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Evaluation of Food Preservatives, Low Toxicity Chemicals, Liquid Fractions of Plant Extracts and their Combinations as Alternative Options for Controlling Citrus Post-harvest Green and Blue Moulds *in vitro*

[1]Emad I. Hussein, [1]Ghassan J.M. Kanan, [1]Khalid M. Al- Batayneh, [2]Khalaf Alhussaen, [1]Wesam Al Khateeb, [1]Janti Qar, [3]Jacob H. Jacob, [1]Riyadh Muhaidat and [4]Mohamed I. Hegazy

[1]Department of Biological Sciences, Yarmouk University, Irbid, Jordan
[2]Department of Plant Production and Protection, Faculty of Agriculture, Jerash, University, Jerash, Jordan
[3]Department of Biological Sciences, Al al-Bayt University, Mafraq, Jordan
[4]Department of Microbiology, Faculty of Agriculture, Zagazig University, Egypt

Corresponding Author: Emad I. Hussein, Department of Biological Sciences, Yarmouk University, Irbid, Jordan

ABSTRACT

The post-harvest moulds *Penicillium digitatum* and *Penicillium italicum* are important plant pathogens and spoilage-causing molds especially against citrus fruits. If not treated, post-harvest moulds can cause enormous economic losses during storage and marketing. Therefore, more investigations are needed to examine new antifungal agents against such fungi. In this work, we aimed to evaluate the antifungal activity of some plant extracts (namely, Harmal seeds (*Peganum harmala* L.), cinnamon bark (*Cinnamomum cassia* L.) and sticky fleabane leaves (*Inula viscosa* L.), food preservatives (namely, sodium benzoate, sodium molybdate, ammonium heptamolybdate tetrahydrate, potassium carbonate and sodium bicarbonate) and their mixtures, i.e., plant extracts and food preservatives against *P. digitatum* and *P. italicum*. Both disc agar diffusion method and broth dilution methods was used to evaluate the antifungal activity of the plant extracts and food preservatives. Results revealed that methanolic fractions of cinnamons' bark and sticky fleabane leaves showed the highest efficacy. MIC values of 150 and 37.5 μg mL^{-1} were obtained with cinnamons' fraction against *P. italicum* and *P. digitatum*, respectively. Sodium benzoate was the most effective against tested fungal species. The obtained MIC values against *P. digitatum* and *P. italicum* were 37.5 and 75 μg mL^{-1}, respectively. Mixtures of tested chemicals showed synergistic effects against both fungal species. Mixtures of sodium benzoate and fractions of either cinnamon or sticky fleabane reflected synergistic effects against *P. italicum* and antagonistic effects against *P. digitatum*. Inhibition zones against *P. italicum* ranged between 38-57 mm.

Key words: Plant extracts, food preservatives, minimum inhibitory concentration, *Penicillium italicum*, *Penicillium digitatum*

INTRODUCTION

The post-harvest green and blue moulds *Penicillium digitatum* (Pers: Fr) Sacc. and *Penicillium italicum,* respectively, are considered universal diseases that lead to the spoilage of

almost all kinds of mature citrus fruits (Plaza *et al*., 2004; Prusky *et al*., 2004; Samson *et al*., 2004). Significant economic losses caused by post-harvest pathogens during storage and marketing are greater than what most people believe. These unavoidable losses between the farm gate and the consumer are currently of big concern (Soylu *et al*., 2005; Smilanick *et al*., 2005). Citrus industry relies heavily on the extensive use of chemical fungicides as a common practice for the control of post-harvest fungal decay of citrus fruits (McGrath, 2001; Bouzerda *et al*., 2003; Tripathi and Dubey, 2004). However, the consumer demands for fungicide-free products and the increasing fungal resistance for fungicides necessitates the search for alternative control options (Obagwu and Korsten, 2003; Soylu *et al*., 2005; Lee *et al*., 2004; Ikeura *et al*., 2011; Bhyan *et al*., 2007; Reddy *et al*., 2010).

Plant extracts and their essential oils are one of the non-synthetic chemical control options that have recently received attention for controlling plant diseases (Soylu *et al*., 2005; Abad *et al*., 2007; Nahunnaro, 2008; Zaker and Mosallanejad, 2010; Hasan *et al*., 2005). Seeds and roots extracts of Harmal (*Peganum harmala*) have been shown to contain a variety of active alkaloids, including harmaline which was reported as a very strong antifungal agent (Telezhenetskaya and D'yakonov, 2004). In addition, eugenol and cinnamaldehyde from cinnamon bark have consistently been reported to have antifungal activity (Delaquis *et al*., 2002). Other studies were carried out to elucidate the biological activity of sticky fleabane extracts (Wang *et al*., 2004). All types of *Inula viscosa* extracts have been proved to exert significant antifungal activity. The extracts from oily leaves paste showed *in vitro* antifungal activity against some dermatophytes, *Candida* spp. and Downy mildew (Cafarchia *et al*., 2002; Cohen *et al*., 2006). Furthermore, the sesquiterpene lactone isolated from *I. viscosa* flowers was found to possess an *in vitro* activity against *Microsporum canis, Microsporum gypseum* and *Trichophyton mentagrophytes* (Abu-Zarga *et al*., 1998; Cafarchia *et al*., 2002).

Recently, many chemical compounds were tested as alternative control options for citrus post-harvest diseases either alone or in combination with physical or biological treatments (Palou *et al*., 2001; Reddy *et al*., 2007; Bonjar, 2004; Bonjar and Nik, 2004). Over a wide range of commonly used chemicals, sodium bicarbonate, potassium sorbate, sodium benzoate, sodium carbonate and ammonium molybdate were the most promising fungicides alternatives to control *Penicillium* species (Palou *et al*., 2002b; Smilanick *et al*., 2002). Soda ash (sodium carbonate) has been used for over 70 years to control post-harvest decay of citrus fruit in California (Palou *et al*., 2001). It has been reported that soda ash reduces the incidence of green mould by over 90% (Smilanick *et al*., 1997). In addition, the combination of sodium bicarbonate and fungicides was used to manage post-harvest green and blue moulds of citrus in California (Smilanick *et al*., 1999; Ismail and Zhang, 2004). However, sodium bicarbonate has partially controlled the green mould and other fungal diseases of citrus fruits (Larrigaudiere *et al*., 2002; Smilanick *et al*., 1999; Palou *et al*., 2001; Larrigaudiere *et al*., 2002). Although potassium sorbate was reported to delay rather than to stop green mould's infection, it has been reported to act (at low levels) synergistically along with heat and fungicides in controlling the disease. Therefore, sorbate has commercially substituted sodium bicarbonate for improving the performance of fungicides in retarding the development of fungal resistant isolates (Palou *et al*., 2002a; Smilanick *et al*., 2005). Moreover, Buazzi and Maeth (1992), indicated that sodium benzoate which is usually used as food preservative, has prevented the growth of many microorganisms including yeasts, bacteria and moulds. Sodium and ammonium molybdate that are applied as molybdenum sources for foliar and soil applications (i.e., as fertilizers) have effectively controlled the growth of green and blue moulds

of citrus fruits (Palou *et al*., 2002a). In addition, the use of antifungal mixtures which selectively had different modes of action is considered a highly recommended practice in avoiding the risks of pathogens resistance. The purpose of this study is to investigate potentially novel treatments for combating pathogenic fungi to citrus fruits, namely, *Penicillium digitatum* and *Penicillium italicum,* the green and blue moulds, respectively. Our emphasis was placed on identifying combined treatments of novel and existing antifungal agents which may exhibit potent synergistic effect against fungal pathogens. Novel antifungal agents include mixtures of plant liquid fractions (natural molecules), combinations of food preservatives and mixtures of both plant fractions and food preservatives.

MATERIALS AND METHODS

Origin of fungal isolates: Two wild-type species of *Penicillium*, *Penicillium digitatum* and *Penicillium italicum,* were isolated from mature spoiled orange (*Citrus sinensis* L.) and lemon (*Citrus limon* L.) fruits and identified using morphological and physiological characters. Fruits were obtained from the local market Irbid-Jordan.

Growth media: The *Aspergillus nidulans* complete (CM) medium described previously by Cove (1966) and modified by Al-Najar (2007) was used to achieve optimal growth conditions for the tested fungal isolates.

Purification of fungal isolates: Conidiospores from each isolate were grown for 7 to 10 days at 20-25°C on CM plates (Al-Najar, 2007) to confirm their purity and identity.

A suspension of conidiospores was prepared in 5 mL physiological saline/Tween 80 (0.05%) solution at a concentration of approximately 10^8 spores per milliliter. Aliquots of 100 μL from a dilution of 10^{-6} or 10^{-7} were plated again on complete media in order to get a single colony as a source of pure culture (Zhang *et al*., 2004).

Tested decay control chemicals: Five decay control chemicals were tested for their effect on mould growth: Sodium benzoate ($C_7H_5O_2Na$), sodium molybdate ($MONa_2O_4 2H_2O$), ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}4H_2O$), potassium carbonate (KCO_3) and sodium bicarbonate ($NaHCO_3$). Various concentrations (20, 30, 40, 80, 120, 160, 200, 240, 280, 320 and 400 μg mL^{-1}) of the chemicals were used in order to determine their influence on the growth of *P. digitatum* and *P. italicum* isolates. Each concentration of the different chemicals was aseptically added to agar wells. Conidiospores suspension from each tested fungal isolate was prepared as described above. Petri dishes of complete media (chemical and plant extract free media) were inoculated with conidial suspension (200 μL) loaded onto the surface of the plate and spread over the whole plate. Three plates were used for each tested concentration per chemical. Control treatments were set up using sterile distilled water and/or dimethylsulphoxide (DMSO) and each treatment was repeated at least twice. The inoculated plates were incubated for 5 days at 20 or 25°C as required. The diameter of inhibition zone was measured in two directions at right angles to each other (Schroeder and Bullerman, 1985).

Plant extracts preparation: Extracts of three plant materials were tested: Harmal seeds (*Peganum harmala* L.), cinnamon bark (*Cinnamomum cassia* L.) and sticky fleabane leaves (*Inula viscosa* L.). Each plant material was dried in shade, ground to a fine powder using liquid

nitrogen and extracted (48 h) with absolute ethanol in Soxhlet extractor (Ndukwe *et al*., 2006). The solvent was removed using rotary evaporator (Heidolph, VV2000) under reduced pressure at temperatures below 55°C. The resulting crude extracts were stored at -20°C until tested. Stock solutions and serial dilutions of each extract were prepared in dimethylsulphoxide (DMSO) (Ambrozin *et al*., 2004) and used as control.

