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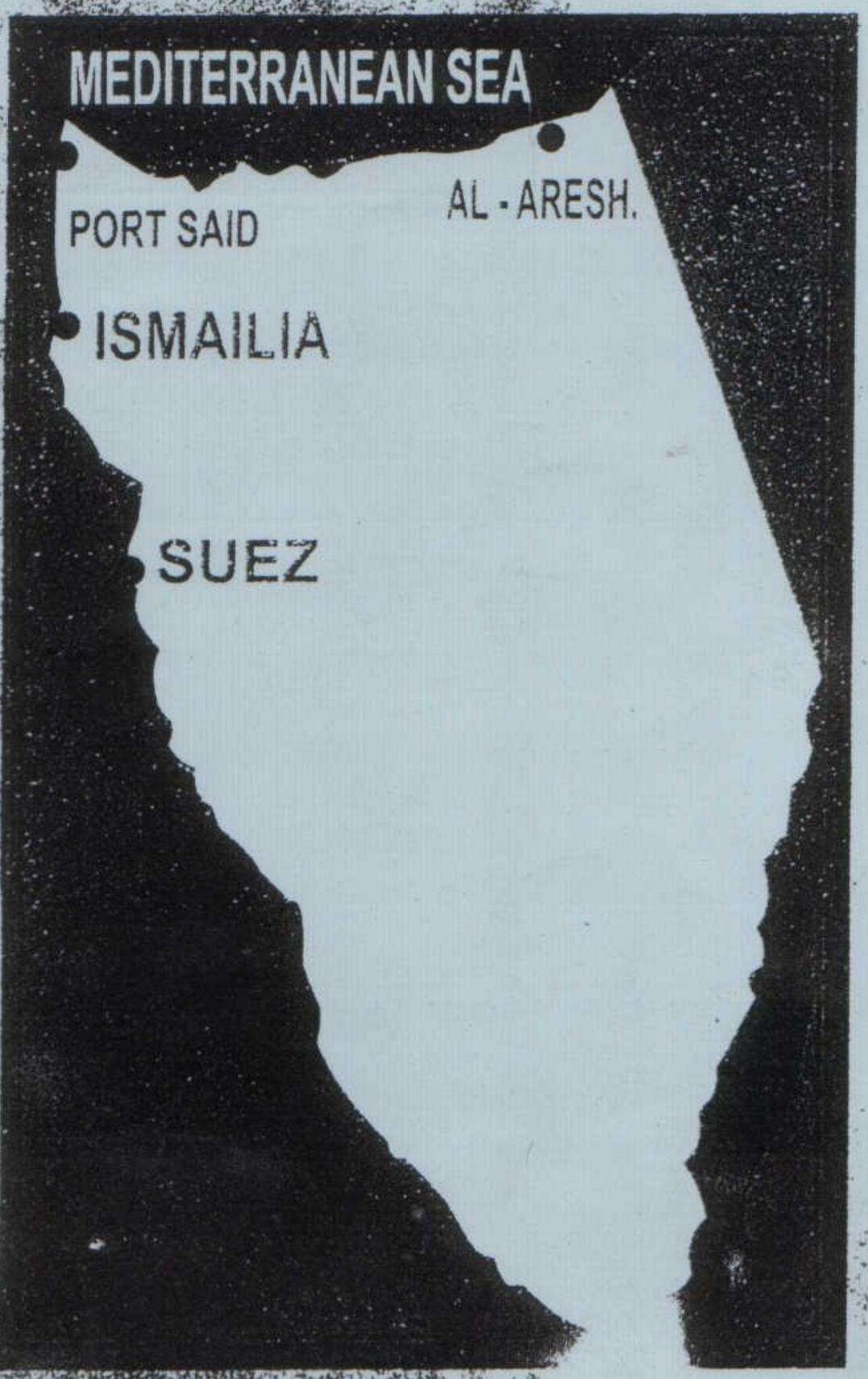

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RELATINSHIP BETWEEN CHEMICAL COMPOSITION AND INHIBITORY EFFECT OF SOME PLANT EXTRACTS ON FOOD-CONTAMINATING FUNGI

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ABSTRACT

Two different plants collected from Sinai namely *Olea europea* and *Cleome droserifolia* are chemically analyzed. Both hydroalcoholic and mother liquor extracts are prepared from each plant and analyzed for certain phenolic compounds such as flavonoids, saponins, tannins and terpens. Alkaloids and resins are also detected in tested extracts. The antifungal activity of the two prepared extracts is also investigated against food-contaminating fungi. The tested fungi are *Rhizopus nigricans*, *Aspergillus niger*, *Penicillium expansum*, *Potrytis cinerea* and *Aspergillus fumigatus*.

