ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

"Antifungal efficacy and chemical composition of essential oil from the Egyptian sweet orange peel (Citrus sinensis, L)"

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Manuscript Info

Manuscript History:

Received: 17 August 2015
Final Accepted: 26 September 2015
Published Online: October 2015

Key words:

Fruit peel - Citrus sinensis, Essential oil–Antimicrobial-Antifungal Mycotoxins.

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Abstract

Fruits and vegetables wastes and their by-products are formed in great amounts during industrial processing and hence represent a serious problem, as they exert harmful impact on environment. So they need to be managed or they can be utilized. The present study was conducted to investigate in vitro antifungal activities of the essential oil of the Egyptian sweet orange peel. The sample of fresh orange peels was subjected to hydro-distillation for 3 h using a Clevenger-type apparatus and distillates of essential oil was dried over anhydrous sodium sulfate, filtered and stored at -4°C until analyzed. Gas chromatography (GC) and Gas chromatography-mass spectrometry (GC-MS) were used to determine the aromatic compounds of peel oil of sweet orange fruit in this study. The identification of the oil constituents was based on the comparison of their retention indices relative to (C6-C24) n-alkanes either with those of published data or with authentic compounds. And also identified using their MS data compared to those from the NIST mass spectral library and published mass spectra. Antifungal activity was carried out by measuring changes in mold growth, PH, and aflatoxin(s) contents in Yeast Extract Sucrose broth media (YES). Results showed that total of 24 compounds were identified and the major components were limonene (86.75%), linalool (1.96%), α-pinene (1.63%), trance-limonene oxide (1.39%), and γ-terpinene (1.03%). The volatile oil showed antifungal activities against *Aspergillus flavus*, and *Aspergillus paraciticus* under in vitro condition. In conclusion, the potential antifungal activity of essential oil present in the Egyptian sweet orange peel could be applied as food preservative.

INTRODUCTION

Peels of various fruits and vegetables are generally considered as waste product thrown away by us, formed in great amounts during industrial processing, and hence represent a serious problem, as they exert harmful impact on environment. So they need to be managed or they can be utilized (**Duda and Tarko, 2007).** Citrus fruits and their by-products are of high economic and medicinal value because of their multiple uses, such as in the food industry, cosmetics and folk medicine. Peels, seeds and pulps of *Citrus* processing industry left after juice extraction, are corresponding to about 50% of the raw processed fruit and can be used as a potential source of valuable by-products (**Silalahi, 2002; Saidani et al., 2004**). Different studies conducted on peels revealed the presence of important constituents, which can be used for pharmacological or pharmaceutical purpose. Number of components having

activities like antioxidant, antimicrobial, antiinflammatory, antiproliferative etc. have been isolated from different peels **(Sawalha et al., 2009 and Velazquez et al., 2013).**

Until recently, essential oils (Eos) have been studied mostly from the viewpoint of their flavour and fragrance chemistry only for flavouring foods, drinks and other goods. Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers, and their exploitation for potential multi-purpose functional use **(Ormancey, et al., 2001 and Sawamura, 2000).**

Citrus peel essential oils (Eos) are reported to be one of the rich sources of bioactive compounds namely coumarins, flavonoids, carotenes, terpenes and linalool etc. (**Mondello et al., 2005**). Recently, Citrus peel essential oils have also been searched for their natural antioxidant and antimicrobial properties (**Tepe et al., 2006; Jayaprakasha et al., 2008 and Viuda et al., 2008**). Due to their great nutraceutical and economic importance, numerous investigations have been performed aimed at identifying the chemical composition, antimicrobial activities of the essential oils from peel of different citrus species. There have been many studies conducted to determine the chemical composition in essential oil of orange peel. For example, the essential oils of orange peel from Iran which have been identified include limonene (92.42%) and β-myrcene (3.89%) **(Yaghoub et al., 2006).** Oranges grown in Italy had the main components of limonene (93.67%) and β-myrcene (2:09%) in essential oil **(Verzera et al., 2004).** Extensive studies on the chemical composition of various *C. sinensis* species have also been conducted.