Fractionation of plant crude extracts: Each crude extract was fractioned into three parts (aqueous, hexane and methanolic fraction). The emulsions that may form between layers were also tested. Each crude extract sample was fractionated with (1:1) ratio of water/dichloromethane (v/v). The resultant aqueous fraction was further extracted with dichloromethane and then concentrated to dryness using rotary evaporator. This dichloromethane fraction was subsequently partitioned with (1:1) n-hexane/90% methanol. The hexane and methanol fractions produced were concentrated to dryness using rotary evaporator and kept in sterile containers at 4°C until used. Each fraction type was prepared at the required concentration (μg mL^{-1}) by dissolving in dimethylsulphoxide then tested for its antifungal activity (Souza-Fagundes *et al*., 2002).

Antifungal assay of plant crude extracts, their fractions, decay control chemicals and their combinations: Aliquot of 100 μL spore suspension (*ca*. 1×10^8 spores mL^{-1}) of each tested isolate was streaked in radial patterns on the surface of complete media plates. Stock solutions of each crude extract or liquid fraction were filter sterilized through a 4 μm Millipore filter (Soylu *et al*., 2005). Agar wells (6 mm in diameter) were made in solidified complete growth media. Each tested concentration from the plants crude extracts or their liquid fractions (20, 50, 100, 200, 400 and 600 μg mL^{-1}) or the five tested chemicals (20, 30, 40, 80, 120, 160, 200, 240, 280, 320 and 400 μg mL^{-1}) were loaded into the agar wells. In addition, the following pair-wise combinations were tested using agar well diffusion method: combinations from each crude extract and each of its liquid fractions (200 μg mL^{-1} of each), combinations of crude extracts or fractions referring to different plant species (200 μg mL^{-1} of each), combinations made between tested chemical substances (160 μg mL^{-1} of each) and combinations (each of 200 μg mL^{-1}) between chemicals and each of the tested plant extracts or fractions. DMSO was used as control for the ethanolic extracts or fractions. The cultured plates were incubated for 3-5 days at 20 or 25°C as required. The radius of the inhibition zone was measured in two directions at right angles to each other. Experiments were carried out with three replicates per treatment and each treatment was repeated at least twice (Ndukwe *et al*., 2006). The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of the extract, the fraction, or the tested chemical that was able to inhibit any visible fungal growth after 48 h in liquid cultures. The fungistatic or fungicidal effects of each tested material was determined by streaking on complete plates with 200 μL spore suspension taken from the liquid culture that specified the MIC value of that substance (Obagwu and Korsten, 2003).

Statistical analysis: The Minimum Inhibitory Concentration (MIC), the correlation coefficient values and the significance values at $p \leq 0.05$ or 0.01 levels (2-tailed) for tested chemical substances, plant crude extracts, their fractions and the mixtures made were calculated by regression analysis for the relationship between the size of fungal inhibition zone (mm) and the concentration (μg mL^{-1}) of the evaluated substances (Log value). Microsoft Excel 2003 software and the SPSS program version 15 were used for data analysis.

RESULTS

***In vitro* sensitivity of *P. italicum* and *P. digitatum* to different concentrations of plant extracts and their liquid fractions:** Data of the regression analysis for the relationship between size of fungal growth inhibition zone (mm) and concentrations of plant (cinnamon bark; sticky fleabane leaves and Harmal seeds) crude extracts and their liquid fractions are shown in Table 1. There was a significant correlation (at either the 0.01 or the 0.05 level of significance) between the tested concentrations of plant crude extracts and mean inhibition zones of both fungal species (*P. italicum* and *P. digitatum*) (Table 1). An exception to this pattern was shown with Harmal's seed crude extract, where no significant correlation was found between tested concentrations and *P. italicum* inhibition zones ($r = 0.806$; $p = 0.053$). Cinnamon's bark crude extract was found to be the most effective against both fungal species (Table 1). The obtained MIC values against *P. digitatum* and *P. italicum* were 75 and 187.5 μg mL^{-1}, respectively. In contrast, Harmal's seed extract was the least effective against both fungal species (with inhibition zones of approximately 18 to 20 mm) and the obtained MIC values were within the range of 400 to 450 μg mL^{-1}. Regarding the inhibition effect of plant's liquid fractions, there was a significant correlation between tested concentrations of fractions and mean inhibition zones of both *Penicillium* species (Table 1). Exceptions to this pattern were hexane fraction ($r = 0.797$; $p = 0.058$) and water fraction ($r = 0.783$; $p = 0.066$) of sticky fleabane against *P. italicum* and *P. digitatum*, respectively. Furthermore, no significant correlations were noticed with Harmal's hexane fraction against *P. italicum* ($r = 0.798$; $p = 0.057$) and *P. digitatum* ($r = 0.803$; $p = 0.054$). Additionally, the layer between Harmal's fractions reflected no significant correlation against *P. italicum* ($r = 0.806$; $p = 0.053$). On the other hand, methanolic fractions of cinnamon bark and sticky fleabane leaves showed the highest inhibitory efficacy against both fungal species where, the largest inhibition zones were obtained with these fractions (Table 1). In addition, MIC values of 150 and 37.5 μg mL^{-1} were obtained with cinnamon methanolic fraction against *P. italicum* and *P. digitatum,* respectively. Also, MIC values of 375 and 75 μg mL^{-1} were obtained with sticky fleabane methanolic fraction against the same species, respectively. In contrast, none of the tested concentrations (within a range of 20 to 600 μg mL^{-1}) of cinnamons' water fraction reflected inhibitory effect against both fungal species.

***In vitro* sensitivity of *Penicillium* species to combinations of cinnamon fractions, sticky fleabane fractions and fractions of both plant species:** Since significant inhibitory effects were obtained with most of the tested plant extracts at concentrations of 200 μg mL^{-1} or more, attention was turned to evaluate mixtures of extracts which may display potent synergistic effects against fungal pathogens. The investigated treatments included: combinations of 1:1 ratio (200 μg mL^{-1} each) between fractions of the same plant material and combinations between fractions of different plants. The obtained results (Fig. 1) indicate that most of the combinations made between cinnamon's bark crude extract and its fractions resulted in antagonistic effects against both citrus-post harvest fungal pathogens. Exceptions to this pattern were the combinations made between cinnamon's crude extract and its methanolic (against *P. italicum*) and hexane fractions (against *P. digitatum*) where inhibition zone diameters were approximately similar to those obtained by the crude extract itself (Fig. 1). However, the combinations between cinnamon's methanolic and hexane fractions or cinnamon's methanolic and water fractions have maintained or preserved the inhibitory effect of methanolic fraction against both fungal species (i.e., inhibition zone diameters similar to those obtained by methanolic fraction (around 42 mm)

Table 1: *In vitro* sensitivity of two wild-type fnngal isolates, *Penicillium italicum and P. digitatum*, to different concentrations (µg m^{-1}) of three plant crude extracts and their liquid fractions

Extract source[a]	Range of conc. (µg mL^{-1})[b]	Range of inhibition [c] zone (mm) Mean±SD	MIC [d]	Corr. value [e](r)	Sig. value[f]	Fnngal isolate
Cinn/crude	20-100 200-600	10.5±2.12 to 18.5±3.53	187.5	0.981**	0.001	*P. italicum*
		29.5±2.12 to 35.5±3.54				
Cinn/crude	20-100 200-600	18.2±1.12 to 23.5±2.12	75	0.952**	0.003	*P. digitatum*
		39±1.41 to 45±2.85				
Cinn/met	20-100 200-600	16.5±1.71 to 21.25±3.2	150	0.941**	0.005	*P. italicum*
		23.5±3.53 to 33.5±2.4				
Cinn/met	20-100 200-600	23±1.4 to 29.5±2.12	37.5	0.968**	0.001	*P. digitatum*
		43.5±2.2 to 55.5±1.76				
Cinn/hex	20-100 200-600	0.0 to 0.0	300	0.903*	0.014	*P. italicum*
		14.5±0.71 to 18.5±2.1				
Cinn/hex	20-100 200-600	0.0 to 0.0	300	0.908*	0.012	*P. digitatum*
		17.5±0.71 to 24±1.10				
Cinn/wat	20100 200-600	0.0 to 0.0 0.0 to 0.0				*P. italicum*
Cinn/wat	20-100 200-600	0.0 to 0.0 0.0 to 0.0				*P. digitatum*
Stick/c rude	20-100 200-600	0.0 to 0.0	375	0.913*	0.011	*P. italicum*
		9.5±2.12 to 16±1.41				
Stick/crude	20-100 200-600	0.0 to 17±1.41	150	0.990**	0.000	*P. digitatum*
		29.5±3.54 to 38±2.14				
Stick/met	20-100 200-600	0.0 to 0.0	375	0.917*	0.010	*P. italicum*
		11.5±0.71 to 20.5±3.51				
Stick/met	20-100 200-600	19.5±2.12 to 22±5.7	75	0.940**	0.005	*P. digitatum*
		35±1.41 to 44±2.83				
Stick/hex	20-100 200-600	0.0 to 0.0 0.0 to 18±1.44	375	0.797	0.058	*P. italicum*
Stick/hex	20-100 200-600	0.0 to 0.0 18±2.16 to 27±2.38	375	0.912*	0.011	*P. digitatum*
Stick/wat	20-100 200-600	0.0 to 0.0 9.5±2.12 to 18.5±2.22	375	0.915*	0.011	*P. italicum*
Stick/wat	20-100 200-600	0.0 to 0.0 0.0 to 26.5±2.22	300	0.783	0.066	*P. digitatum*
Harm/crude	20-100 200-600	0.0 to 0.0 0.0 to 20.5±2.33	400	0.806	0.053	*P. italicum*
Harm/crude	20-100 200-600	0.0 to 0.0 13.5±1.4 to 18.5±1.14	450	0.908*	0.012	*P. digitatum*
Harm/met	20-100 200-600	0.0 to 0.0 12±1.41 to 24.5±3.45	450	0.908*	0.012	*P. italicum*
Harm/met	20-100 200-600	0.0 to 0.0 13.5±1.4 to 22.5±1.23	450	0.912*	0.011	*P. digitatum*
Harm/hex	20-100 200-600	0.0 to 0.0 0.0 to 13±1.11	600	0.798	0.057	*P. italicum*
Harm/hex	20-100 200-600	0.0 to 0.0 0.0 to 17.5±2.16	600	0.803	0.054	*P. digitatum*
Harm/wat	20-100 200-600	0.0 to 0.0 12±1.34 to 24±2.88	375	0.914	0.011	*P. italicum*
Harm/wat	20-100 200-600	0.0 to 17.25±0.5	375	0.908*	0.012	*P. digitatum*
		17.5±2.6 to 21.5±1.75				
Harm/bet	20-100 200-600	0.0 to 0.0 0.0 to 19.5±1.77	375	0.806	0.053	*P. italicum*
Harm/bet	20-100 200-600	0.0 to 15.75±0.5				
		17±0 to 22.5±2.25	375	0.930**	0.007	*P. digitatum*