The obtained results revealed that both of the two tested extracts have great effect on examined fungi. The hydroalcoholic extract exhibit pronounced antifungal activity than that caused by the mother liquor. The hydroalcoholic extract showed to be fungicidal material while the mother liquor is fungistatic agent. The measured values of the minimum inhibitory concentrations and the minimum fungicidal concentrations are found between 700-1350 µg/ml for the two tested extracts. The percentages of growth diameter inhibition of tested fungi in solid medium are found between 4.9-18.2% and 14.1-29.6% for *Cleome droserifolia* and *Olea europea*, respectively. The inhibition percentages are found less when using liquid cultures since the reduction (%) of the fungal growth are found between 1.4-12.5% for *Cleome droserifolia* while it is about 8.8-22.8% in case of *Olea europea*.

INTRODUCTION

The contribution of biotechnology in our life have opened the doors to new possibilities in antimicrobial preparations whereby only certain constituents of the plants extract will be utilized. Fungal growth on cheese, to a lesser extent, and other fermented dairy products is a problem for the cheese manufacturer during aging and for retailer and consumer, as well, during refrigerated storage. Fungi cause major economic problems and some of these fungi are capable to produce toxic metabolites, therefore the inhibition of fungal growth is of significant importance. Because of the active natural products having active antimicrobial effects are more specific in their actions, readily biodegradable and have lower, if not, harmful effects on the consumer health than the synthetic chemicals. Therefore, the world now is returning to the use of natural products both in food industries and in the field of medicine. Because many plants produce secondary metabolites which are important sources of pesticides, microbicides, and pharmaceutical drugs as well, hence many authors pointed out that plants can be useful in the treatment and/or control of human, animal and plant diseases. Some of these products have been tested for their antimicrobial properties against, viruses (Nakayama *et al*., 1990), fungi (Ansari and Shrivastava, 1991), and bacteria (Diker *et al*., 1991). Heisey and Gorham (1992) found that the extracts of 54 plant species have the ability to inhibit the growth of yeast, i.e. *Candida albicans*; bacteria, i.e. *Streptococcus mutans*; and fungi, i.e. *Trichophyton rubrum*. *Cleome droserifolia* (Sammwa) is a wild plant found all over the desert of Egypt. Ten methylated flavonoids from aerial parts of four *Cleome droserifolia* species were isolated (Sharaf *et al*., 1992). Yang *et al*. (1990) identified five flavonoids from the aerial parts of *Cleome droserifolia* grown in Egypt. *Olea europea* (Olive) is known also in Egypt. The inhibitory effect of different extracts of these two plants were examined against some bacterial strains of three bacterial families, *Enterobacteriaceae*, *Bacillaceae* and *Micrococcaceae* (El-Fadaly *et al*., 1997).The aim of this work was to determine whether the extracts of tested plants collected from the Sinai inhibit the growth of fungal strains having economic importance in food spoilage. In addition, the effect of these extracts, either fungistatic or fungicidal action was also distinguished.

MATERIALS AND METHODS

I. Used materials:

Plant samples:

The aerial parts of *Cleome droserifolia* (Forssk) Del. (Cleomaceae), which refers as Sammwa and the leaves of *Olea europea* Linn. (Oleaceae), which is known as olive were collected from south and north of Sinai, respectively. These plant samples were air-dried, milled, kept in a polyethylene bags and preserved in deep freezer till use.

Fungal strains and cultivation media:

The food-contaminating fungi used in this investigation were taken from Prof. Dr. Reichenbach, Research Group on Microbial Secondary Metabolites, Division of Microbiology, GBF, Braundschweig, Stockheim, Germany.Potato Dextrose Agar (PDA) was used for maintaining the fungal cultures at 5°C till use. For the disc diffusion method, linear growth of fungi and determination of minimum fungicidal concentrations (MFCs), Czapek-Dox's agar medium was used. To determine the minimum inhibitory concentrations (MICs) values, double strength of Czapek-Dox's solution was used.

