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Evaluation of the antimycotic activity of some plant extracts on the predominant fungal strains isolated from Ras cheese surface

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ABSTRACT

Crude extracts of 24 kinds of herbs and spices were screened *in vitro* for their antimycotic activities against nine mould and two yeast strains commonly found on Ras cheese surface. The primary screening using aqueous and acetonic extracts showed high antifungal activity of caraway, cinnamon, cumin, fenugreek, galangal, marjoram and miswak extracts. Ethanolic extract of these plants in addition to ambrosia and rosemary were further investigated for its antimycotic activities. The results showed a dose-dependent effect and high correlations (0.8-1.0) between the antifungal effects of these extracts and its concentration in the culture media. The examined extracts (0.3%) resulted in 88-100% of the antifungal activity of control (50 ppm delvocid). A prominent and maximum effect of cinnamon at concentration of (0.2%) was obtained. Among the examined fungi strains; *Aspergillus* spp., *Penicillium* spp. and *Saccharomyces cerevisiae* were more sensitive to the tested extracts. These findings strengthen the possibility of using some ethanolic extracts of the tested plants as an alternative to chemicals to prevent the fungal growth on Ras cheese surface. However further studies on Ras cheese treatment remain necessary.

Key words: Herbs, spices, Ras cheese, antimycotic activity, Delvocid.

INTRODUCTION

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Ras cheese is the main Egyptian hard cheese that is rather similar to the Greek, Kefalotyri. It is probable that the basic Ras cheese originated in the Balkans, then originated in Egypt during the early stage of the Egyptian industrial renaissance after 1878 (Abou-Donia, 2002). This cheese under certain circumstances is liable to become contaminated with moulds and their metabolites. In general, Ras cheese is an excellent substrate for mould growth during ripening and storage especially under the uncontrolled hygienic conditions in the small cheese pilots that widely spread in different rural regions in Egypt. There is no doubt that fungi growth creates great problems in Ras cheese industry, not only for the economical losses through the development of discoloration, poor appearance and off-flavours in cheese, but also for its public health hazards. The risk of eating such contaminating cheese results from the production of toxic metabolites known as mycotoxins, which may cause dangerous acute and chronic diseases to consumers (Edwards, 1973 and Bajaj and Ghosh, 1975). Therefore, prevention of fungi growth is a matter of challenge to the cheese producers; they commonly use either antifungal preservatives such as weak acids (sorbic acid) or antibiotics (natamycin) to inhibit fungi growth in cheese or on its surface. Sorbates tends to have an adverse effect on the appearance quality of the cheese, as well as their potency is not constant against all mould strains (Lueck and Remmert, 1980). On the other hand, objections were raised against the use of natamycin (delvocid) as it leads to the development of mould strains resistance to the permitted level of natamycin (Brul and Coote, 1997) beside its relatively high price. Therefore, the use of natural preservatives to control mould spoilage in cheese has been studied by many researchers in the recent years. Plants contain a vast array of secondary metabolite with different biotechnological and antifungal applications. Well known examples of these compounds include flavonoids, phenols, phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenie glycosides and glucosinolates. The use of different extracts of herbs and spices for controlling fungi growth in Ras cheese and other sour cheeses such as Labneh cheese were the target of many studies (Hassan and El-Deeb, 1988, Abou Dawood, 1996, 1999 and 2002, Abdel-Kader, *et al*., 2001 and Zommara and Rashed, 2005). The inconsistent results for the antimycotic activity of some plants among studies may be attributed to differences in the composition of plant extract due to agronomic conditions and harvest time that make the comparison between different studies more difficult (Mishra and Dubey, 1994 and Guillén and Cabo, 1996). The present study was designed to screen the

antifungal activity of 24 kinds of herbs and spices using the disc diffusion assay method, the most widely used method to determine microbial growth inhibition *in vitro*. The antifungal effect of water, acetone and ethanol extracts of the plant materials were investigated on the most predominant fungal strains commonly isolated from Ras cheese surface.

MATERIALS AND METHODS

Materials investigated for their antifungal activity:

Twenty-four kinds of plants, spices and herbs (Table1) were obtained from the local market at Alexandria city and the botany farm of the Faculty of Agriculture, Kafr El-Sheikh University. All plant materials were dried and ground to fine powders before use in a high-speed micro mill. Extractions of water, acetone (80 %) and ethanol (80 %) of each plant material (100 g) were carried out as described by Scott and Mckibben, (1978). The extracts were filtered through 0.45 µm filter (Nalgen Aerican filter) for sterilization. The concentration of total soluble solid (TSS) in the ethanolic extracts were determined using a Reffractometer (RR 12, NRO 5116, Poland). Delvocid (Gist brodcades, NV, Holland) was used in a concentration of 50 ppm as a control (Nilson*, et al.,* 1975).