The antimicrobial activity of sweet orange (*Citrus sinensis*) has not been thoroughly investigated, and little is known about its activity against toxin-producing fungi, such as *Aspergillus flavus* and Aspergillus parasiticus. In our search for commercially useful citrus essential oils, we selected to study the essential oil of sweet orange (Citrus sinensis) peel, as this species has not been investigated for its chemical composition and any biological activity. So, the general objective of the present study was to determine the in-vitro anti-fungal activity against two pathogenic fungal strains in order to establish the time survival kinetics of these micro-organisms when incubated with increased concentration of essential oils.

2. Materials and Methods

2.1- Plant materials and isolation of essential oil

Fully fresh sweet orange Citrus *sinensis* L. fruits obtained from ministry of agriculture, Egypt. The fruits were peeled off carefully with the help of a sharp knife to avoid any damage of oil glands. The sample of fresh orange peels was subjected to hydro-distillation for 3 h using a Clevenger-type apparatus. Distillates of essential oil was dried over anhydrous sodium sulfate, filtered and stored at -4°C until analyzed (**Hussain et al., 2008**). The yield of volatile oils was weighted and calculated in g / 100 g dry plant.

2.2- Gas chromatographic (GC) analysis

GC analysis was performed by using Hewlett- Packard model 5890 equipped with a flame ionization detector (FID). A fused silica capillary column DB-5 (60 mx 0.32 mm id,) was used. The oven temperature was maintained initially at 50°C for 5 min, then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate of 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The retention indices (Kovats index) of the separated volatile components were calculated using hydrocarbons (C_6-C_{24}, AldrichCo.) as references.

2.3- Gas chromatographic - mass spectrometric (GC-MS) analysis:

The analysis was carried out by using a coupled gas chromatography Hewlett-Packard model (5890) / mass spectrometry Hewlett-Packard MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400 a.m.u. The GC condition was carried out as mentioned above. The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology) and compared with those of authentic compounds and published data**.** The quantitative determination was carried out based on peak area integration. **(Adams, 1995).**

2.4- Compounds identification

The identification of the oil constituents was based on the comparison of their retention indices relative to (C_6-C_{24}) *n*-alkanes either with those of published data or with authentic compounds. Compounds were also identified using their MS data compared to those from the NIST mass spectral library and published mass spectra (**Adam, 2001**).

2.4- Sensitivity testing (Antibacterial and antifungal activities).

Microorganisms and Cultures:

The cultures used in this study were obtained from the Mycotoxins Lab, Microbial Lab, National research Centre, Egypt. The fungal strains of *Aspergillus parasiticus* NRRL 2999 and *A. flavus* (local strain) were checked for purity and identity according to **Raper and Fennel (1955)** using nutrient Yeast Extract Sucrose media (YES). The inoculated YES media were inoculated at 28 ± 1°C for 10 days before evaluating the efficacy of the studied essential oils.

Antifungal activity testing:

Yeast extract sucrose (YES) broth medium which is known to support mold growth and toxin production **(Davis et al., 1966)** was used as a basal medium in this study. Fifty millimeter of medium was dispensed into each of a series of 250-ml Erlenmeyer flasks. The medium was autoclaved at 121°C for 15 min., and the cooled. Simultaneously, the Eos of orange peel were prepared at concentrations of 0.0, 0.05, 0.1, 0.15, 0.2, or 0.25% of the Eos orange peel, was added. Each group of flasks, were incubated at 28^0C. The parameters of mycelial dry weight, pH and aflatoxin(s) production were carried –out at the intervals of 3, 7, and 10 days in different treatments incubated at 28^0C.

Mycotoxins standard:

Aflatoxins (B1 and G1) were obtained from Sigma Chemical Co. St. Louis MO, USA.