[a]Extract source: Cinn: Cinnamon (*Cinnamomum cassia*) bark, stick: Sticky fleabane leaves (*Inulla viscosa*), Harm: Harmal (*Peganum harmala*) seeds, crude: Crude extract, met: Methanolic fraction, hex: Hexane fraction, wat: Water fraction, bet: Between layers fraction, [b]A range of concentrations (20, 50 100, 200, 400 and 600 µg mL^{-1}) of extract was nsed against tested fungal isolates, [c]Fungal inhibition zone was determined from the mean diameter (mm) zone±SD of three independent tests nsing agar well diffusion method, [d]Miuimal inhibitory concentration of the extract, [e]Correlation coefficient values, [f]Level of significance at the 0.05 and 0.01 levels (2- tailed), **Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed)

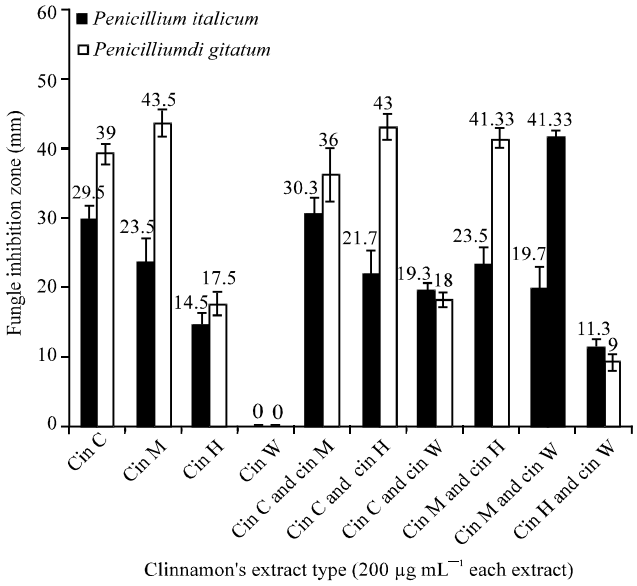

Fig. 1: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolate s obtained by 200 µg mL^{-1} of cinnamon different fractions, Cin: Cinnamon, C: Crude extract, C: Crude extract, M: Methanolic fraction, H: Hexane fraction; W: Water fraction

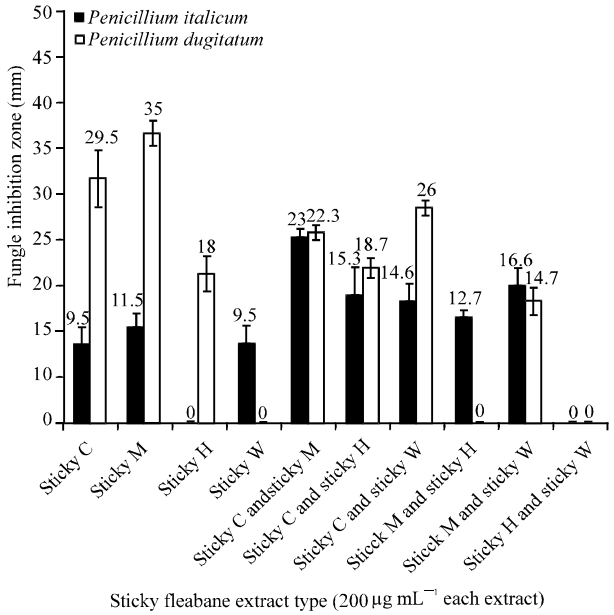

Fig. 2: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by 200 µg mL^{-1} of sticky fleabane (*Inulla viscosa*) crude extract, its liquid fractions and combinations of these fractions. C: Crude extract, M: Methanolic fraction, H: Hexane fraction and W: Water fraction

were obtained) (Fig. 1). The combined concentrations (200 µg mL^{-1} each) of sticky fleabane crude extract and its hexane fraction resulted in synergistic effects against *P. italicum* (Fig. 2). The mean

inhibition zone obtained by the above mentioned combination was approximately 16 mm as compared to 0.0 and 9 mm obtained by the crude extract and its hexane fraction, respectively. However, the combination of sticky fleabane methanolic and hexane fractions has preserved the inhibitory effect obtained by the methanolic fraction against *P. italicum* (Fig. 2), where inhibition zones of approximately 12 mm were obtained. Mixtures between sticky fleabane crude extract and either it's methanolic or water fraction resulted in additive effects against *P. italicum*. Moreover, mixtures of sticky fleabane methanolic and water fractions had also generated additive effects against *P. italicum*. Regarding the effects of the remaining possible combinations made between sticky fleabane extract and its liquid fractions, all have generated antagonistic effects against both fungal species tested (Fig. 2). The mixture of methanolic and hexane fractions resulted in no inhibitory effect against *P. digitatum,* as compared to approximately 35 and 18 mm inhibition zones obtained by each fraction alone, respectively. Also, the combination made between sticky fleabane hexane and water fractions showed no inhibitory effect against both fungal species, although hexane fraction was relatively effective (approximately 18 mm) against *P. digitatum* but not *P. italicum* (no inhibitory effect). In addition, the water fraction generated 9 mm inhibition zone against *P. italicum* but no activity was detected against *P. digitatum* (Fig. 2).

Mixtures made of cinnamon's crude extract and sticky fleabane (each of 200 μg mL^{-1}) or its methanolic fraction generated inhibition zones against *P. digitatum* of similar sizes (i.e., approximately in the range of 32 to 39 mm) to these obtained by each extract individually (Fig. 3). Also, mixtures of cinnamon's methanolic fraction and the crude extract of sticky fleabane or its methanolic fraction have generated inhibition zones (in the range of 35 to 44 mm) against *P. digitatum*, with similar sizes to those obtained by each extract alone. The combinations made between cinnamon's methanolic fraction and that of sticky fleabane resulted in inhibition zones against *P. italicum* of similar sizes (approximately 24 mm) to those obtained by cinnamon's

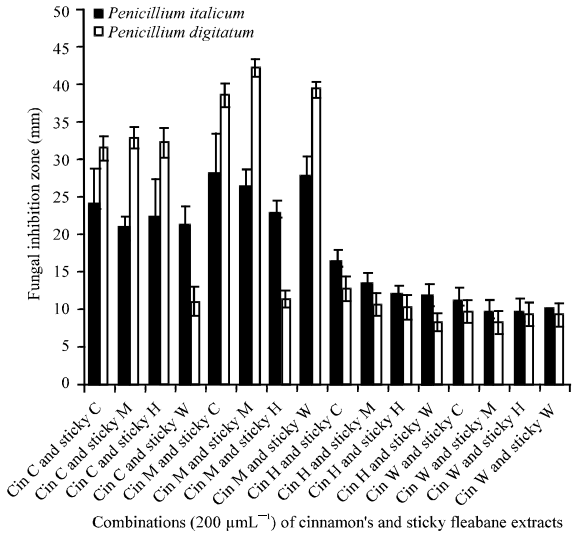

Fig. 3: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by 200 μg mL^{-1} of all possible combinations between cinnamon and sticky fleabane extracts and fractions, Cin: Cinnamon (*Cinnamomum cassia*) bark, sticky: Sticky fleabane (*Inulla viscosa*) leaves, C: Crude extract, M: Methanolic fraction, H: Hexane fraction, W: Water fraction

fractions when tested individually (Fig. 3). In addition, the combinations made between cinnamon's hexane fraction and all fractions of sticky fleabane generated inhibition zones against *P. italicum* of approximately equal sizes to these obtained by cinnamon's hexane fraction (i.e., zones in the range of 12 to 15 mm). In contrast, the combinations (each of 200 μg mL^{-1}) made between cinnamon's and sticky fleabane water fractions and between cinnamons water fraction and the crude extract of sticky fleabane have maintained the inhibition levels obtained by the sticky fleabane extract (i.e., inhibition zones of approximately 10 mm) against *P. italicum*. Similar sized zones to those obtained by cinnamons' methanolic fraction (approximately 25 mm) were generated against *P. italicum* by combined concentrations (each of 200 μg mL^{-1}) of cinnamons' methanolic fraction and sticky fleabane crude or water fraction, namely, 27 mm (Fig. 3). Surprisingly, synergistic effects were obtained by combined concentrations of cinnamons water and sticky fleabane hexane fraction against *P. italicum* where, inhibition zones of approximately 8 mm were obtained as compared to "no inhibition" by individual fractions. The same synergistic effect was obtained against *P. digitatum* by combinations of cinnamon and sticky fleabane water fractions (Fig. 3). The remaining combinations among cinnamons and sticky fleabane fractions all generated antagonistic effects towards both fungal species as compared to the effect of individually tested fraction (Fig. 3).

***In vitro* sensitivity of *Penicillium* species to combinations of cinnamon fractions and harmal fractions:** There was a considerable synergistic effect from the combined concentrations of Harmals' crude extract and each of its methanolic, hexane and the layer between fractions against *P. italicum* (inhibition zones were almost 15 mm) as compared to "no inhibition" exerted by each fraction alone. In addition, synergistic effects of the mixture of harmal seeds crude extract and its hexane fraction against *P. digitatum* were observed, where inhibition zones of approximately 21 mm were generated as compared to no inhibition by hexane fraction alone. However, additive effects against *P. digitatum* were resulted from the combination between harmals' crude extract and its methanolic (zones of 25 mm as compared to 13 mm obtained by each fraction alone) or water fractions (zones of 33 mm as compared to 14 and 18 mm by their individual fraction, respectively). Also, the combinations made between the layer formed between harmal fractions and each of the crude extract or its methanolic fraction generated inhibition zones against *P. digitatum* of approximately similar sizes to those obtained by each fraction individually (Fig. 4).