Fungi strains:

Isolates of fungal strains of *Penicillium camemberti* (*P. camemberti*), *Penicillium roqueforti* (*P. roqueforti*), *Aspergillus flavus* (*A. flavus*), *Aspergillus parasiticus* (*A. parasiticus*), *Aspergillus niger* (*A. niger*), *Mucor spp., Fusarium reseom* (*F. reseom*), *Cladosporium herbarum* (*C. herbarum*), *Alternaria tenins* (*Alt. tenins*), *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Geotrichum candidum* (*G. candidum*) were previously isolated and used in this study.

Determination the antimycotic activity:

The paper disc diffusion assay was used for *in vitro* determination of the antimycotic activity of the extracts on basis of hyphal radial growth rate of filamentous moulds and colony formation of yeasts in the presence or absence of the extracts. Filamentous mould or yeast cultures were separately inoculated in 250 ml flasks with 100 ml sterilized PDA medium according to Difco (1983). Then, it was poured to Petri dishes (9 cm) and set on a straight surface allowing the media to solidify. A 5 mm diameter filter paper disc (inhibitor free) was dipped into

each extract by means of clean, sterilized dry forceps. Excess fluid was removed from the disc by touching it against the side of the flask. The disc was placed on the surface in the center of a Petri dish contained the PDA medium inoculated with approximately 10^6 spores or cfu /ml for moulds and yeasts, respectively. A sample was considered to contain a fungi growth inhibitory substance when a clear zone was formed around the disc. The diameter of the clear zone is proportional to the antimycotic activity of a treatment. The diameter of the inhibition zone around the discs was measured after incubation at 26ºC for 48 and 24 hours for moulds and yeasts, respectively. The values were expressed in millimeters for the mean of 5 replicates for each tested sample according to Quiroga *et al.,* (2001). The radial growth of mycelia in all plates was measured and the percentage of growth inhibition was calculated from the mean values as maintained by (Reyes Chilpa *et al.,* 1997).

$$\text{Inhibition \%} = \frac{\text{Mycelia growth in control - Mycelia growth in plant extracts}}{\text{Mycelia growth in control}} \text{ X } 100$$

Statistical analysis:

Data are expressed as the mean value for five replicates per treatment, and statistical analysis was evaluated by the statistical analysis system (SAS, 1994*)* soft-ware programs. Significance differences among treatments were determined by Duncan's multiple range test at $P \leq 0.05$ (Duncan, 1955).

RESULTS

A primary screening of the antimycotic activity of herbs and spices on the common mould strains isolated from the surface of Ras cheese discs was carried out. Portions of 100 g of the plant materials (Table 1) were extracted either by ethanol or acetone and examined for its antimycotic activities using the paper disc diffusion assay as previously described. The total moulds growth inhibition zone diameters (mm) by individual extracts are illustrated in Fig. (1). The results showed that all the examined plant materials were effective to inhibit moulds growth. The order in the acetonic extracts was cinnamon (74.0), galangal (63.0), cumin (69.3), caraway

(68.8), fenugreek (68.6), miswak (68.2), marjoram (67.0), anise (66.8), sage (66.0) and visnaga (65.8). However the total inhibition zone diameters of the water extracts were cinnamon (68.6), cumin (66.4), caraway (66.4), miswak (63.1), anise (63.0), galangal (63.0), sage (62.8), fenugreek (62.4), Ambrosia (62.2) and chili (61.4). On the other hand, *P. roqueforti*, *P. camembrti*, *A. parasiticus*, *A. flavus*, *A. niger* and *Mucor spp*. were more sensitive than *F. reseom*, *Alt. tenius* and *C. herbarum* for the tested extracts with significantly ($P<0.05$) higher antimycotic activities of the acetonic extracts than the water extracts. (Fig. 2).