II. Chemical analysis:

Chemical and phytochemical analysis of the plants :

Moisture, ash, minerals, crude fiber, crude protein and crude lipids were determined according to the methods of A.O.A.C. (1990). Total carbohydrates and total soluble sugars were determined according to Mgnetski *et al.* (1959). Tannins, terpenes, alkaloides, saponins, flavonoids, glycosides, cardenolides and resins were detected after El-Badrawy (1996).

Extraction of the hydroalcoholic extract:

A weight of 100 grams of each milled plant sample was macerated in 500 ml of methanol overnight at room temperature, then filtered and the methanolic crude extract was collected. Another portion of 500 ml of methanol was added to the plant residue and boiled for two hours under refluxing and then filtered. In the same manner a volume of 500 ml portion of methanol : water (1:1) mixture was added to the residue and left overnight at room temperature, and filtered. The plant residue was finally boiled with 500 ml of methanol : water (1:1) solvent and filtered. All the obtained filtrates were collected together to form the hydro-alcoholic extract, which was subjected to rotary evaporator to remove the solvents. The obtained crude extract was studied for its antifungal activity.

Successive extraction of hydroalcoholic extract with organic solvents:

Fifty gram of each hydroalcoholic extract was subjected to a sequence of successive extractions by using selective organic solvents according to their polarities as follows; petroleum ether (60-80°C), diethyl ether, ethyl acetate, and butanol. The residue after each extraction was re-extracted with the next solvent. The remaining aqueous residue, which refers as mother liquor was studied for its antifungal activities.

III. Microbiological procedures:

Inoculum preparation:

The fungi used in this study were grown on slopes of potato dextrose agar (PDA) at 28°C for 7 days. The culture was then washed with 0.1% peptone solution to prepare spores suspension. The latter was diluted further to obtain about 10^4 - 10^5 spores/ml to inoculate the solid medium in Petri dishes. One ml of this dilution was added to 14 ml of melted solid Czapek-Dox's medium, poured into Petri dishes and incubated at 28°C for 7 days to be used to inoculate the liquid culture.

Determination of the Minimum Inhibitory Concentrations (MICs):

The MIC value for each representative fungal strain was examined in liquid medium amended with the test extract using a step-wise broth method as described by Fitzgerald *et al.* (1992). After 7 days incubation, the test tube in which no growth can be recorded should contain the lowest inhibiting concentration of the tested extract. Three replicates were prepared for each fungal strain. To verify the nature of the effect of tested extracts if temporary or permanent, appropriate subculturing from MIC tubes were applied on plates of Czapek-Dox's agar medium without plant extract. After an incubation period of 7 days, it was possible to determine the minimal fungicidal (FMC) and/or fungistatic concentration (Gardner and Provine, 1984).

Measurements of inhibition zone:

Holes were punched with a cork borer (6 mm) in Czapek-Dox's agar medium plates freshly seeded with 1 ml of standard inoculum of fungal spores for each strain as mentioned above. The holes were then filled with MIC values of the tested extract of the two plants examined. The plates were kept at 5°C for one hr to allow diffusion of the extract through the agar media. After incubation for 7 days at 28°C, the diameter of clear zones were measured and recorded. The plates rechecked thereafter daily up to one week to make sure that no further growth was appeared in the area of clear zones. The antifungal activity was further expressed in term of the diameter of inhibition zone surrounding the well (Collins and Lyne, 1985). Similarly, the control prepared with the same solvent (tested material free) which did not inhibit any of the fungi tested.

Effect of plant extracts on fungal growth:

In solid medium, discs of 0.6 cm in diameter were cut off the edge of 7 days old cultures of the tested fungi and a single disc was placed in the center of a Petri dish containing Czapek-Dox's medium supplemented with the tested extract by half value of MIC. Three replicates were prepared for each fungal strain tested, which then incubated at 28°C. The linear growth of the tested fungi was recorded after the 7^{th} day. Control devoided of the tested extract for each treatment was conducted (Bollen, 1972).In liquid culture, sterile

Czapek-Dox's medium amended with a half value of MIC of each extract was prepared for all fungi tested. Medium was dispensed in 250 ml Erlenmeyer flasks of 50 ml aliquots per each flask in triplicates. Medium was used without additional extracts as control. Each flask was inoculated with a standard inoculum of one disc of 0.6 cm in diameter of 7 days old Petri dishes of fungal cultures. The flasks were then statically

incubated at 28°C for 7 days after which, the produced mycelial mat were filtered and washed twice with distilled water, dried
in an oven at 80°C to a constant weight (Ansari and Shrivastava, 1991). The fungal growth expressed as mycelium dry weight (MDW).

