	0.18 ± 0.03 (H.S)	0.11 ± 0.03 (H.S)	0.41 ± 0.03 (S)	0.41± 0.02 (S)	0.40 ± 0.04 (S)
Laurus nobilis (Laurel)	0.37 ± 0.01 (H.S)	0.39 ± 0.02 (S)	0.33 ± 0.05 (N.S)	0.43 ± 0.05 (N.S)	0.43 ± 0.05 (N.S)	0.43 ± 0.05 (N.S)
Persea Americana (Avocado)	0.31 ± 0.01 (H.S)	0.32 ± 0.01 (H.S)	0.15 ± 0.01 (H.S)	0.42 ± 0.02 (S)	0.41± 0.03 (S)	0.40 ± 0.03 (S)
Zingiber officinale (Ginger)	0.30 ± 0.00 (H.S)	0.29 ± 0.02 (S)	0.13 ± 0.00 (H.S)	0.41± 0.02 (S)	0.40 ± 0.03 (S)	0.40 ± 0.03 (S)

(H.S) Highly significant ($p \leq 0.01$); (S) significant ($p \leq 0.05$); and (N.S) non significant ($p > 0.05$).
The obtained data were manipulated statistically by means of program for statistical analysis (Microstate- software) in which the equations of the hypothesis tests, including standard deviation, T-statistics value and probabilities (P) were used.

Table 2. The effect of plant extracts on mycelial growth of *Fusarium oxysporum* (g/50 ml medium).

Treatment	Aqueous extract (CDW)			Aqueous extract (BDW)		
	0.5%	1%	2%	0.5%	1%	2%
Control	0.52 ± 0.00	0.52 ± 0.00	0.52 ± 0.00	0.52 ± 0.00	0.52 ± 0.00	0.52 ± 0.00
Cinnamomum zeylanicum (Cinnamon)	0.40 ± 0.01 (H.S)	0.33 ± 0.03 (S)	0.31 ± 0.01 (H.S)	0.52 ± 0.1 (N.S)	0.52 ± 0.1 (N.S)	0.52 ± 0.1 (N.S)
Cymbopogon proximus (Halfa barr)	0.30 ± 0.01 (H.S)	0.22 ± 0.01 (H.S)	0.13 ± 0.005 (H.S)	0.49 ± 0.03 (S)	0.48 ± 0.02 (S)	0.48 ± 0.02 (S)
Laurus nobilis (Laurel)	0.43 ± 0.02 (S)	0.40 ± 0.01 (H.S)	0.33 ± 0.01 (H.S)	0.52 ± 0.1 (N.S)	0.52 ± 0.1 (N.S)	0.52 ± 0.1 (N.S)
Persea Americana (Avocado)	0.38 ± 0.01 (H.S)	0.27 ± 0.01 (H.S)	0.19 ± 0.03 (S)	0.50 ± 0.05 (N.S)	0.49 ± 0.05 (N.S)	0.49 ± 0.05 (N.S)
Zingiber officinale (Ginger)	0.35 ± 0.01 (H.S)	0.19 ± 0.03 (S)	0.16 ± 0.005 (H.S)	0.49 ± 0.02 (S)	0.48 ± 0.03 (S)	0.48 ± 0.02 (S)

(H.S) Highly significant ($p \leq 0.01$); (S) significant ($p \leq 0.05$); and (N.S) non significant ($p > 0.05$).
The obtained data were manipulated statistically by means of program for statistical analysis (Microstate- software) in which the equations of the hypothesis tests, including standard deviation, T-statistics value and probabilities (P) were used.

tion). The absorbency was read at 235 nm using a spectrophotometer (Spekol). One unit of pectin lyase (1 U) was defined as the amount of the enzyme that releases 1 µ mol of 4,5-unsatmated digalacturonic acid per min.