Figure (3) represents the total inhibition zone diameters of water and acetonic extracts of the tested plant materials on the growth of *S. cerevisiae* and *G. candidum*. Most of the plant extracts with high antimycotic activities against moulds were also effective to inhibit growth of the examined yeast strains. The average growth inhibition zone diameters (mm) for the most effective acetonic extracts in descending order were cinnamon (19.0), cumin (19.0), fenugreek (18.6) caraway (18.2), galangal (17.8), marjoram (17.8), liquorice (17.6), miswak (17.4), sage (17.0) and visnaga (17.0). However, the figures for the water extracts were cumin (18.6), cinnamon (17.4), fenugreek (17.4), miswak (17.2), anise, (17.0) caraway (17.0), chamomile (17.0), galangal (17.0), marjoram (16.6) and liquorice (16.6). The acetonic extracts were significantly ($P<0.05$) effective to inhibit the yeast growth than the aqueous extracts (Fig. 4). The data also showed that both yeast strains were more sensitive for the tested extracts (Fig. 4) than the examined moulds (Fig. 2). The growth inhibition zone diameters (mean±SD) with the yeasts was (212±0.7) and (198±1.4) compared to (179±2.3) and (168±3.6) with the moulds for acetonic and water extracts, respectively.

The results from the primary screening reveal that caraway, cinnamon, cumin fenugreek, galangal, marjoram and miswak were more effective than the other plants in suppressing growth of the tested mould and yeast strains. Therefore ethanolic extracts of these plants in addition to ambrosia and rosemary were further investigated for its antimycotic activity on the previously mentioned fungi strains. Ambrosia and rosemary were chosen according to a primary antifungal investigation of their ethanolic extracts (data not shown). Figure 5 illustrates the growth inhibition percentage of the tested moulds by 0.1, 0.2 and 0.3% ethanolic extracts of the selected plants compared to delvocid (50 ppm). Delvocid was found to inhibit the growth of all the investigated moulds by about 90% and served as a control treatment. All ethanolic extracts were

effectively suppressed the growth of the investigated mould strains (Fig. 5). These extracts showed growth inhibition effect with correlation coefficient of (0.84-1) and mean±SD of (55±10), (84±9.8) and (93±4.2) for the percent of growth inhibition by 0.1, 0.2 and 0.3% ethanolic extracts, respectively. The increase of plant extract concentrations from 0.1% to 0.2% significantly (P<0.05) suppressed the growth of moulds, however increasing the concentrations to 0.3% had no significant effect. Relative to delvocid (100%) and in descending order, the 0.3% ethanolic extracts resulted in moulds growth inhibition (%) as the follows: Cinnamon (100), cumin (98), ambrosia (95), galangal (94), caraway (93), fenugreek (91), rosemary (88), miswak (88) and marjoram (88). However, the maximum growth inhibition effect of cinnamon was obtained at concentration of 0.2% which was as effective as 50 ppm of delvocid. On the other hand, the combined antifungal effect of the examined plant extracts on the tested moulds showed that *Penicillum* and *Aspergillus* strains were more sensitive than the other mould strains especially at the 0.3% concentration (Fig. 6).

The effect of 0.1, 0.2 and 0.3% of the investigated plant ethanolic extracts on the growth of *S. cerevisiae* and *G. candidum* are illustrated in Fig. (7). All the investigated plant extracts were effective to inhibit the growth of the mentioned yeasts. Furthermore, the increase of the extract concentration was positively correlated (0.88-1) to its growth inhibition effect. The most effective plants at concentration of 0.3% compared to 50 ppm of delvocid (100%) was in descending order, 88 (cinnamon), 85 (galangal), 84 (caraway), 82 (cumin), 81 (marjoram). However, the other plants inhibited the yeast growth by 67-70%. On the other hand, *S. cerevisiae* was more susceptible to the treatment by the plant extracts than *G. candidum*, as shown in (Fig. 8).

DISUCSSION

Initial screenings of plants for possible antimicrobial activities typically begin by using crude aqueous or alcohol extractions and can be followed by various organic extraction methods (Cowan, 1999). In the present study an initial screening of aqueous and acetone extraction of crude extracts of 24 kinds of herbs and spices plant materials to study its antifungal activities, against several fungi strains commonly found on Ras cheese surface during ripening and cold storage, was carried out. In fact all the examined plants showed different levels of antifungal

activities which were more abundant in its acetonic extracts. The most effective plants (either on its aqueous or acetonic extracts) were caraway, cinnamon, cumin fenugreek, galangal, marjoram and miswak. Zommara and Rashed (2005) found that aqueous extracts of galangal, cina, samolia, red pepper, cinnamon, turmeric, cumin, henna and sage were effective to inhibit the growth of 73-82% of the isolated mould isolated from Ras cheese surface; however extracts of ambrosia, aloe, cumin, fenugreek, thyme and anise were more effective to inhibit the growth of the isolated yeasts. A wide range of studies demonstrated the antifungal effect of herbs and spices and identified many antimycotic compounds such as coumarins in caraway, (Scheel, 1972) cinnamaldehyde, eugenol and cinnamic acid in the essential oil of cinnamon (Guynot, *et al*., 2003 and Jham *et. al*., 2005), essential oil of galangal (Tripathi *et al*., 1983), borneol, camphor, carvacrol, eugenol, farnesol, flavonoids, geraniol, limonene, linalool, oleanolic acid, thymol and ursolic acid in marjoram (Esiyok, *et al*., 2004). On the other hand, water extract of miswak contains potential antimicrobial anionic components such as chloride, sulfate, thiocyanate and nitrate (Darout *et al*., 2000).