Antifungal analysis:

Mycelial of cultures were carefully overlaid with chloroform (50 ml) and kept 24 hr in dark then several steps were carried out according to the method of **Bauer et al., (1983).** Quantitative determination of aflatoxins B1 and G1 was performed on silica gel D.G-plates according to the **AOAC methods (1980).**

Calculations:

***Average rate of net aflatoxin (μg/day) or mycelial dry weight (g/day) produced in one day by the mould during the period were calculated as follow**:

Average rate = net (aflatoxin or mycelial dry weight produced after incubation period.) / Incubation period.

***Average rate of increase or decrease in mycelial dry weight (g/day) =**

(Net mycelial dry weight at a period B - net mycelial dry weight at a period A) / Period (B-A).

***Average rate of accumulation or degradation in aflatoxin (μg/day) during incubation period =** (net aflatoxin produced at a period B - net aflatoxin produced at a period A) / Period (B-A).

***Average rate of net aflatoxin (μg/day) produced by 1 gram of mycelia dry weight in one day was calculated by equation: A/B**

where: A= average rate of net aflatoxin preduced in 1 day during icubation period

B= average rate of net mycelial dry weight produced in 1 day during incubation period

***The precentage of inhibitory or stimulatory effect of Eos of orange peel was calculated as follow:**

% Inhibition or stimulation of aflatoxin production = (D- C) / C.

where:C=average rate of net aflatoxin produced by 1 gram of dry mycelium in 1 day by the mould in a medium containing no extract (control).

D=average of rate of net aflatoxin produced by 1 gram of dry mycelium in 1 day by the mould in a medium containing extract.

Statistical analysis

Significant differences between treatments and strains sensitivity were analyzed using the M-STAT programmed at 99% level of confidence. Mean separation test was done using the Least Significance Difference (LSD) **(Nissen, 1990).**

3. Results and Discussion

3.1. Chemical componants of essential oil of the Egyptian sweet orange (Citrus *sinensis*) peels

The *Citrus* peels, commonly treated as agro-industrial waste, are a potential source of valuable secondary plant metabolites and essential oils (**Andrea et al., 2003**). *Citrus* peel essential oils are reported to be one of the rich sources of bioactive compounds namely coumarins, flavonoids, carotenes, terpenes and linalool etc. (**Mondello et al., 2005**).

In this study, Gas chromatography (GC) and Gas chromatography-mass spectrometry (GC-MS) were used to determine the aromatic compounds of peel oil of the Egyptian sweet orange fruit. Total of 24 compounds were identified (table 1) and the major components were limonene (86.75%), linalool (1.96%), α-pinene (1.63%), trance-limonene oxide (1.39%), and γ-terpinene (1.03%). Some other compounds were nonanal (0.87%) and terpinolene, α-farnesene (0.77%, for each). Limonene contributes to the aromatic odor of the oil and hence the plant belongs to the limonene chemotype.. Our results revealed that essential oil contents from peels of *Egyptian sweet orange (Citrus sinensis*) was 0.33±0.01 g/100 g fresh peels. Our results are in agreement with the findings of many researches **Sawamura et al. (2005)** found that limonene, neral and geranial were the major oil components of four different varieties of C. sinensis Eos, while b-pinene and g-terpinene were completely absent in C. reticulata. **Minh Tu et al. (2002)** observed maximum limonene (95.1%) content in C. reticulata Blanco var. tangerine EO from Vietnam and **Dharmawan et al. (2007)** observed 89.6% limonene content in freshly-squeezed juice also. Most volatile compounds detected in sweet orange peel-oil were also found in many citrus oils, such as α-pinene, myrcene, octanal, linalool, γ-terpinene, terpiolene, citronellal, and neral. These compounds were previously reported in other citrus oils but their aroma and antimicrobial activities were not described (**Sawamura et al., 2005; Tu et al., 2002 and Choi et al., 2000)**. Recently, Citrus peel essential oils have also been searched for their natural antioxidant and

antimicrobial properties **(Kuate et al., 2006; Tepe et al., 2006; Dharmawan et al., 2007; Jayaprakasha et al., 2008; Viuda et al., 2008 and Velazquez et al., 2013).**

Table 1. Chemical composition of the Eos of Egyptian orange peel.