Protease assay

Protease activity was determined according to Kunitz (1947) using casein (1%) as a substrate. The reaction mixture containing 1 ml of enzyme solution and 1 ml of 1% casein in 0.05 M citrate phosphate buffer, pH 6.0 was incubated at 30˚C for 20 min. The reaction was stopped with 3 ml of 10% trichloroacetic acid (TCA) and the mixture centrifuged at 5000 rpm for 10 min. The optical density of the supernatant was measured at 280 nm. One unit of protease was defined as the activity that produced an increase in optical density of 1.0 in 20 min at 280 nm.

RESULTS

The antifungal effects of the studied plant extracts [*Cymbopogon proximus* (Halfa barr), *Cinnamomum zeylanicum* (cinnamon), *Laurus nobilis* (laurel), *Persea americana* (avocado) and *Zingiber officinale* (ginger)] by either CDW or BDW on the tested fungi [*Alternaria alternata* f. sp. *lycopersici* and *Fusarium oxysporum* f. sp. *lycopersici* were compared with the control (Tables 1 and 2). The results showed that the growth inhibition of the tested fungi produced by plants extracted with CDW at concentration ranging from 0.5 - 2% were significantly different from control values, while plants extracted with BDW showed a generally non significant effect against

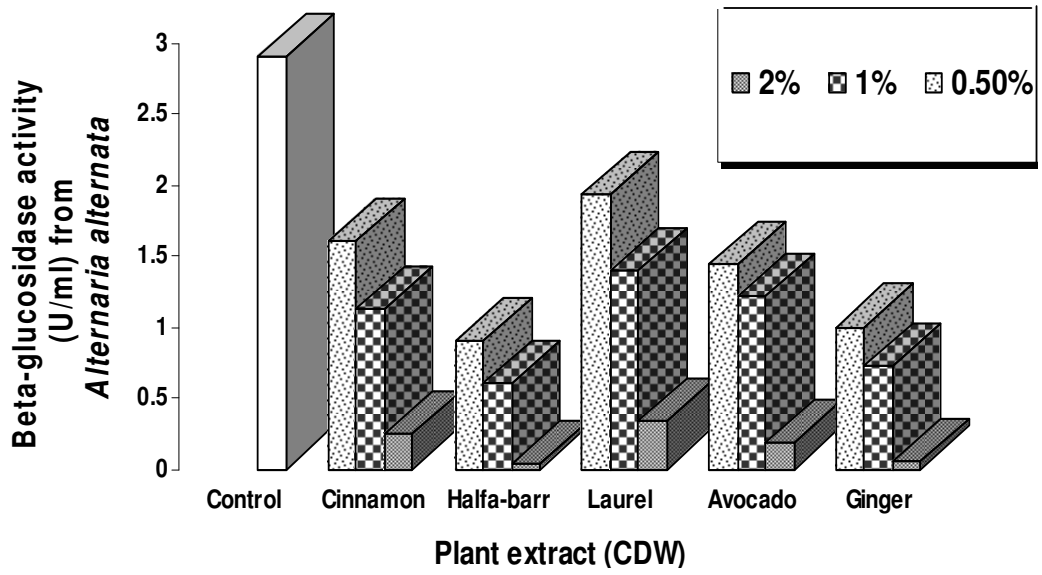

Figure 1. The effect of plant extracts (CDW) with 3 different concentrations (0.5, 1 and 2%) on the production of β-glucosidase (U/ml) from *Alternaria alternate*.