Cowan (1999) stated that nearly all of the identified active components from plants against microorganisms are aromatic or saturated organic compounds and often obtained through initial ethanol or methanol extraction. Therefore, three concentrations (0.1, 0.2 and 0.3%) of ethanolic extracts of caraway, cinnamon, cumin fenugreek, galangal, marjoram, miswak, ambrosia and rosemary were further investigated for its antifungal activity. The results showed a dose-related effect of the extracts. Cinnamon, cumin, caraway and galangal had the highest antimycotic activity compared to the other tested plants. The effect of 0.2% of cinnamon extract was equivalent to that obtained by 50 ppm of delvocid. However, the other plant extracts (0.3%) showed antimycotic effect equivalent to 88-98% of that of delvocid. In this respect, the antifungal activity of the essential oils from several aromatic species from the Lauraceae family including cinnamon was investigated against seventeen micromycetes (Simic *et al*., 2004). Among the fungal species tested were food poisoning and food spoilage fungi, and plant and animal pathogens. Cinnamon showed the strongest antifungal activity. The main component of the essential oil of cinnamon was *trans*-cinnamaldehyde (Abad *et al*., 2007). Also, Guynot, *et al*., (2005) investigated the antifungal effect of 20 essential oils against the most important moulds in terms of spoilage of bakery products (*Eurotium* spp., *Aspergillus* spp. and *Penicillium* spp.). They

found that cinnamon leaf, rosemary, thyme, bay and clove essential oils exhibited some antifungal activity against all isolates which was depended on water activity (a_w) and pH values in the used media.

During Ras cheese ripening and storage, the most widespread and probably most important moulds, in terms of biodeterioration of Ras cheese, are species of *Aspergillus* and *Penicillium* (El-Essawy *et al*., 1984, Abdel-Rahman and El-Bassiony, 1985, Hassan and El-Deeb, 1988, Hassan, *et al.*, 2004, Zommara and Rashed, 2004). In the present study the examined ethanolic extracts were found to have a prominent growth inhibition effect on the tested mould strains. This may give an advantage to be used in Ras cheese treatment to prevent cheese surface spoilage. Further investigations must be carried out to ensure the suitability of applying these plant extracts to control the fungi growth on Ras cheese surface without affecting its sensory characteristics.

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Table (1): Common and scientific names of plants, spices and herbs used in the study.

Common name	**Scientific name**	. Arabic d. name	**Part used**
Aloe	*Aloe vera*	الصبار	Leaves
Ambrosia	*Ambrosia maritima*	الدمسيسة	Leaves
Anise	*Pimpinella anisum L.*	الينسون	Seeds
Black pepper	*Piper nigrum L.*	الفلفل الأسمر	Seeds
Caraway	*Carum carvi*	الكراوية	Seeds
Chamomile	*Matricaria chamomilla*	البابونج	Flowers
Chili	*Capsicum frutescens L.*	الفلفل الأحمر	Peels
Cina	*Artemisia cina*	الشيح	Flowers
Cinnamon	*Cinnamomum zeylanicum*	القرفة	Bark
Cloves	*Syzygium aromaticum*	القرنفل	Flowers
Cumin	*Cuminum cyminum*	الكمون	Seeds
Fenugreek	*Trigonella foeum-graecum L.*	الحلبة	Seeds
Galangal	*Alpinia galanga L. willd*	الخلنجان	Rhizome
Ginger	*Zingiber officinale*	الجنزبيل	Rhizome
Henna	*Lawsonia inermis*	الحنة	Leaves
Liquorice	*Glycyrrhiza glabra L.*	العرقسوس	Roots
Marjoram	*Origanum majorana*	البردقوش	Leaves
Miswak	*Salvadora persica*	الأراك	Bark
Nigella	*Nigella sativa L.*	حبة البركة	Seeds
Rosemary	*Rosmarinus officinalis L.*	الحصالبان	Leaves
Sage	*Salvia officinalis L.*	المرمرية	Leaves
Thyme	*Thymus vulgaris*	الزعتر	Leaves