Furthermore, the combination made between harmals' water fraction and the layer between fractions showed inhibition zones against *P. digitatum* with sizes approximately similar to those obtained by each fraction alone. The remaining possible combinations made between harmal's fractions resulted in antagonistic effects as compared to each fraction alone (Fig. 4).

Mixtures of cinnamons water fraction and each of the crude extract, hexane fraction and the layer between harmals fractions generated synergistic effects against *P. italicum*, where inhibition zones between 8-15 mm were obtained as compared to no inhibition using individual fractions (Fig. 5). However, mixtures between cinnamon's methanolic fraction and each of harmal's fractions were found to reserve the same level of inhibition of cinnamon's fraction alone against *P. italicum* (in the range of 22 to 25 mm). Furthermore, the inhibition zones against *Penicillium* species obtained from the combinations between cinnamon's hexane fraction with harmal extract or any of its liquid fractions were of similar sizes (in the range of 13-18 mm) compared to those obtained by cinnamon's hexane fraction alone. In contrast, the remaining possible combinations between fractions of cinnamon and harmal have reflected antagonistic effects, where reduced inhibition

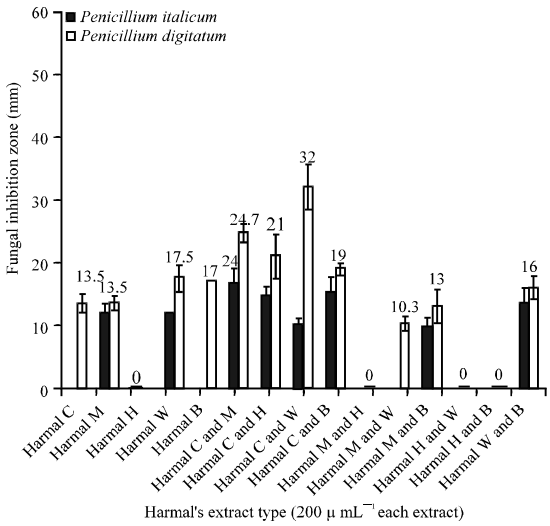

Fig. 4: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by 200 µg mL^{-1} of harmal (*Peganum harmala*) seed crude extract, its liquid fractions and combinations of these fractions, C: Crude extract, M: Methanolic fraction, H: Hexane fraction, W: Water fraction, B: Between layers fraction

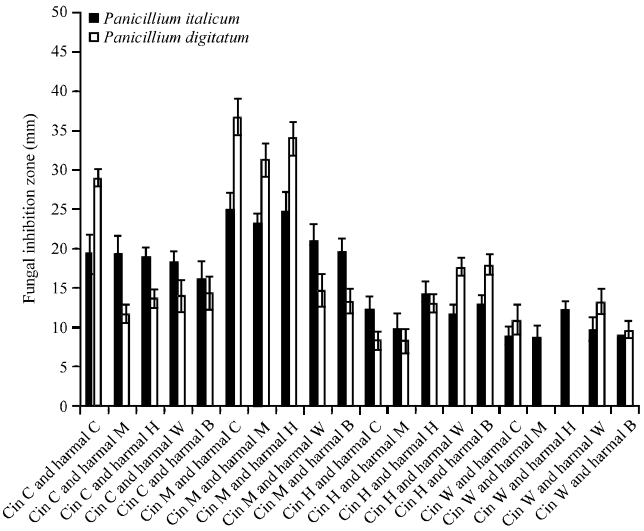

Fig. 5: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by 200 µg mL^{-1} of all possible combinations between cinnamon and harmal extracts and fractions, Cin: Cinnamon (*Cinnamomum cassia*) bark, harmal: Harmal (*Peganum harmala*) seeds, C: Crude extract, M: Methanolic fraction, H: Hexane fraction, W: Water fraction and B: Between layers fraction

zones (in the range of 8-36 mm) were obtained from such combinations as compared to zones in the range of 18-44 mm resulted from any of the cinnamon's fractions alone (with the exception of cinnamon's water fraction, where no inhibition zones were noticed).

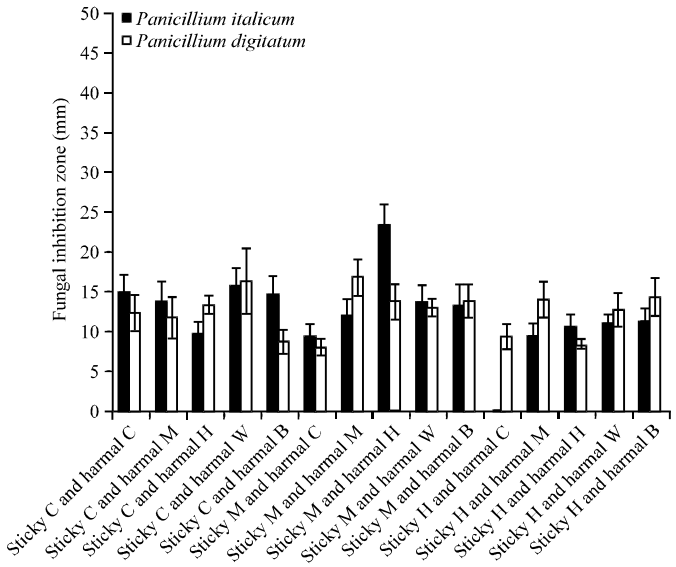

Fig. 6: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by 200 μg mL^{-1} of all possible combinations between sticky fleabane and harmal extracts and fractions, sticky denotes sticky fleabane (*Inulla viscosa*) leaves, harmal denotes harmal (*Peganum harmala*) seeds, C: Crude extract, M: Methanolic fraction, H: Hexane fraction, W: Water fraction and B: Between layers fraction

***In vitro* sensitivity of *Penicillium* sp. to combinations of sticky fleabane fractions and harmal fractions:** Results presented in Fig. 6, indicate that mixtures of sticky fleabane leaves and harmal seeds hexane fractions have generated synergistic effects against both *Penicillium* species. The obtained inhibition zones were approximately 11 mm as compared to no inhibition effect for single extracts (Fig. 2, 4). Also, mixtures of sticky fleabane methanolic fraction and harmal hexane fraction exerted synergistic effects against *P. italicum*. However, mixtures from sticky fleabane methanolic fraction and any of the harmals' fractions maintained the same inhibitory level of the sticky fleabane fraction (inhibition zone of 11-13 mm) compared to those obtained by the sticky fleabane fraction alone (Fig. 2). The remaining combinations between sticky fleabane and harmals' fractions showed antagonistic effects against *P. digitatum*, where smaller sized zones by such combinations were obtained (Fig. 6).

***In vitro* sensitivity of *Penicillium* isolates to various concentrations of low toxicity-food preservatives:** A significant correlation (at the 0.01 level of significance) between tested concentrations (in the range of 20 to 400 μg mL^{-1}) of all examined food preservative chemicals and mean inhibition zones (mm) of both *Penicillium* species was obtained (Table 2). An exception to this trend was Na-molybdate, where the correlation between tested concentrations and inhibitory zones of *P. italicum* was not significant ($r = 0.464$; $p = 0.150$). Na-benzoate was the most effective against both fungal species. The MIC values against *P. digitatum* and *P. italicum* were 37.5 and 75 μg mL^{-1}, respectively. Furthermore, NH_4-molybdate and K-carbonate showed the next level of strength after Na-benzoate against the tested fungal species (MIC values approximately 175 μg mL^{-1}). Na-bicarbonate and Na-molybdate were the least effective against the tested fungal species (Table 2), where no inhibition zones against *P. digitatum* were obtained with

Table 2: *In vitro* sensitivity of two wild-type fungal isolates, *Penicillium italicum and P. digitatum*, to different concentrations (μg mL^{-1}) of five chemical substances used as food preservatives

Chemical substance[a]	Range of conc (μg mL^{-1})[b]	Range of Inhibition[c] zone (mm) mean±SD	MIC[d]	Corr. value[e] (r)	Sig. value[f]	*Fungal isolate*
Na-benzoate	20-120 160-400	0.0-20±1.44	75	0.464	0.150	*P. italicum*
		20.0±2.12 to 28.5±1.6				
Na-benzoate	20-120 160-400	0.0-24±2.45	37.5	0.914**	0.000	*P. digitatum*
		24.5±2.34 to 62.5±2.12				
Na-molybdate	20-120 160-400	0.0-0.0 0.0-22.5±1.32	200	0.635*	0.036	*P. italicum*
Na-molybdate	20-120 160-400	0.0-0.0 0.0-0.0				*P. digitatum*
NH_4-molybdate	20-120 160-400	0.0-14±1.67	275	0.925**	0.000	*P. italicum*
		14±1.67 to 17.5±1.12				
NH_4-molybdate	20-120 160-400	0.0-15±2.77	175	0.973**	0.000	*P. digitatum*
		24.5±2.45 to 37±2.56				
K-carbonate	20-120 160-400	0.0-15.5±2.11	175	0.973**	0.000	*P. italicum*
		18±1.33 to 35.5±3.11				
K-carbonate	20-120 160-400	0.0-15±1.44	175	0.973**	0.000	*P. digitatum*
		17±2.11 to 26±2.18				
Na- bicarbonate	20-120 160-400	0.0-0.0 0.0 to 17±1.55	275	0.742**	0.009	*P. italicum*
Na- bicarbonate	20-120 160-400	0.0-0.0 0.0 to 13±1.23	350	0.808**	0.003	*P. digitatum*

a: Five chemical substance were tested against two wild-type fungal isolates representing two *Penicillium* species indicated above. b: a range of concentrations (20, 30, 40, 80, 120, 160, 200, 240, 280, 320 and 400 μg mL^{-1}) of each chemical substance was nsed against tested fungal isolates. c: Fungal inhibition zone was determined from the mean diameter (mm) zone±SD of three independent tests nsing agar well diffusion method. d: Minimal inhibitory concentration of the tested chemical substance. e: correlation coefficient values. f: level of significance at the 0.05 and 0.01 levels (2- tailed). ** Correlation is significant at the 0.01 level (2-tailed).*Correlation is significant at the 0.05 level (2-tailed)

Na- molybdate at a range of concentrations from 20 to 400 μg mL^{-1}. Moreover, no inhibition zones were obtained with either Na-molybdate or Na-bicarbonate within a range of concentrations from 20 to 160 μg mL^{-1}.