these fungi (Tables 1 and 2). These results were in agreement with Masuduzzaman et al. (2008) who observed that cold water extract showed better performance compared with boiled water extract. This may be due to a fact that some of the essential compounds for the inhibittory agent might have been spoiled during preparation of extracts in boiled water. In Table 1, the growth of *Alternaria alternata* was highly inhibited by all the tested concentrations (0.5 - 2%) of CDW extracts of *Cymbopogon proximus* (Halfa barr) 0.29 - 0.11 g/50 ml medium compared with control (0.43 g/50 ml medium), the corresponding inhibition ranging from 32.6 - 74.4%, followed by *Zingiber officinale* (ginger) 0.30 - 0.13 g/50 ml medium (30.2 - 69.8%), *Persea Americana* (avocado) 0.31 - 0.15 g/50 ml medium (27.9 - 65.1%) *Cinnamomum zeylanicum* (cinnamon) 0.35 - 0.23 g/50 ml medium (18.6 - 46.5%) and *Laurus nobilis* (laurel) 0.37 - 0.33 g/50 ml medium (14.0-23.3%). In Table 2, the growth of *Fusarium oxysporum* was highly inhibited by all the tested concentrations (0.5 - 2%) of CDW extracts of *Cymbopogon proximus* (halfa barr) 0.30 - 0.13 g/50 ml medium compared with the control (0.52 g/50 ml medium), the corresponding inhibition ranging from 42.3 - 75%, followed by *Zingiber officinale* (ginger) 0.35 - 0.16 g/50 ml medium (inhibition ranging from 32.7 - 69.2%) *Persea Americana* (avocado) 0.38 - 0.19 g/50 ml medium (inhibition ranging from 26.9 - 63.5%), *Cinnamomum zeylanicum* (Cinnamon) 0.40 - 0.31 g/50 ml medium (inhibition ranging from 23.1 - 40.4%) and *Laurus nobilis* (Laurel) 0.43 - 0.33 g/50 ml medium (inhibition ranging from 17.3 - 36.5%).

Concerning the effect of plant extract (CDW) on the production of certain essential hydrolytic enzymes from *Alternaria alternata* (β-gulcosidase, pectin lyase and protease), β-gulcosidase from *Alternaria alternata* was strongly inhibited by 2% of all tested plant extracts (88.6 - 98.9% inhibition), *Cymbopogon proximus* (halfa barr) was the most potent plant that caused strong inhibition at all the tested concentrations (0.5 - 2%) 0.90 - 0.03 U/ml compared with control (2.90 U/ml), the corresponding % of inhibition ranging from 68.97 - 98.9%, followed by ginger (65.5 - 98.3% inhibition) while avocado, cinnamon and laurel at 1 and 2% had moderate inhibitory effect on this enzyme (Figure 1). Figure 3 shows that pectin lyase from *Alternaria alternata* was completely inhibited by halfa barr and ginger at all tested concentrations (0.5 - 2%) and strongly inhibited by avocado, cinnamon and laurel (98.1 - 85.0% inhibition). Figure 5 also shows that there were strong inhibition of protease from the same fungus by halfa barr followed by ginger, avocado, cinnamon and laurel at all the tested concentrations at 0.5% (79.3 - 58.0% inhibition), at 1% (84.7 - 62.7% inhibition) and at 2% (93.3 - 84.7% inhibition).

Concerning the effect of plant extracts with CDW on the production of certain essential hydrolytic enzymes (β-gulcosidase, pectin lyase and protease) from *Fusarium oxysporum*, β-gulcosidase production was strongly inhibited by halfa barr at concentrations of 0.5 and 1% (68.0 and 82.3 % inhibition, respectively) followed by ginger at the same concentrations (62.3 and 76.0% inhibition, respectively), while it was completely inhibited by the 2 extracts at 2% concentration (Figure 2). Avocado at 2% had a strong inhibitory effect (91.7%), while cinnamon and laurel had a moderate inhibitory effect. Figure 4 shows that pectin lyase was completely inhibited by halfa barr at all the tested concentrations and strongly inhibited by ginger (92.8 - 97.6%) and avocado (78.8 - 95.2%), followed by cinnamon and laurel. The effect of plant extracts on protease production was similar to that showed