Turmeric	*Curcuma longa*	الكركم	Rhizome
Visnaga	*Ammi visnaga L.*	الخلة	Leaves

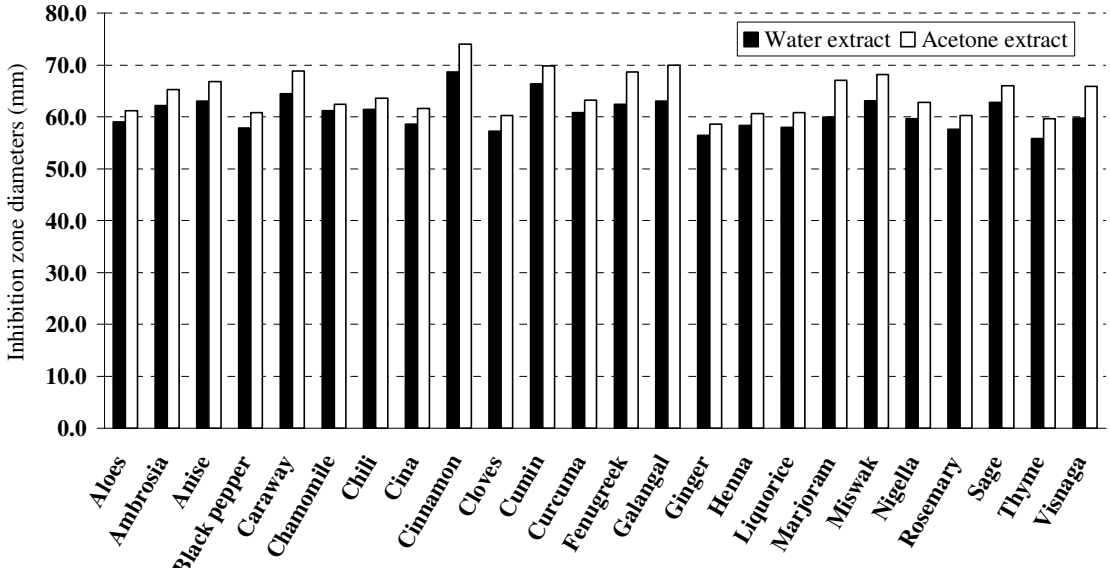

Fig. (1). Combined growth inhibition of common moulds isolated from Ras cheese surface by different water and acetone plant extracts. See materials and methods for moulds names.

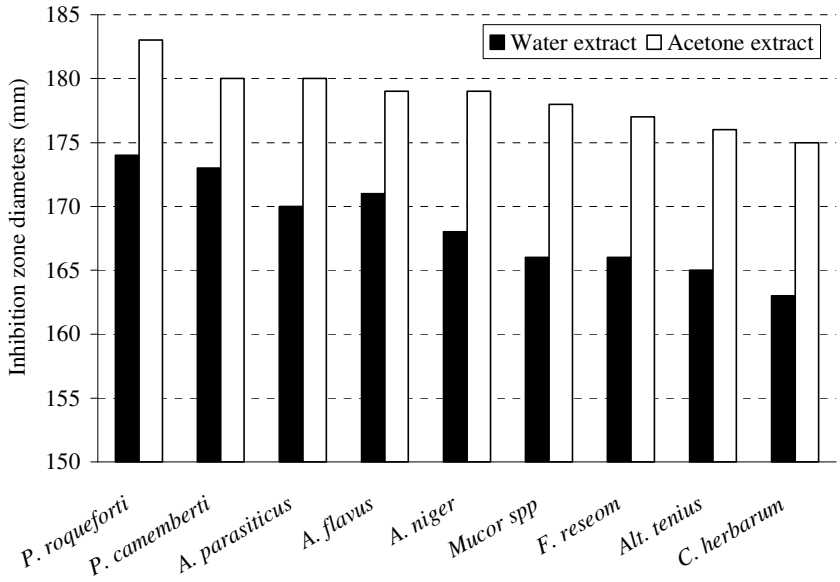

Fig. (2). Combined effect of plant water and acetone extracts on the common moulds isolated from Ras cheese surface. See table (1) for plant names.