***In vitro* sensitivity of *Penicillium* isolates to various combinations of low toxicity-food preservatives:** Substantial synergistic killing effects against both fungal species from almost all possible combinations made between various tested chemicals (each of 160 μg mL^{-1}) were obtained (Fig. 7). The combinations between Na-benzoate and the remaining chemicals generated inhibition zones of approximately 55 mm against *P. italicum*, while the inhibition zones from each chemical alone were in the range of 0.0 to 18 mm. Furthermore, inhibition zones in the range of 37 to 63 mm against both fungal species were obtained from combinations made between Na-molybdate or NH_4-molybdate and the remaining chemicals. Moreover, synergistic effects against both *Penicillium* species from the combined concentrations of carbonate and bicarbonate were observed which were in the range of 53 mm to 55 mm compared to zones of 0.0 to 18 mm obtained by each chemical, respectively.

***In vitro* sensitivity of *Penicillium* isolates to various combinations of cinnamon's extracts and low toxicity-food preservatives:** Synergistic effects against *P. italicum* from all combinations made between Na- benzoate and cinnamons' extract or its liquid fractions were generated (Fig. 8). In contrast, antagonistic effects by these combinations against *P. digitatum* were noticed. Zones of inhibition against *P. italicum* by benzoate and cinnamon fractions were in the range of 38 to 57 mm, whereas, zones obtained by each substance alone were in the range of

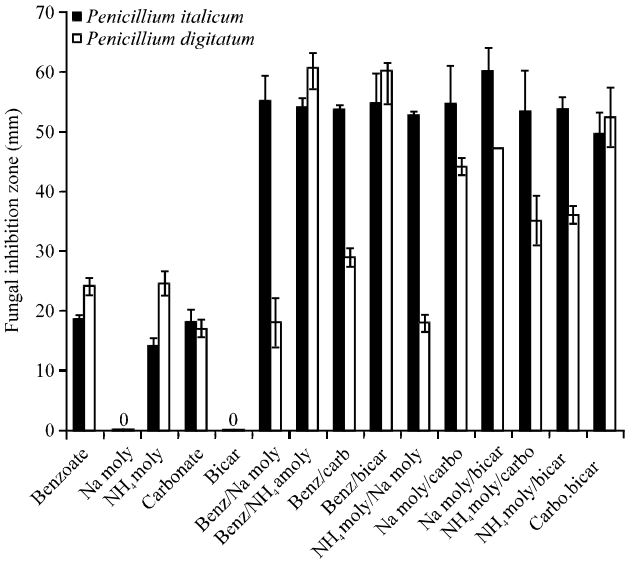

Fig. 7: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by 160 μg mL^{-1} of each chemical type (as single) and combination of two substances (each of 160 μg mL^{-1}), tested chemical substances are: Na-benzoate, Na- molybdate, NH_4-molybdate, K-carbonate and Na-bicarbonate tested as singles and pair-wise combinations

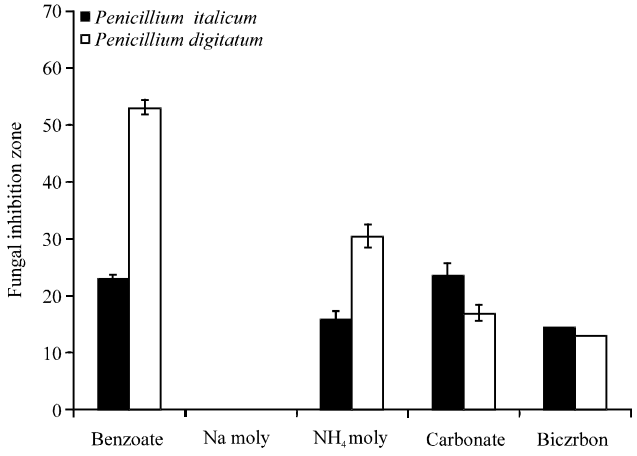

Fig. 8: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by five chemical substances (200 μg mL^{-1} of each chemical alone). Tested chemical substances are: Na-benzoate, Na-molybdate, NH_4-molybdate, K-carbonate and Na-bicarbonate, addition of Na-molybdate generated no inhibition

0.0 to 24 mm (Fig. 1, 8). In contrast, the antagonistic effects generated by these combinations against *P. digitatum* showed inhibition zones in the range of 18 to 22 mm. Such mixtures clearly reduced the efficacy of benzoate alone (Fig. 9) against *P. digitatum* (approximate zone of 53 mm). Furthermore, synergistic effects against both fungal species from combinations between Na-molybdate and cinnamon's liquid fractions were obtained. Inhibition zones by such combinations were in the range of 42-53 mm compared to zones in the range of 0.0 to 24 mm exerted by each

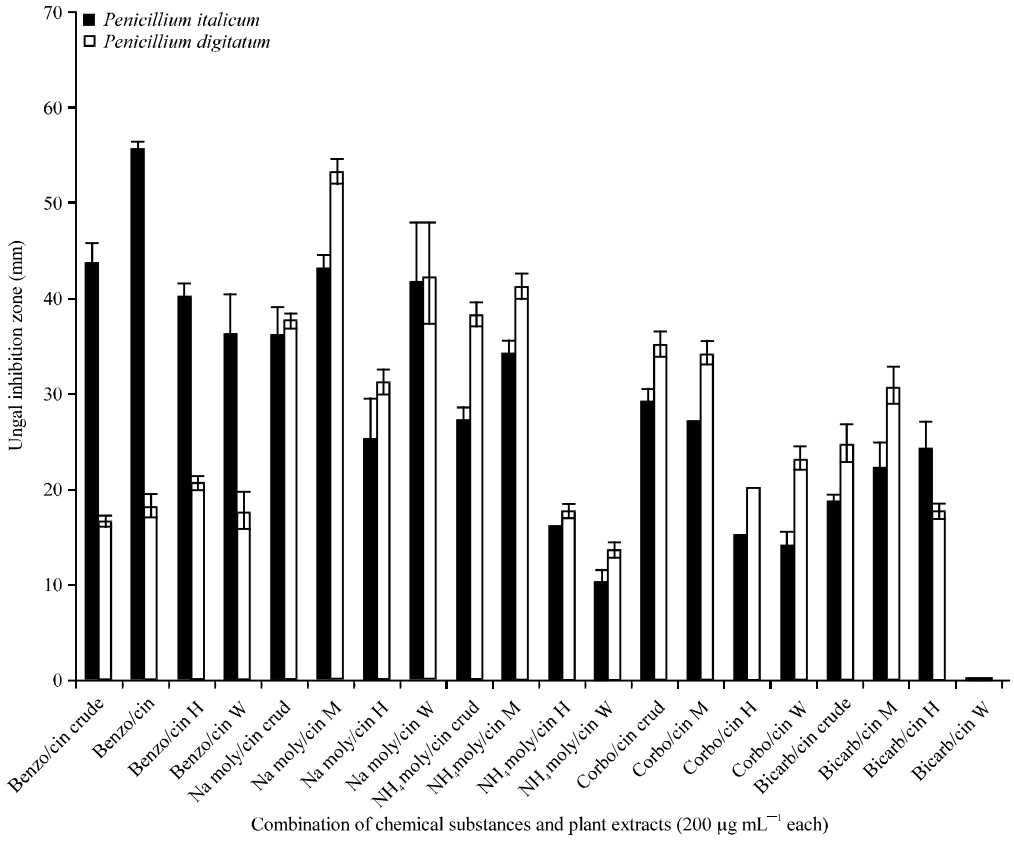

Fig. 9: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by combinations of cinnamon crude extract or its liquid fractions and chemical substances (200 µg mL^{-1} each) Tested chemical substances are: Na-benzoate, Na-molybdate, NH_4-molybdate, K-carbonate and Na-bicarbonate, each chemical substance was tested in a combination with cinnamon crude extract and its liquid fractions: cin crude: Cinnamon crude extract, cin M: Cinnamon methanolic fraction, cin H: Hexane fraction, cin W: Water fraction

substance alone. Moreover, the combination made between Na- molybdate and cinnamons' water fraction has enhanced the inhibitory effect against both fungal species considerably, where zones of approximately 43 mm were obtained (Fig. 9) compared to no inhibitory effect by each individual substance (Fig. 1, 8). An exception to this synergistic pattern was in the activity obtained by the mixture of Na-molybdate and cinnamon's crude extract (approximate zone of 37 mm), where the addition of Na-molybdate (which generated no inhibition) did not negatively influence the activity of cinnamon's extract (inhibition zones were in the range of 30-39 mm). The mixtures made between NH_4-molybdate or Na-carbonate and cinnamon's fractions exerted inhibitory effects (against both fungal species) that were similar to those obtained by cinnamons' fractions when tested individually, i.e., the activity of cinnamon fraction was maintained and has not been influenced by the combined chemicals. An exception to this was the combination of Na-carbonate and cinnamon's water fraction (no inhibition zones when tested individually), where a synergistic effect against *P. digitatum* was obtained (i.e., double sized zones of 28 mm). In contrast, the