RESULTS AND DISCUSSION

I. Chemical and phytochemical analysis:

It is obvious from Table (1) that *Cleome droserifolia* has higher content of moisture, ash, fiber and carbohydrates than that of *Olea europea* sample. On the other hand, the higher levels of total protein and lipids are found in *Olea europea* sample. It is also observed from the same Table that the leaves of *Olea europea* have higher levels of potassium, zinc and cadmium than that of *Cleome droserifolia*. The levels of sodium, iron and manganese are higher in *Cleome droserifolia*, which has more than five times of iron.
From Table (2), it could be noticed that hydroalcoholic extract of the two samples contains flavonoids, saponins, tannins, terpens, alkaloids and resins. On the other hand, only saponins and tannins were detected in the mother liquor extract of the two plant samples.

Table 1. Chemical analysis and mineral content of the tested plants.

	Chemical analysis (g/100d dry weight)					
	Moisture	Ash	Fiber	Protein	Lipids	Carbohydrates
Olea europea	6.14	6.24	24.2	9.7	4.5	15.7
Cleome droserifolia	12.6	12.08	28.1	8.6	3.8	21.7

	Mineral content (mg/100g dry weight)							
	P	K	Na	Fe	Zn	Mn	Cu	Cd
Olea europea	250	241	32	31	13	2.5	0.41	0.39
Cleome droserifolia	252	161	84	175	11	4.1	0.44	0.12

Table 2. Phytochemical screening of the prepared extracts.

Constituents	*Olea europea*		*Cleome droserifolia*	
	Hydroalcoholic	M. liquor	Hydroalcoholic	M. liquor
Flavonoids	+	-	+	-
Saponins	+	+	+	+
Tannins	+	+	+	+
Terpens	+	-	+	-
Alkaloids	+	-	+	-
Resins	+	-	+	-

II. Values of MICs:

Data listed in Table (3) show more precisely the inhibiting effect of tested extracts by means of MICs expressed in μg ml^{-1}. Tabulated results represent the lowest concentration of the extract which is capable of totally inhibiting the fungal growth. The hydroalcoholic extract of the two tested plants prove to be more effective on fungal growth than the mother liquor. The number of MICs values appear to be less in case of *Olea europea* than that of *Cleome droserifolia* with *Aspergillus niger, Aspergillus fumigatus* and *Potrytis cinerea*. Identical number of MICs values are obtained with both *Rhizopus nigricans* and *Penicillium expansum*. From MICs, it could be noted that the relative order of the fungal sensitivity towards the two tested plants is *Aspergillus niger, Aspergillus fumigatus, Penicillium expansum* and *Potrytis cinerea*.
The inhibitory effect of these extracts of the two tested plants may be due to the presence of some native compounds which are extracted by the solvent used. The intracellular content of Na^+ in bacteria is chiefly maintained by a tightly coupled Na^+/H^+ exchange system which can be blocked and consequently alters the cellular pH which in turn prevents proliferation of cells (Giunta *et al.*, 1984). The pronounced antifungal activity of these plants could be attributed either partially or completely to the presence of some minerals as found by the chemical analysis (Table 1).

III. Fungicidal and fungistatic (MFC):

Results recorded in Table (3) indicate that the hydroalcoholic extract of the two tested plants have fungicidal effect against all tested fungi. These data, however, reveal that the extract kill fungi, therefore, they have an irreversible and permanent effect. Meanwhile, the mother liquor have fungistatic action against the fungal strains. This is clearly obvious since the fungal growth is detected after reseeding samples of the MICs tubes on the plates containing plant extract-free medium. The explanation of this phenomenon is that the concentration of plant extract inhibit the growth but after the fungi can persists and resume again the growth once the agent is removed in spite of the value of MIC of each fungus.

Table 3. Values of minimum inhibitory concentrations and corresponding values of diameter of growth inhibition of food-contaminating fungi as affected by the two tested extracts of investigated plants.

Tested Fungi		*Olea europea*			*Cleome droserifolia*		
		MIC	MFC	DIZ	MIC	MFC	DIZ
Aspergillus niger	H	700	-	12.4	800	-	14.5
	M	800	+	9.8	950	+	9.2
Aspergillus fumigatus	H	700	-	14.5	800	-	16.3
	M	800	+	10.6	950	+	10.6
Rhizopus nigricans	H	950	-	16.2	950	-	18.2
	M	1100	+	10.4	1100	+	12.8
Penicillium expansum	H	1000	-	18.6	1000	-	12.6
	M	1250	+	10.2	1250	+	8.4
Potrytis cinerea.	H	1100	-	16.8	1200	-	12.2
	M	1300	+	10.7	1350	+	8.9

MIC: Minimum inhibitory concentration, μg ml^{-1}.
MFC: Minimum fungicidal concentration, μg ml^{-1}.
DIZ: Diameter of inhibition zone of growth, mm.