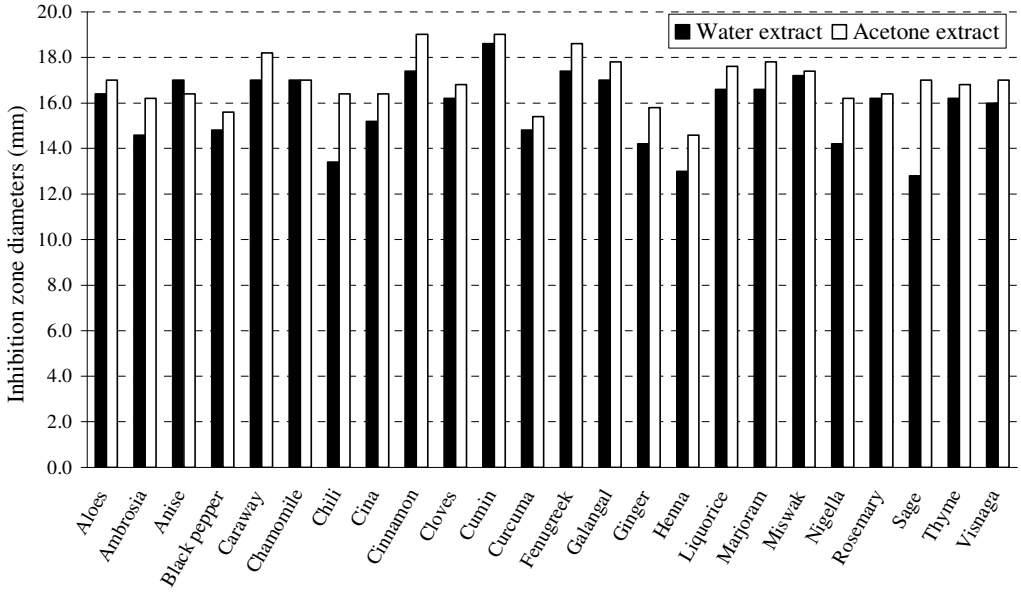

Fig. (3). Combined growth inhibition of *S. cerevisiae and G. candidum* isolated from Ras cheese surface by different water and acetone plant extracts.

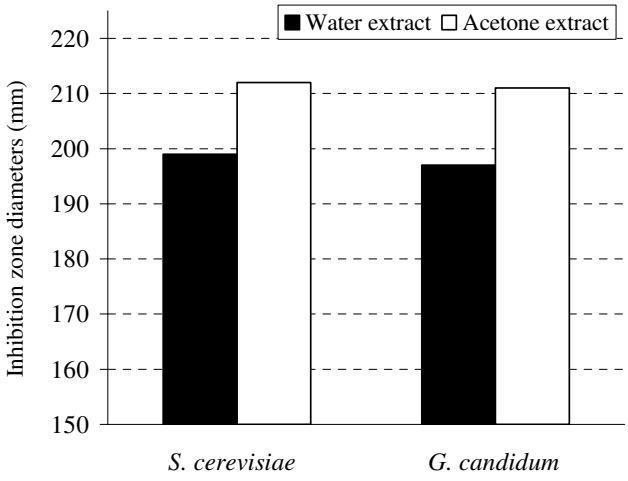

Fig. (4). Combined growth inhibition effect of different plants water and acetone extracts on *S. cerevisiae and G. candidum*.