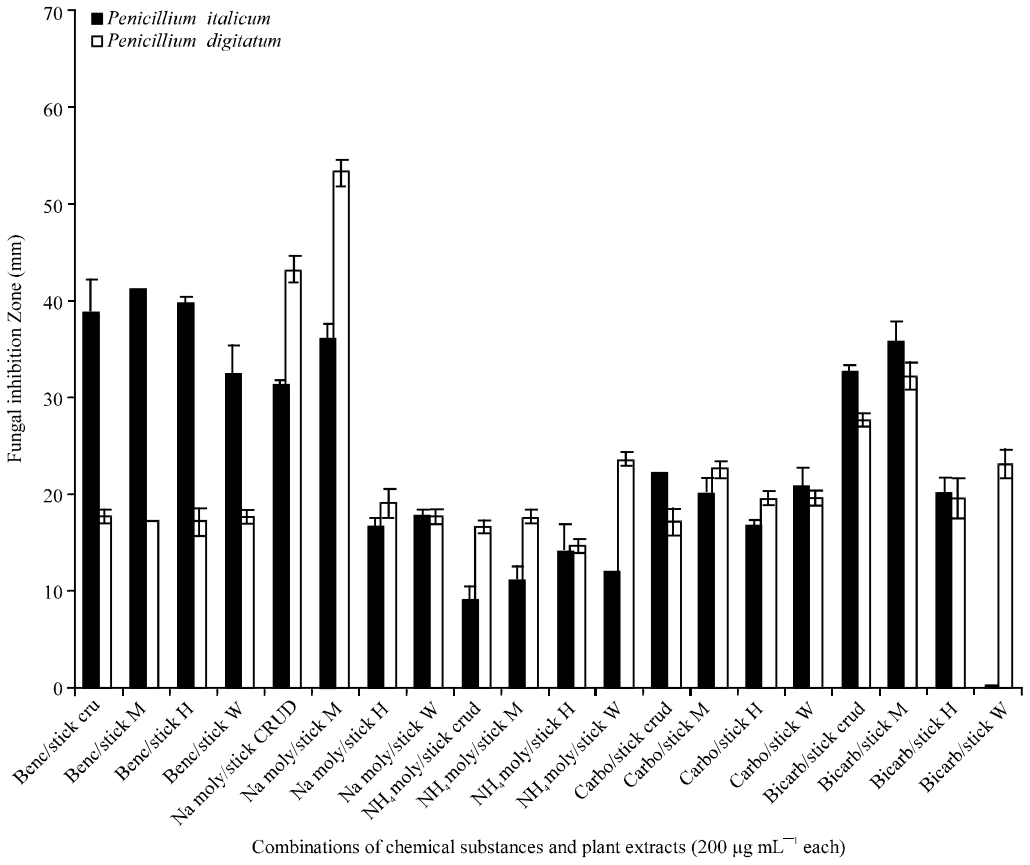

Fig. 10: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by combinations of chemical substances and plant extract or its liquid fractions (200 µg mL^{-1} each), tested chemical substances are: Na-benzoate, Na-molybdate, NH_4-molybdate, K-carbonate and Na-bicarbonate, each chemical substance was tested in combination with sticky fleabane crude extract and its liquid fractions: Stick cru: Sticky fleabane crude extract, stick, M: Sticky fleabane methanolic fraction, stick H: Hexane fraction, stick W: Water fraction

addition of cinnamon's water fraction to Na- bicarbonate had completely abolished the effect of bicarbonate, where no inhibitions by this combination against both fungal species were obtained.

***In vitro* sensitivity of *Penicillium* isolates to various combinations of sticky fleabane extract and low toxicity-food preservatives:** The combinations made between Na-benzoate and any of the sticky fleabane fractions had synergistic effects against *P. italicum* and antagonistic effects against *P. digitatum* (Fig. 10). The obtained inhibition zones against *P. italicum* by mixtures of benzoate and either methanolic or hexane fraction of sticky fleabane leaves were approximately of 42 mm in size compared to zones of 0.0 to 22 mm generated by each substance alone (Fig. 2 and 8). An exception to this was the activity of mixtures of benzoate and either the crude extract or water fraction of sticky fleabane, where additive effects were monitored against *P. italicum* (Fig. 10). The obtained antagonistic effects against *P. digitatum* by benzoate and sticky fleabane fractions indicated that the combined plant fractions have negatively influenced the

activity of benzoate. The obtained inhibition zones by benzoate alone (approximately 53 mm) were greatly reduced to approximately 18 mm in all tested combinations. In contrast, all combinations of Na-molybdate and sticky fleabane fractions had synergistic effects against both fungal species. The obtained inhibition zones by these combinations ranged from 16 to 35 mm against *P. italicum* and from 18 to 54 mm against *P. digitatum*. However, the obtained zones by each substance alone against *P. italicum* and *P. digitatum* ranged from 0.0 to 11 and 0.0 to 35 mm, respectively. The combinations made between Na- bicarbonate and sticky fleabane fractions had synergistic effects against *P. italicum*, whereas activities against *P. digitatum* were similar to those obtained by the plant fractions alone (Fig. 10). However, antagonistic effects against both fungal species were generated from combinations of NH_4-molybdate or K-carbonate and sticky fleabane fractions.

***In vitro* sensitivity of *penicillium* isolates to various combinations of harmal extracts and low toxicity-food preservatives:** The combinations between benzoate and either harmal's crude extract or its hexane fraction exerted synergistic effects against *P. italicum* (inhibition zones were in the range of 35 to 38 mm) (Fig. 11), while zones in the range of 0.0 to 22 mm were obtained by each substance alone (Fig. 4, 8). The combinations of benzoate and harmal's methanolic fraction, also generated additive effects against *P. italicum* (approximately 32 mm inhibition zone).

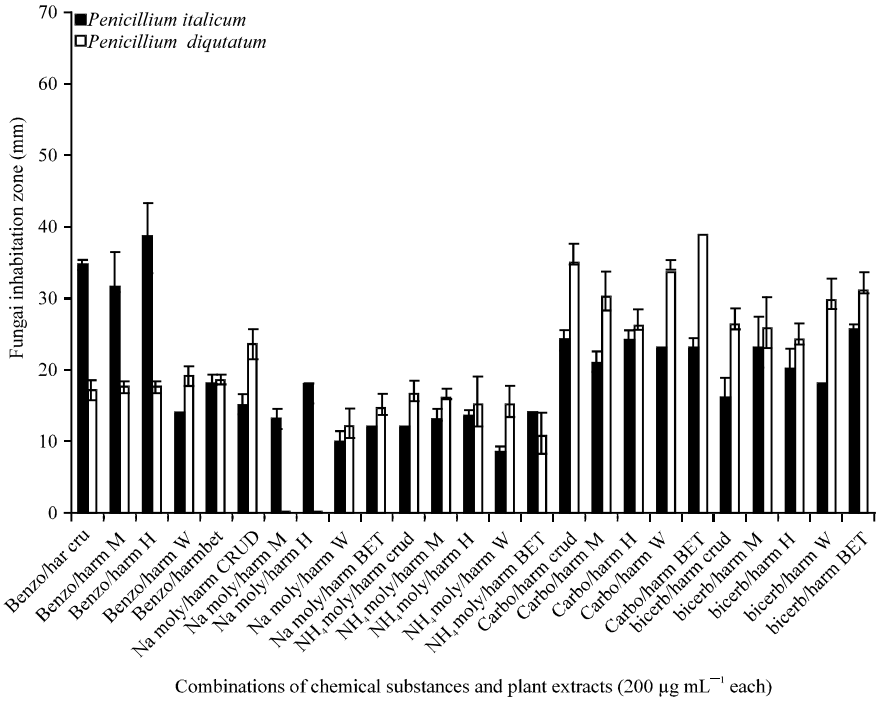

Fig. 11: Inhibition zone of *Penicillium italicum* and *P. digitatum* wild-type isolates obtained by combinations between each chemical substance (200 µg mL^{-1}) and harmal extract or liquid fraction (200 µg mL^{-1}), tested chemical substances are: Na-benzoate, Na- molybdate, NH_4-molybdate, K-carbonate and Na-bicarbonate, each chemical substance was tested in a combination with harmal crude extract and its liquid fractions: Harm cru denotes, harmal crude extract, harm M: Harmal methanolic fraction, harm H: Hexane fraction, harm W: Water fraction and harm bet: The layer between fractions

In contrast, the above mentioned mixture generated antagonistic effects against *P. digitatum* (approximately 17 mm inhibition zone) where the plant fraction greatly reduced the activity obtained by benzoate alone (approximately 53 mm inhibition zone). Furthermore, synergistic effects against *P. italicum* were generated by mixtures of Na-molybdate and each of harmals crude extract, hexane fraction or the layer between fractions (Fig. 11). Inhibition zones of approximately 14 mm were obtained by these combinations as compared to zero zones by any of the tested substances individually. In contrast, the combinations between Na-molybdate and either methanolic or hexane fraction of harmal generated no inhibitory effect against *P. digitatum*. In contrast, mixtures of carbonate or bicarbonate and a harmal's fraction generated additive effects against *P. digitatum*, where inhibition zones in the range of 28 mm to 38 mm were obtained. An exception to this pattern was the combination between carbonate or bicarbonate and harmal's hexane fraction, where synergistic effects against *P. digitatum* were obtained (inhibition zones in the range of 24 to 26 mm) compared to 0.0 to 18 mm by each substance alone (Fig. 4, 8). However, the combinations between carbonate or bicarbonate and harmal's fractions generated inhibitory zones (in the range of 22 to 24 mm) against *P. italicum* of similar sizes to those obtained by each chemical alone. Furthermore, the combinations of NH_4-molybdate and harmal's fractions exerted antagonistic effects against *P. digitatum* (i.e., half-sized zones of 16 -18 mm), whereas inhibition zones of similar sizes (i.e., inhibition zones of 13-16 mm) to these obtained by NH_4-molybdate alone against *P. italicum* were obtained.