A K^+/Na^+ antiporter which posses can cause marked changes in the intracellular concentrations of sodium and potassium in these microorganisms (Giunta *et al.*, 1986). The measuring of MICs and MFCs values lead to detect the real effect of the extract *in vivo* against the microorganisms (Helena and Per-Anders, 1993). The tested plants have both K and Na in different concentration as can be seen in Table (1).
The superiority of the hydroalcoholic extracts of the two tested plants over the mother liquor may be due to the presence of some certain compounds such as flavonoids, terpens, alkaloids and resins as shown in Table (2).

IV. Values of inhibition zone:

The antifungal activities of the two tested extracts of the two plants are also examined by measuring the diameter of inhibition zones. Obtained results are also recorded in Table (3). Examined fungi show different susceptibility against tested extracts. Data prove that the hydroalcoholic extract of the two tested plants are more effective than the mother liquor. This is clear by the large diameter of inhibition zones measured by using solid medium. Recorded results also illustrated that the efficiency of the solvents used in extracting antifungal substances differed with the plant materials (Yong Long and Mabry, 1981). Paster *et al.* (1988) stated that the presence of caffeic acid and catechin in olive plants lead to the reduction of bacterial count to the extent of being non-detectable. Fleming *et al.* (1973) reported that green olive fruit extracts contain large quantities of phenols which showed antimicrobial activity.

V. Evaluation of the effect of plant extracts on the fungal growth:

Following the linear growth of tested fungi grown on solid medium, the inhibitory effect of hydroalcoholic and mother liquor extracts in cultivation media supplemented with the half value of MIC is examined. All tested fungi show similar growth starting point as results of the presence of the extract since they all exhibited initially no growth up to the 3rd day of incubation. This period may be explained as an adaptation period required for the fungal growth in a micro-environment containing antifungal agent. After that, poor vegetative growth is observed which normally go further up to the 9th day to give measurable growth. Results obtained of measured growth are recorded in Tables (4) for the two tested plants . The efficiency of these extracts on fungal growth inhibition are also calculated and listed in the same Tables. Paster *et al.* (1990) found that 400 μg/ml of origanum oil lead to complete prevention of the growth of either *Aspergillus niger* or *Aspergillus flavus*.

Table (4): Percentages radial growth inhibition of the tested fungi as affected by the two extracts of the examined plants after 10 days incubation in solid medium.

Tested fingi		*Olea europea*				*Cleome droserifolia*			
		Tested extract conc. (µg/ml)	Growth diameter (cm)			Tested extract conc. (µg/ml)	Growth diameter (cm)		
			Cont.	Treated	%		Cont.	Treated	%
Aspergillus niger	H	350	8.5	6.8	20.0	400	8.5	7.0	17.7
	M	400	8.5	7.3	14.1	475	8.5	7.2	15.3
Aspergillus fumigatus	H	350	8.2	6.2	24.4	400	8.2	7.4	9.8
	M	400	8.2	6.8	17.1	475	8.2	7.8	4.9
Rhizopus nigricans	H	475	7.4	5.8	21.6	475	7.4	6.2	16.2
	M	550	7.4	6.1	17.6	550	7.4	6.8	8.1
Penicillium expansum	H	500	8.8	6.2	29.6	500	8.8	7.2	18.2
	M	625	8.8	6.7	23.9	625	8.8	7.6	13.6
Potrytis cinerea	H	550	6.8	5.1	25.0	600	6.8	5.9	13.2
	M	675	6.8	5.4	20.6	650	6.8	6.2	8.8

The inhibition effect of tested extracts are also investigated for the fungal growth in liquid culture. The liquid culture containing the half concentration of MICs of each extract required for each fungus. Obtained results are recorded in Table (5) for *the two tested plants*. Tabulated data prove that the extracts of *Olea europea* are much higher in their effect on all tested fungi than that obtained by the extracts of *Cleome droserifolia*. At the same time the hydroalcoholic extract is superior over the mother liquor in its effect for the two tested plants. It could be concluded that the hydroalcoholic extract and mother liquor of the two tested plant are good inhibitors both as fungicidal factor and fungistatic agent, respectively. So, these results suggest the use of these obtained materials in preventing the growth of food-contaminating fungi.