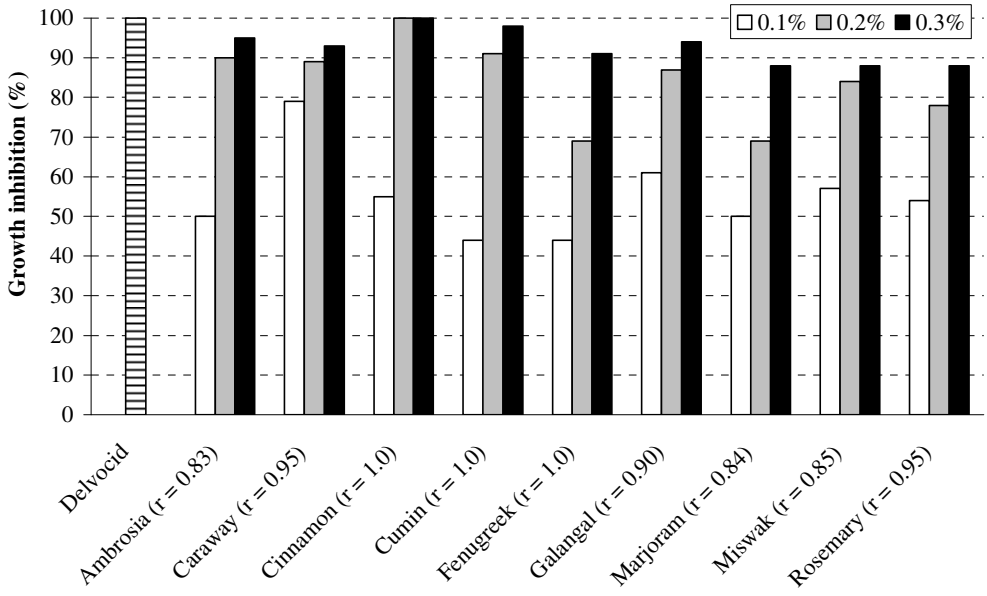

Fig. (5). Combined growth inhibition (%) of common moulds isolated from Ras cheese surface by different plant ethanol extracts compared to 50 ppm of delvocid. See materials and methods for moulds names. (r = Correlation coeffecient)

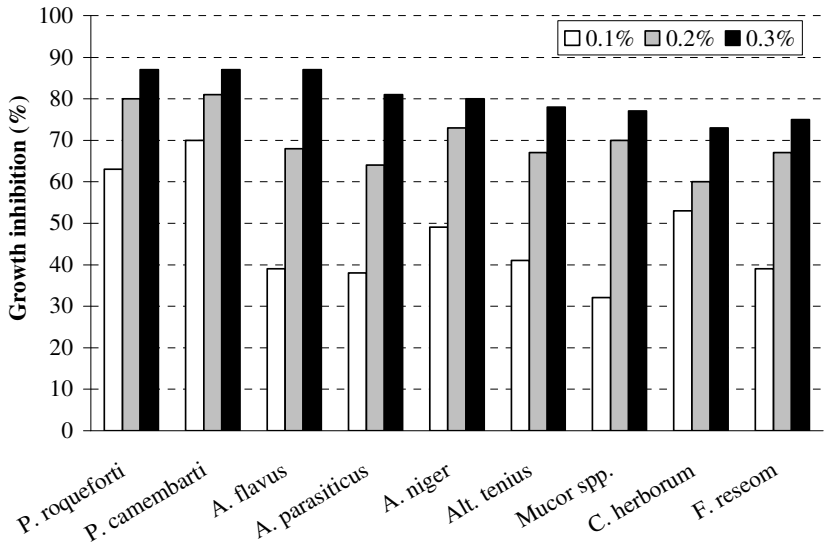

Fig. (6). Combined growth inhibition effect of plant ethanolic extracts on the moulds isolated from Ras cheese surface. See Fig. 5 for plant names.

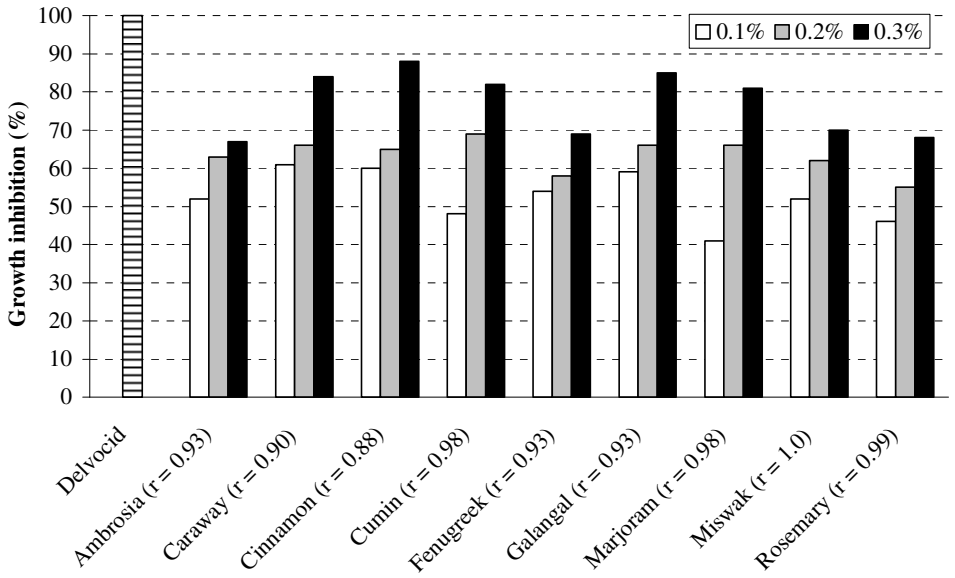

Fig. (7). Combined growth inhibition (%) of *S. cerevisiae* and *G. candidum* by different plant ethanol extracts compared to 50 ppm of delvocid. (r = Correlation coeffecient)