DISCUSSION

Results of this study indicate that cinnamon's bark, sticky fleabane leaves extracts and their methanolic fractions have shown the highest efficacy against both fungal species tested. Cinnamon's extract showed MIC values of 75 and 187.5 μg mL^{-1} against *P. digitatum* and *P. italicum*, respectively. Whereas, sticky fleabane extract revealed MIC values of 150 and 375 μg mL^{-1} against the same tested species, respectively. These findings disagreed with that obtained by Al-Najar (2007), where none of the mentioned extract types has resulted in complete inhibition of fungal growth within a range of concentrations from 50 to 520 μg mL^{-1}, although such extracts were the most effective (among other tested extracts) against tested isolates (*in vitro*) of both fungal species. In contrast, previous results from the *in vivo* study against *P. italicum* isolates revealed that the same extract types have completely inhibited the growth of all *P. italicum* tested isolates that infect orange rather than lemon fruits (Al-Najar, 2007). Concerning the effect of cinnamon's and sticky fleabane methanolic fractions, the results indicate that MIC values of 37.5 and 75 μg mL^{-1} were obtained against *P. digitatum* with the above mentioned plant extracts, respectively. Also, these fractions have generated MIC values of 150 and 375 μg mL^{-1} against *P. italicum,* respectively. Clearly, the results obtained previously with the same fractions (Al-Najar, 2007; Kanan and Al-Najar, 2008, 2009) are consistent with our findings, where these fractions have completely inhibited the growth of both fungal species tested. The IC_{50} values obtained with cinnamons fraction against several *P. digitatum* isolates were within the range of 5-23 μg mL^{-1}, whereas, IC_{50} values within the range of 27.25- 31.75 μg m^{-1} were obtained with sticky fleabane fraction (Kanan and Al-Najar, 2008). In addition, the same liquid fractions have completely inhibited the growth of several *P. italicum* isolates where, IC_{50} values within the range of 11.2- 24 and 25-36 μg mL^{-1} were obtained with cinnamons' and sticky fleabane fractions, respectively (Kanan and Al-Najar, 2009). The combinations (200 μg mL^{-1} each) made between cinnamon's methanolic fraction and that of sticky fleabane or harmal's fractions have maintained

the inhibitory effect obtained by cinnamon's fraction, towards tested fungal species and here, similar sized inhibition zones to those obtained by cinnamon's fraction were generated. The obtained results suggest that the high efficacy of cinnamon's extract is related to the presence of certain bioactive agents which might include cinnamaldehyde, eugenol, cinnamic acid as well as flavonoids, alkaloids, tannins, anthraquinones and phenolic compounds. These active components have been shown previously to have a strong antifungal activity (Inouye *et al*., 2000; Gill and Holley, 2004). However, the detected efficacy may be traced mainly to cinnamaldehyde that works as an inhibitor for enzymes such as **β**-(1,3)-glucansynthase which is involved in chitin and **β**- glucans cell wall components biosynthesis (Cowan, 1999). This possibility coincides with the findings of Rojas *et al*. (1992), who identified such components as active antifungal agents. Furthermore, eugenol and cinnamaldehyde have consistently been reported to be the main components of cinnamon exhibiting high fungitoxic activity (Jham *et al*., 2005). As indicated above, the sticky fleabane (*Inula viscosa*) crude extract and its methanolic fraction possess high efficacy against the tested fungi which is in agreement with previous findings by Kanan and Al-Najar (2008, 2009). Wang *et al*. (2004) proposed that sticky fleabane extract exhibit significant activity against several pathogenic fungal species of various crop plants including dermatophytes and downy mildew. However, the obtained results disagreed with some findings of (Muller-Riebau *et al*., 1995), who found small amounts of antifungal essential oils or phenolics. The antifungal oils and phenolics seem to affects chitin biosynthesis due to high content of flavonoids, phenolic compounds and anthraquinones in methanolic and aqueous extracts of sticky fleabane (Cohen *et al*., 2006). These observations are in agreement with those reported previously by other researchers (Ali-Shtayeh and Abu Ghdeib, 1999; Cafarchia *et al*., 2002; Cohen *et al*., 2006; Al-Najar, 2007). The mixtures of sticky fleabane methanolic and hexane fractions have abolished the inhibitory effect of both fractions against *P. digitatum*. In addition, combining both fractions (sticky fleabane hexane and water) did not show any inhibition effect against the tested fungal species. It can thus be suggested that active components in fractions had antagonistic effect on each other when combined, since their availability in a pair-wise form or in the crude extract has reduced their efficacy. Our results indicate that among tested plant materials, harmal's extract and its liquid fractions were the least effective against both fungal species tested. These findings support previous findings by Kanan and Al-Najar (2009) which showed that none of the harmal's extracts has led to complete inhibition of any of four tested *P. italicum* isolates. However, *P. digitatum* isolates were found to be more susceptible to the plant extracts tested (Kanan and Al-Najar, 2008). The inhibitory activity of the crude extract may be related to high content of alkaloids (harmine, harmaline and tetrahydroharmine) and phenolic compounds, where these compounds may alter fungal cell membrane permeability and thus allow the deficit of macromolecules (Rasooli and Razzaghi-Abyaneh, 2004). This could be due to the ability of phenols to inactivate essential enzymes that react with cell membrane proteins or by disrupt functions of the genetic material (Kartal *et al*., 2003; Telezhenetskaya and D'yakonov, 2004). The *in vitro* treatment of fungal isolates with sodium benzoate was the most effective of all tested chemicals in controlling both *P. digitatum* and *P. italicum*.

These findings coincided with those obtained previously by Al-Najar (2007) where, MIC values from 4 to 50 mM was shown to be effective for complete fungal growth inhibition. Also, our findings agreed with that proposed by Buazzi and Maeth (1992), who stated that benzoate prevented the growth of nearly all examined microorganisms. However, the effectiveness of sodium benzoate was shown to be pH dependent because its undissociated form is mostly accountable for the

antimicrobial activity, since most organic acid salts are effectively inhibitory at pH 5-5.5 and lower (the PKa value of potassium benzoate is 4.2) (Palou *et al*., 2002a). Ammonium molybdate and potassium carbonate were ranked as the second most effective (after Na-benzoate) against tested fungal species (MIC values of approximately 175 μg mL^{-1}). Results obtained with the molybdate agreed with findings of Palou *et al*. (2002a) who declared that 5 mM NH_4-molybdate totally controlled both blue and green moulds on all cultivars of orange fruits. Furthermore, Al-Najar (2007) proposed that potassium molybdate has significantly inhibited the growth of four tested strains of *P. digitatum*, where the toxicity of molybdate has resulted in the formation of dark bluish-petroleum, color in the inoculation site, when a concentration of 400 mM was used. As compared to the activity of NH_4-molybdate, Na-molybdate showed no inhibition effect against both fungal species tested within a concentration range of 20-400 μg mL^{-1}. However, these findings disagreed with findings of Al-Najar (2007) who proposed that MIC values in the range of 250-500 mM (IC_{50} values from 47.5-350 mM) were required to inhibit the growth of *P. italicum* isolates. Also, these results disagreed with findings of Palou *et al*. (2002a) who stated that both sodium and ammonium molybdate have effectively controlled the growth of green and blue moulds of citrus fruits. However, results of this study indicate that the synergistic effects against tested pathogenic fungi were obtained by activity of the following mixtures (200 μg mL^{-1} each): sodium and ammonium molybdate mixture; mixtures of either sodium or ammonium molybdate and any of the remaining tested chemicals, mixture of sodium molybdate and cinnamons' fractions and mixtures of carbonate and bicarbonate.

The generated synergistic effects by mixtures of substances having different origins and modes of actions may propose that these mixtures leave the fungus unable to disrupt binding of the mixture to their protein at the binding site. In addition, the used mixtures may render the efflux mechanisms inefficient to pump the toxic substances out of the cell. Furthermore, the tested mixtures may inhibit certain metabolic pathways that usually aid in converting the toxic materials into non-toxic forms, i.e., the mixture might inhibit specific proteins that usually can detoxify the substance.

CONCLUSION

Results of this study indicate that cinnamon's bark extract was the most efficient against tested fungal species (MIC values of approximately 75 and 187.5 μg mL^{-1} were obtained against *P. digitatum* and *P. italicum*, respectively). Methanolic fractions of cinnamon's bark and gluey fleabane leaves extract exhibited the highest value against both fungal species tested. However, cinnamon's fraction was more efficient at lower concentrations (MIC values of approximately 37.5 μg mL^{-1}). Mixtures of cinnamon's methanolic fraction and either hexane or water fraction of cinnamon have maintained the efficiency of the methanolic fraction against both fungal species. The synergistic effects against *P. italicum* were obtained from mixtures of sticky fleabane extract and its hexane fraction, whereas additive effects against the same species were obtained by mixtures of sticky fleabane extract and its methanolic or water fraction. Also, a synergistic effect against *P. italicum* were obtained by mixtures of harmal extract and each of its methanolic, hexane or layer between fractions compared to no inhibitory effect by each extract type alone. The same synergistic effect was generated against *P. digitatum* by mixtures of harmal's extract and its hexane fraction, although no inhibition was achieved by hexane fraction alone. However, additive effects were obtained against *P. digitatum* by mixtures of harmal's extract and either its methanolic (zones of approximately 25 mm) or water fraction (zones of approximately 33 mm). Na-benzoate

was the most effective (MIC values in the range of 75 μg mL^{-1}- 37.5) against fungal species tested. NH_4-molybdate and K-carbonate were ranked the second in terms of efficacy against tested fungal species (MIC of approximately 175 μg mL^{-1}). All combinations between tested chemicals have generated synergistic inhibitory effects against both fungal species. Combinations made between Na-benzoate and either cinnamon's or sticky fleabane fractions generated synergistic effects against *P. italicum* and antagonistic effects against *P. digitatum*. The combinations made between Na-molybdate and cinnamon's liquid fractions resulted in synergistic effects against both fungal species. The mixture of Na-molybdate and cinnamon's water fraction has enhanced the inhibitory effect of both substances against fungal species tested, where zones of approximately 43 mm were obtained as compared to no inhibition effect of each individual substance. All combinations of Na-molybdate and sticky fleabane fractions have reflected synergistic effects against both fungal species. The mixture of Na-benzoate and harmal's methanolic fraction generated additive effects against *P. italicum* and antagonistic effects against *P. digitatum* where, the plant fraction has significantly reduced the activity of benzoate. Synergistic effects against *P. italicum* were also obtained by the combination of either Na-benzoate or Na-molybdate with harmal's extract or its hexane fraction.

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REFERENCES

Abad, M.J., M. Ansuategui and P. Bermejo, 2007. Active antifungal substances from natural sources. ARKIVOC., 2007: 116-145.

Abu-Zarga, M.H., E.M. Hamed, S.S. Sabri, W. Voelter and K.P. Zeller, 1998. New sesquiterpenoids from the Jordanian medicinal plant *Inula viscose*. J. Nat. Prod., 61: 798-800.

Al-Najar, R.A., 2007. Selection and evaluation of alternatives to synthetic fungicides for the control of post-harvest citrus fruits rot caused by *Penicillium italicum* (blue mould) in Jordan. M.Sc. Thesis, Mutah University, Jordan.

Ali-Shtayeh, M.S. and S.L. Abu Ghdeib, 1999. Antifungal activity of plant extracts against dermatophytes. Mycoses, 42: 665-672.

Ambrozin, A.R., P.C. Vieira, J.B. Fernandes, M.F. da Silva, S. Albuquerque, 2004. Trypanocidal activity of Meliaceae and Rutaceae plant extracts. Mem. Inst. Oswaldo Cruz., 99: 227-231.

Bhyan, S.B., M.M. Alam and M.S. Ali, 2007. Effect of plant extracts on *Okra mosaic virus* incidence and yield related parameters of Okra. Asian J. Agric. Res., 1: 112-118.