Table (5): Reduction percentages of mycelial growth of the tested fungal strains as a results of examined extract addition after 10 days incubation in liquid culture.

Tested fingi		*Olea europea*				*Cleome droserifolia*			
		Tested extract conc. (µg/ml)	MDW			Tested extract conc. (µg/ml)	MDW		
			Cont.	Treated	%		Cont.	Treated	%
Aspergillus niger	H	350	180.0	152.3	15.4	400	180.0	160.5	10.00
	M	400	180.0	160.8	10.7	475	180.0	167.2	7.1
Aspergillus fumigatus	H	350	195.5	156.5	19.9	400	195.5	182.3	6.8
	M	400	195.5	165.3	15.5	475	195.5	186.4	4.7
Rhizopus nigricans	H	475	170.6	145.9	14.5	475	170.6	159.6	6.5
	M	550	170.6	155.6	8.8	550	170.6	168.2	1.4
Penicillium expansum	H	500	205.8	175.9	14.5	500	205.8	188.7	8.3
	M	625	205.8	182.3	11.4	625	205.8	192.6	6.4
Potrytis cinerea	H	550	220.9	170.6	22.8	600	220.9	193.3	12.5
	M	675	220.9	185.4	16.1	650	220.9	198.2	10.3

MDW: Mycelial dry weight mg/50 ml of cultivation medium.

Since the plant samples used in this study are collected from uncultivated areas (untreated soil), therefore the antagonistic actions obtained are attributed to the extracted plant constituents, since these plants are free from any chemical residues. So, these results can suggest the possibility to use tested plant, either in the agricultural field or in the field of food processing after obtaining high purity antimicrobial substances. Finally, since the risk of residues left in the foodstuffs limits the use of antibiotic agents, only a restricted number of chemicals (non-antibiotic drugs) have been approved now as widely accepted safe materials (Helena and Per-Anders, 1993). The same problem have been discussed in the case of crops and seeds because of the need to use preservative chemicals to prolong the storage period. Consequently, efforts have been made to evaluate the preservative action of natural substances, e.g. plant constituents or plant extracts.

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الملخص العربى

العلاقة بين التركيب الكيماوى والتأثير التثبيطي لبعض المستخلصات النباتية على الفطريات الملوثة للأغذية

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أخذ نباتين من منطقة شمال سيناء أحدهما الزيتون والأخر يسمى بالسموة وتم تحليلهما كيماويا . تم تحضير المستخلص المائى الكحولىوالمستخلص الرئيسى من كل منهما وتم تحليلهما من حيث المركبات الفينولية مثل الفلافونويدز والصبونين والتانين والتربينز . كذلك تم الكشف عن الألكالويدزوالريزينز كذلك تم الكشف عن النشاط المضاد للنمو الفطرى باستخدام الفطريات الملوثة للأغذية مثل :

Rhizopus nigricans, Aspergillus niger, Pencillium expansum , Potrytis cinerea and Aspergillus fumigatus

أثبتت النتائج المتحصل عليها أن كل من المستخلصين كان لهم تأثير مثبط قوى على نمو الفطريات المستخدمة .للمستخلص المائى الكحولى تأثير مضاد للفطريات أكثر مما سببه المستخلص الرئيسى كذلك بين المستخلص المائى الكحولى أن له تأثير مميت على الفطريات المختبرة بينما المستخلص الرئيسى كان له تأثير مثبط فقط على النمو الفطرى . وقد تم تقدير أقل تركيز مثبط وأقل تركيز مميت للمستخلصات المستخدمة التى تراوحت بين ٧٠٠-١٣٥٠ ميكروجرام /مل لكل من المستخلصين تحت الدراسة . وجدت النسبة المئوية لقطر هالة التثبيط للفطريات المختبرة فى بيئات صلبة تتراوح بين ٤,٩-١٨,٢% , ١٤,١-٢٩,٦% لكل من السموة والزيتون على الترتيب كانت النسبة المئوية للتثبيط أقل عند استخدام البيئات السائلة حيث كانت النسبة المئوية لتثبيط النمو الفطرى بين ١,٤-١٢,٥% لنبات السموة بينما كانت٨,٨ -٢٢,٨% فى حالة نبات الزيتون