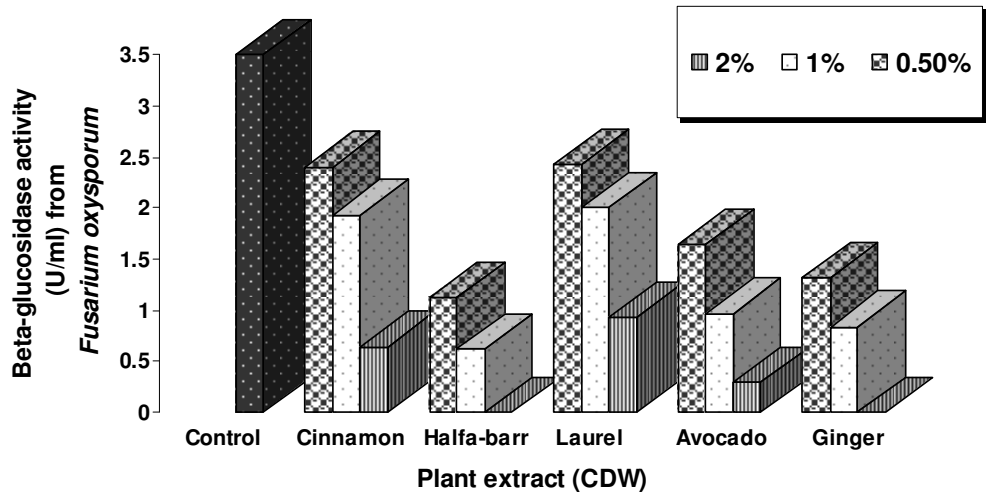

Figure 2. The effect of plant extracts (CDW) with 3 different concentrations (0.5, 1 and 2%) on the production of β-glucosidase (U/ml) from *Fusarium oxysporum*.

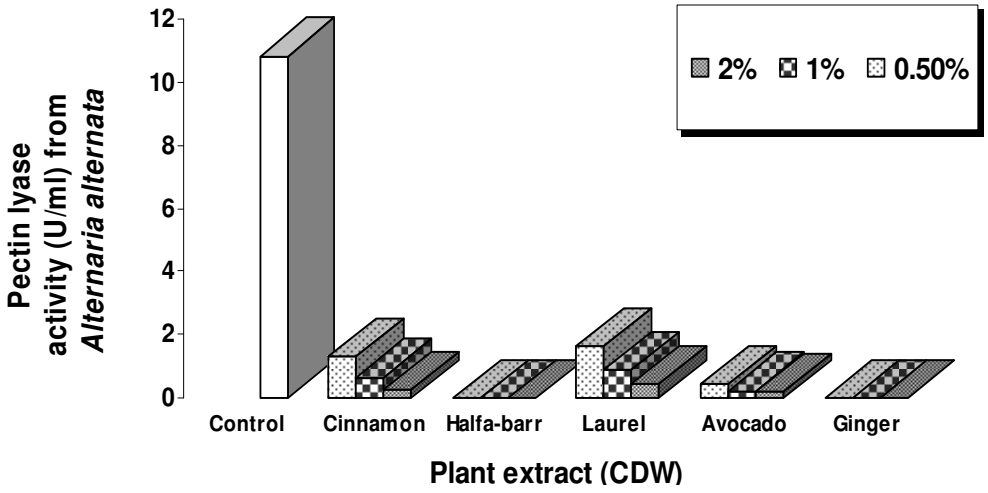

Figure 3. The effect of plant extracts (CDW) with 3 different concentrations (0.5, 1 and 2%) on the production of pectin lyase (U/ml) from *Alternaria alternate*.

on β- gulcosidase production except that at 2% concentration of halfa barr and ginger, the protease was not completely inhibited (Figure 6).

DISCUSSION

According to the results of Tables 1 and 2, extracts of halfa barr and ginger were the most effective to inhibit the growth of the tested fungi followed by avocado, cinnamon and laurel. The present results are in conformity with other studies, where halfa barr was shown to be the most potent among many tested plant extracts against *Fusarium verticillioides* and *Aspergillus flavus* (El-Assiuty et al., 2006). Proximadiol, 5α-hydroxy-β-eudesmol, 1β-hydroxy-β-eudesmol, 1β-hydroxy-α-eudesmol, 5α-hydroperoxy-β-eudesmol and 7α,11-dihydroxycadin-10(14)-ene were isolated from the unsaponifiable fraction of extract of *Cymbopogon proximus.* These components were shown to be powerful antimicrobial agents (El-Askary et al., 2003). Abd Malek et al., (2005) investigated several essential oils from the fresh rhizomes of *Zingiber officinale* which had high inhibitory effect on fungal growth. The major components of the rhizome oils were found to be zingiberene (16.70%), (E,E)-α-farnesene (13.10%) and geranial (7.60%) Sharma et al., (2002). Simi et al. (2004) stated that the essential oil of *cinnamon* showed more antifungal activity than the essential oil of laurel. The present study showed that halfa barr was the most efficient and it might be a promising material to control