Bonjar, G.H.S. and A.K. Nik, 2004. Antibacterial activity of some medicinal plants of Iran against *Pseudomonas aeruginosa* and *P. fluorescens*. Asian J. Plant Sci., 3: 61-64.

Bonjar, G.H.S., 2004. Screening for antibacterial properties of some Iranian plants against two strains of *Escherichia coli*. Asian J. Plant Sci., 3: 310-314.

Bouzerda, L., H. Boubaker, E.H. Boudyach, O. Akhayat and A.A. Bin Aoumar, 2003. Selection of antagonistic yeasts to green mold disease of citrus in Morocco. Food Agric. Environ., 1: 215-218.

Buazzi, M.M. and E.H. Maeth, 1992. Characteristics of sodium benzoate injury of Listeria monocytogenes. Microbios, 70: 199-207.

Cafarchia, C., N. de Laurentis, M.A. Milillo, V. Losacco and V. Puccini, 2002. Antifungal activity of essential oils from leaves and flowers of *Inula viscosa* (Asteraceae) by Apulian region. Parassitologia., 44: 153-156.

Cohen, Y., W. Wang, B.H.B. Daniel and Y. Ben-Daniel, 2006. Extracts of *Inula viscosa* control downy mildew of grapes caused by *Plasmopara viticola*. Phytopathology, 96: 417-424.

Cove, D.J., 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochem. Biophys. Acta, 113: 51-56.

Cowan, M.M., 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev., 12: 564-582.

Delaquis, P.J., K. Stanich, B. Girard and G. Mazza, 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. Int. J. Food Microbiol., 74: 101-109.

Gill, A.O. and R.A. Holley, 2004. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. Applied Environ. Microbiol., 70: 5750-5755.

Hasan, M.M., S.P. Chowdhury, Shahidul Alam, B. Hossain and M.S. Alam, 2005. Antifungal effects of plant extracts on seed-borne fungi of wheat seed regarding seed germination, Seedling health and vigour index. Pak. J. Biol. Sci., 8: 1284-1289.

Ikeura, H., N. Somsak, F. Kobayashi, S. Kanlayanarat and Y. Hayata, 2011. Application of selected plant extracts to inhibit growth of *Penicillium expansum* on Apple fruits. Plant Pathol. J., 10: 79-84.

Inouye, S., T. Tsuruoka, M. Watanabe, K. Takeo, M. Akao, Y. Nishiyama and H. Yamaguchi, 2000. Inhibitory effect of essential oils on apical growth of *Aspergillus fumigatus* by vapour contact. Mycoses, 43: 17-23.

Ismail, M. and J. Zhang, 2004. Postharvest citrus diseases and their control. Outlooks Pest Manage., 15: 29-35.

Jham, G.N., O.D. Dhingra, C.M. Jardim and V.M.M. Valente, 2005. Identification of major fungitoxic component of cinnamon bark oil. Fitopatologia, 30: 404-408.

Kanan, G.J. and R.A. Al-Najar, 2008. *In vitro* antifungal activities of various plant crude extracts and fractions against citrus post-harvest disease agent *Penicillium digitatum*. Jor. J. Biol. Sci., 1: 89-99.

Kanan, G.J. and R.A. Al-Najar, 2009. *In vitro* and *In vivo* Antifungal Activities of Various plant extracts and their fractions against citrus post-harvest disease agent *Penicillium italicum*. J. plant. Prot. Res., 49: 341-352.

Kartal, M., M.L. Altun and S. Kurucu, 2003. HPLC method for the analysis of harmol, harmalol, harmine and harmaline in the seeds of *Peganum harmala*. J. Pharm. Biomed. Anal., 31: 263-269.

Larrigaudiere, C., J. Pons, R. Torres and J. Usall, 2002. Storage performance of elementines treated with hot water, sodium carbonate and bicarbonate dips. J. Horit. Sci. Biotech., 77: 314-319.

Lee, K.W., H. Everts and A.C. Beynen, 2004. Essential oils in broiler nutrition. Int. J. Poult. Sci., 3: 738-752.

McGrath, M.T., 2001. Fungicide resistance in Cucurbita Powdery Mildew: Experiences and challenges. Plant Dis., 85: 236-245.

Muller-Riebau, F., B. Berger and O. Yegen, 1995. Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in Turkey. J. Agric. Food Chem., 43: 2262-2266.

Nahunnaro, H., 2008. Effects of different plant extracts in the control of yam rot induced by *Rhizopus stolonifer* on stored yam (*Dioscorea* sp.) in Yola, Adamawa State Nigeria. Agric. J., 3: 382-387.

Ndukwe, I.G., J.D. Habila, I.A. Bello and E.O. Adeleye, 2006. Phytochemical analysis and antimicrobial screening of crude extracts from the leaves, stem bark and root bark of *Ekebergia senegalensis* A. Juss. Afr. J. Biotechnol., 5: 1792-1794.

Obagwu, J. and L. Korsten, 2003. Control of citrus green and blue molds with garlic extracts. Eur. J. Plant Pathol., 109: 221-225.

Palou, L., J.L. Smilanick, J. Usall and I. Vinas, 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate and sodium bicarbonate. Plant Dis., 85: 371-376.

Palou, L., J. Usall, J.A. Munoz, J.L. Smilanick and I. Vinas, 2002a. Hot water, sodium carbonate and sodium bicarbonate for the control of postharvest green and blue molds of clementine mandarins. Postharvest Biol. Technol., 24: 93-96.

Palou, L., J. Usall, J.L. Smilanick, M.J. Aguilar and I. Vinas, 2002b. Evaluation of additives and low-toxicity compounds as alternative chemicals for the control of *Penicillium digitatum* and *Penicillium italicum* on citrus fruit. Pest. Manag. Sci., 58: 459-466.

Plaza, P., A. Sanbruno, J. Usall, N. Lamarca, R. Torres, J. Pons and I. Vinas, 2004. Integration of curing treatments with degreening to control the main postharvest diseases of *Calementine mandarins*. Postharv. Biol. Technol., 34: 29-37.

Prusky, D., J.L. McEvoy, R. Saftner, W.S. Conway and R. Jones, 2004. Relationship between host acidification and virulence of *Penicillium spp*, on apple and citrus fruit. Phytopath, 94: 44-51.

Rasooli, I. and M. Razzaghi-Abyaneh, 2004. Inhibitory effects of Thyme oils on growth and aflatoxin production by *Aspergillus parasiticus*. Food Control, 15: 479-483.

Reddy, K.R.N., C.S. Reddy and K. Muralidharan, 2007. Exploration of ochratoxin a contamination and its management in rice. Am. J. Plant Physiol., 2: 206-213.

Reddy, K.R.N., S.B. Nurdijati and B. Salleh, 2010. An overview of plant-derived products on control of mycotoxigenic fungi and mycotoxins. Asian J. Plant Sci., 9: 126-133.

Rojas, A., L. Hernandez, R. Pereda-Miranda and R. Mata, 1992. Screening for antimicrobial activity of crude drug extracts and pure natural products from Mexican medicinal plants. J. Ethnopharmacol., 35: 275-285.

Samson, R.A., K.A. Seifert, A.F.A. Kuijpers, J.A.M.P. Houbraken and J.C. Frisvad, 2004. Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial β-tubulin sequences. Stud. Mycol., 49: 175-200.

Schroeder, L.L. and L.B. Bullerman, 1985. Potential for development of tolerance by *Penicillium digitatum* and *Penicillium italicum* after repeated exposure to potassium sorbate. Appl. Environ. Microbiol., 50: 919-923.

Smilanick, J.I., B.E. Mackey, R. Reese, J. Usall and D.A. Margosan 1997. Influence of the concentration of soda ash, temperature and immersion period on the control of post-harvest green mold of oranges Plant Dis., 81: 379-382.

Smilanick, J.L., D.A. Margosan, F. Mlikota, J. Usall and I.F. Michael, 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial postharvest practices on their efficacy. Plant Dis., 83: 139-145.

Smilanick, J.L., J. Aiyabei, F. Mlikota, J. Gabler, D. Sorenson and B. Mackey, 2002. Quantification of the toxicity of aqueous chlorine to spores of *Penicillium digitatum* and *Geotrichum citriaurantii*. Plant Dis., 86: 509-514.

Smilanick, J.L., M.F. Mansour, D.A. Margosan, F. Mlikota-Gabler and W.R. Goodwine, 2005. Influence of pH and NaHCO3 on the effectiveness of imazalil to inhibit germination of spores of *Penicillium digitatum* and to control green mold on citrus fruit. Plant Dis., 89: 640-648.

Souza-Fagundes, E.M., A.B.R. Queiroz, O.A.M. Filho, G. Gazzinelli, R. Corra-Oliveira, T.M.A. Alves and C.L. Zani, 2002. Screening and fractionation of plant extracts with antiproliferative activity on human peripheral blood mononuclear cells. Mem. Inst. Oswaldo. Cruz., 97: 1207-1212.

Soylu, E.M., F.M. Tok, S. Soylu, A.D. Kaya and Gulsun, 2005. Antifungal activities of the essential oils on post-harvest disease agent Penicillium digitatum. Pak. J. Biol. Sci., 8: 25-29.

Telezhenetskaya, M.V. and A.L. D'yakonov, 2004. Alkaloids of *Peganum harmala*. Unusual reaction of peganine and vasicinone. Chem. Nat. Comp., 27: 471-474.

Tripathi, P. and N.K. Dubey, 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. Postharv. Biol. Technol., 32: 235-245.

Wang, W., B.H. Ben-Daniel and Y. Cohen, 2004. Extracts of *Inula viscose* control downy milew caused by *Plasmopara viticola* in grape-vines. Phytoparasitica, 32: 208-211.

Zaker, M. and H. Mosallanejad, 2010. Antifungal activity of some plant extracts on *Alternaria alternata*, the causal agent of alternaria leaf spot of potato. Pak. J. Biol. Sci., 13: 1023-1029.

Zhang, H.Y., C. X. Fu, X.D. Zheng, D. He, L.J. Shan and X. Zhan, 2004. Effect of *Cryptococcus laurentii* (Kufferath) skinner in combination with sodium bicarbonate on biocontrol of post harvest green mold decay of citrus fruit. Bot. Bull. Acad. Sin., 45: 159-164.