Peak No	KI	Area %	Identified compounds	Identified Methods
1	924	0.21	α-Thujene	KI & MS &St
2	936	1.63	α-Pinene	KI & MS &St
3	973	0.31	Sabinene	KI & MS &St
4	992	0.18	β -Myrcene	KI & MS&St
5	1005	0.09	Octanal	KI & MS
6	1009	0.04	Cis- β -Ocimene	KI & MS
7	1012	0.25	δ-3-Carene	KI & MS&St
8	1036	86.75	D-L-Limonene	KI & MS
9	1071	1.03	γ -Terpinene	KI & MS
10	1092	0.77	Terpinolene	KI & MS
11	1103	1.96	Linalool	KI & MS&St
12	1109	0.87	Nonanal	KI & MS
13	1141	1.39	*trance*- limonene oxide	KI & MS
14	1152	0.08	*iso*- Pulegol	KI & MS
15	1159	0.52	Citronellal	KI & MS&St
16	1165	0.47	*iso* - thujol	KI & MS
17	1208	0.09	Decanal	KI & MS
18	1227	0.18	z- carveol	KI & MS&St
19	1230	0.69	Citronellol	KI & MS
20	1242	0.44	d-carvone	KI & MS
21	1248	0.17	Neral	KI & MS
22	1280	0.38	Geranial	KI & MS&St
23	1362	0.06	β -Elemene	KI & MS
24	1516	0.77	α -Farnesene	KI & MS
Total		99.33%		

Compound listed in the order of elution from a DB5 column, KI = identification based on Kovats index; MS= identification based on comparison of mass spectra; St= identification based on co-injection with authentic compounds.

3.2.Antifungal effects of the EOs of orange peel in a medium (yeast extract broth) incubated at 28˚C.

To demonstrate the activity of the Eos of orange peel in controlling the mould growth and aflatoxin(s) production, relative comparisons were made between the control and treatments and the percentage of inhibition or stimulation of the mould by extract were calculated (tables 2, 3and figures from 1-6).

***3.2.1. Mycelial dry weight*:** As can be seen from table (2) and figures 1 & 2, all of concentrations of the EOs of orange peel extract exhibited a variable antifungal activity against the two tested fungal strains. There was a gradual decrease in growth rate as affected by increasing the EOs of orange peel extract, especially at concentrations of 0.0 up to 0.25 mg/25ml medium during incubation period (10 days). Our results were in the same trend of those reported by **Sultana et al., (2012)** and **Manal et al. (2014).** In regard to data in table (1), the antifungal properties of the tested oil in this study may be a result of its monoterpenes (limonine). In this respect **Sultana et al. (2012)** found that the chemical composition of volatile oil of the fruit peel of *Citrus reticulate* Blanco (Rutaceae) composed mainly monoterpenes (99.1%) constituting l-limonine (92.4%), γ- terpene (2.6 %) and phellandrene (1.8 %), showed antibacterial and antifungal activities against *Aspergillus favus*, *Aspergillus niger*, *Aspergillus fumigates* and *Candida albicans* under in vitro condition. On the other hand, **Sonia et al. (2014)** found antimicrobial and antioxidant activity of orange peel. Also, **Akhilesh et al. (2012)** found that orange (Citrus sinensis) hexane extract was found to be most effective against bacterial pathogens giving a zone of 13 mm, while the cold water extract of orange was effective against fungal pathogens. Results obtained by **Omran et al., (2011),** indicated that decoction extract of orange peel had no antifungal effect and these differences with our results may be due to differences in of the extract tested and /or the microorganism used.

Table (2): Change in mycelial dry weight (gm), and Aflatoxin content (µg) produced by *A. parasiticus* and *A. flavus* in 25 ml YES broth medium containing the Eos of Egyptian orange peel incubated 28 °C for up to 10 days.

a: Initial pH = 5.56 (at zero time of incubation medium).