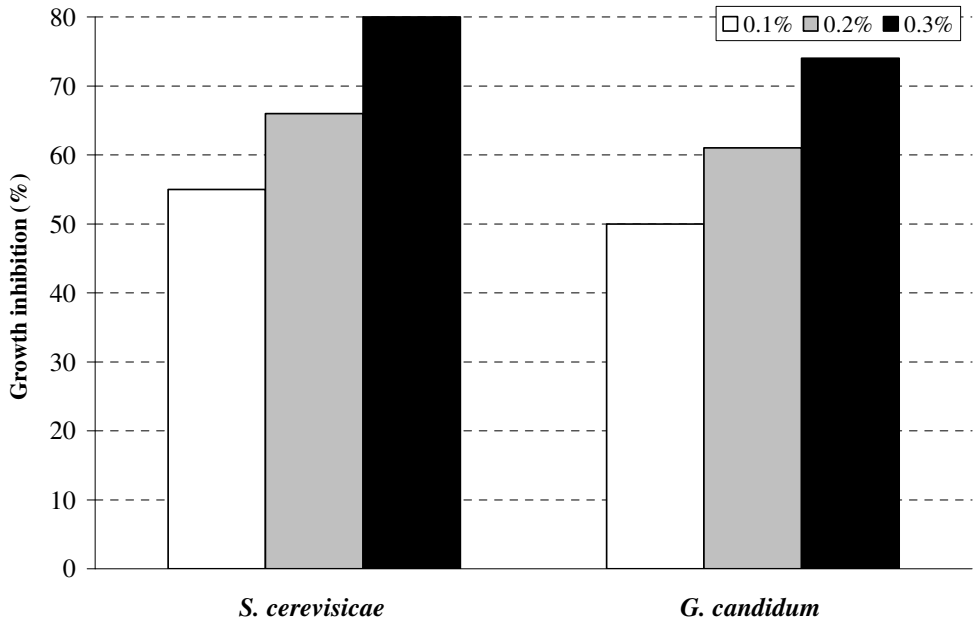

Fig. (8). Combined growth inhibition effect of plant ethanol extracts on the tested yeasts. See Fig. 7 for plant names.

الملخص العربى

تقييم التأثير المضاد للفطريات لبعض المستخلصات النباتيه على الفطريات الشائعه الوجود على سطح الجبن الراس

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تم اختبار مستخلصات ٢٤ نوع من النباتات والأعشاب والتوابل من حيث قدرتها المضاده للفطريات باستخدام ٩ سلالات من الفطريات وسلالتين من الخمائر الشائعة الوجود على سطح الجبن الراس. أوضحت الأختبارات المبدئيه باستخدام المستخلصات المائيه والأسيتونيه وجود نشاط قوى مضاد للفطريات لمستخلصات الكراويه، القرفه، الكمون، الحلبه، الخلنجان،البردقوش والأرك (المسواك) مقارنة بالنباتات الأخرى. تم اختبار المستخلصات الكحوليه للنباتات السابقة بالأضافة لنباتات الدمسيسه والحصالبان من حيث نشاطها المضاد للفطريات. لقد أوضحت النتائج وجود تناسب طردى بين تركيز المستخلص فى البيئة المزرعيه وتأثيره المضاد للفطريات حيث تراوح معامل الأرتباط بينهما من ٠،٨ إلى ١،٠٠. وقد تراوحت نسبة تثبيط نمو الفطريات عند استخدام تركيز ٠،٣ % من هذه النباتات بين ٨٨ إلى ١٠٠% من تلك المتحصل عليها بواسطة ٥٠ جزء فى المليون من مادة الدلفوسيد التجاريه والتى تستخدم على نطاق واسع فى معاملة الجبن الراس لأيقاف نمو الفطريات عليها. ولقد لوحظ تأثير واضح لمستخلص نبات القرفه مقارنة بالمستخلصات الأخرى بلغ حده الأقصى عند تركيز ٠،٢ %، وكانت أكثر الفطريات تأثرا هى التابعة لأجناس اللأسبرجلس والبنسليوم والسكارومايسز. تشجع هذه النتائج على إمكانية استخدام مستخلصات لهذه النباتات كبدائل للمواد الكيماويه فى مقاومة نمو الفطريات على سطح الجبن الراس، إلا أنه يحب إجراء دراسات أخرى عن النشاط المضاده للفطريات لهذه النباتات عن طريق معاملة الجبن الراس.