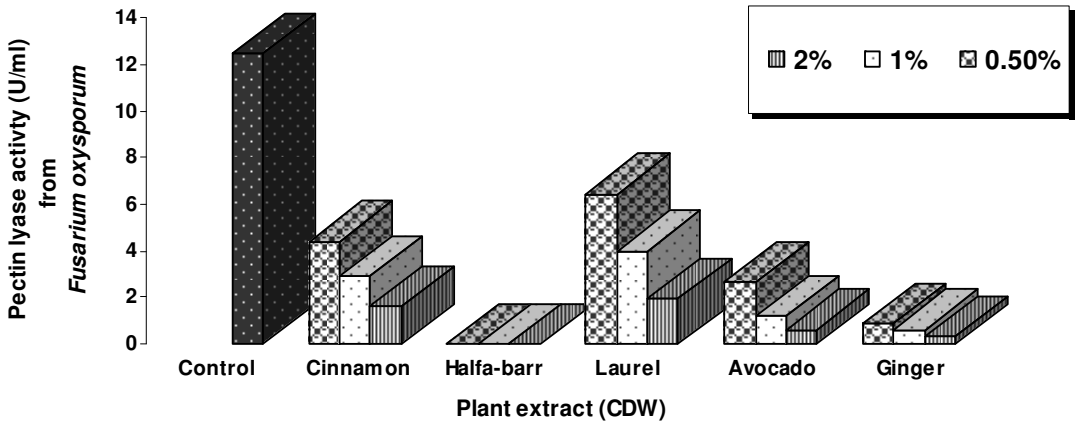

Figure 4. The effect of plant extracts (CDW) with 3 different concentrations (0.5, 1 and 2%) on the production of pectin lyase (U/ml) from *Fusarium oxysporum.*

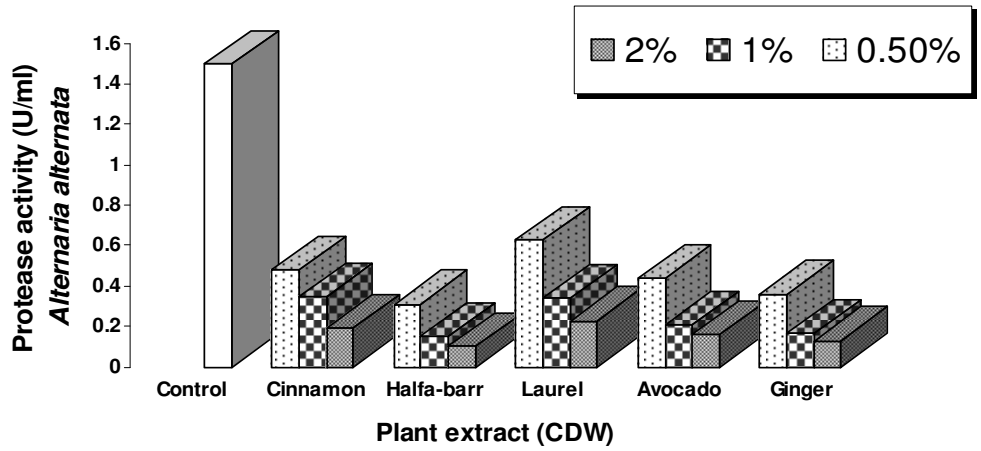

Figure 5. The effect of plant extracts (CDW) with 3 different concentrations (0.5, 1 and 2%) on the production of protease (U/ml) from *Alternaria alternate.*

the studied fungi. It can be concluded that to reduce the dependence on the synthetic fungicides as well as to decrease the higher production costs, plant extracts especially halfa barr and ginger may be used as natural fungicides to control some pathogens in the field.

Concerning the effect of plant extract (CDW) on the production of certain essential hydrolytic enzymes (β-gulcosidase, pectin lyase and protease) from *Alternaria alternata* (Figures 1, 3, 5) and *Fusarium oxysporum* (Figures 2, 4, 6), many pathogenic fungi possess a definite or specific infection pattern in order to infect the host plant. This is usually performed by the synthesis of a characteristic set of polymer-degrading enzymes; cellulases and pectin-degrading enzymes to enable successful establishment of the host-pathogen relationship. If, as in most cases, multiple forms of cell wall- degrading enzymes are formed by the pathogen (Mendgen and deising, 1993), we tried in this work to find a strong inhibitory effect from natural plant extracts which can suppress the mode of action of these dangerous enzymes. Our results were in agreement with that of Bell et al. (1965) who stated that the water-soluble substances isolated from the leaves of 7 plant species [*Lespedeza cuneata* (Sericea), *Vitis rotundifolia* (Muscadine grape), *Diospyros virginiana* (Persimmon), *Cornus florida* (Dogwood), *Rubus strigosus* (Red raspberry), *Rubus occidentalis* (Black raspberry), *Rose odorata* (Rose)] were shown to inhibit the fungal hydrolytic enzymes, pectinase and cellulase. Also, Masuduzzaman et al. (2008) stated that the *Allamanda* leaf extract and separated compounds were shown to possess different inhibitory effects against *Phomopsis vexans, Fusarium sp, Rhizoctina solani, Sclerotiurn rolfsii* and *Phytophthora capsic* and their hydrolytic enzymes.

In conclusion, the findings in this study confirmed that plant extracts can be used as natural fungicides to control pathogenic fungi and thus reduce the dependence on the synthetic fungicides. Halfa barr was found to be the most

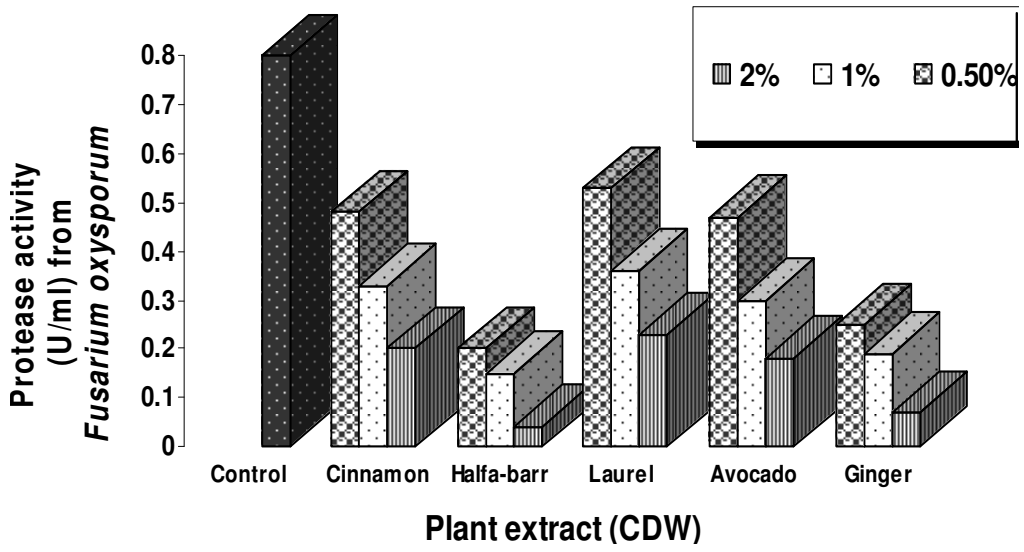

Figure 6. The effect of plant extracts (CDW) with 3 different concentrations (0.5, 1 and 2%) on the production of protease (U/ml) from *Fusarium oxysporum.*

efficient (75% inhibition) and it might be a promising material for controlling these fungi. Finally, this study is only a preliminary one. More studies are still needed in the future to test the antifungal activities of the studied plant extracts on other different fungi.

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