Extract added (mg 25/ml medium)	Incubation time (days)	*A.parasiticus*					*A.Flavus*				
		Mycelial dry weight	[a]pH	Aflatoxin content			Mycelial dry weight	[a]pH	Aflatoxin content		
				B1	G1	Total			B1	G1	Total
0.0		3.1	3.21	120	1385	1505	2.74	3.42	117	1210	1327
0.05		2.6	3.11	105	1187	1292	2.11	3.36	45	840	885
0.1		1.83	5.12	35	185	495	1.85	4.05	85	343	428
0.15	3	1.35	5.38	NT	NT	NT	0.94	5.48	NT	NT	NT
0.2		0.9	5.49	NT	NT	NT	NG	5.23	NT	NT	NT
0.25		NG	5.50	NT	NT	NT	NG	5.15	NT	NT	NT
0.0		2.87	6.95	260	1980	2420	2.53	6.58	235	1712	1947
0.05		2.15	6.68	310	1455	1765	2.35	6.60	215	1530	1745
0.1	7	1.62	6.45	185	635	820	1.74	5.43	75	625	625
0.15		1.28	5.41	NT	NT	NT	0.65	5.27	NT	NT	NT
0.2		0.74	5.38	NT	NT	NT	NG	5.19	NT	NT	NT
0.25		NG	5.25	NT	NT	NT	NG	5.33	NT	NT	NT
0.0		2.5	6.95	383	1120	1503	2.36	6.74	217	980	1197
0.05		1.91	6.55	145	615	760	1.85	6.6	135	655	790
0.1	10	1.65	6.45	55	345	400	1.65	6.38	72	348	420
0.15		1.23	5.64	NT	NT	NT	0.63	5.58	NT	NT	NT
0.2		0.65	5.37	NT	NT	NT	NG	5.44	NT	NT	NT
0.25		NG	5.35	NT	NT	NT	NG	5.51	NT	NT	NT

NG: No growth.
NT: Not detected.

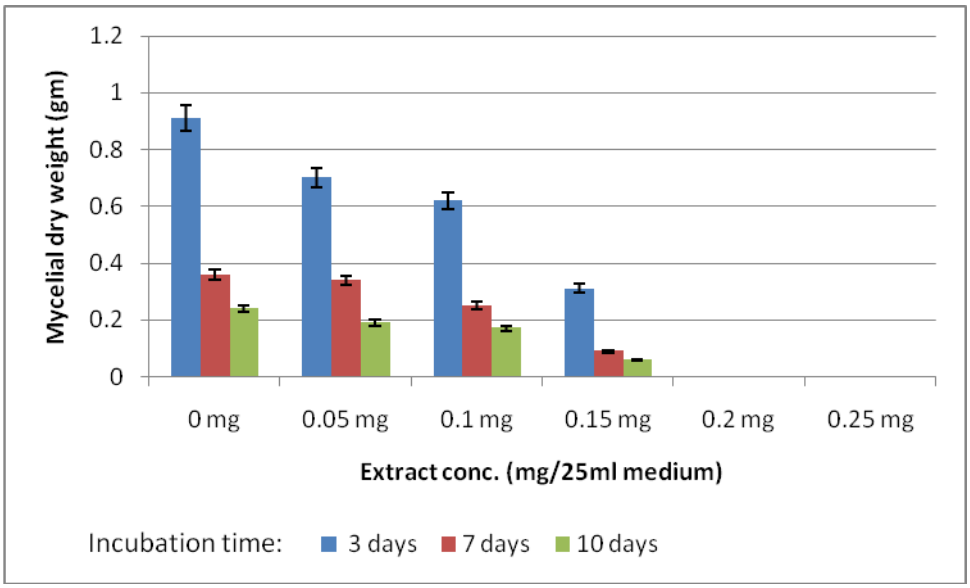

Figure 1. Change in mycelial dry weight (g/day) produced by *A. flavus* in 25 ml YES broth medium containing the Eos of the Egyptian orange peel incubated 28°C for up to 10 days.

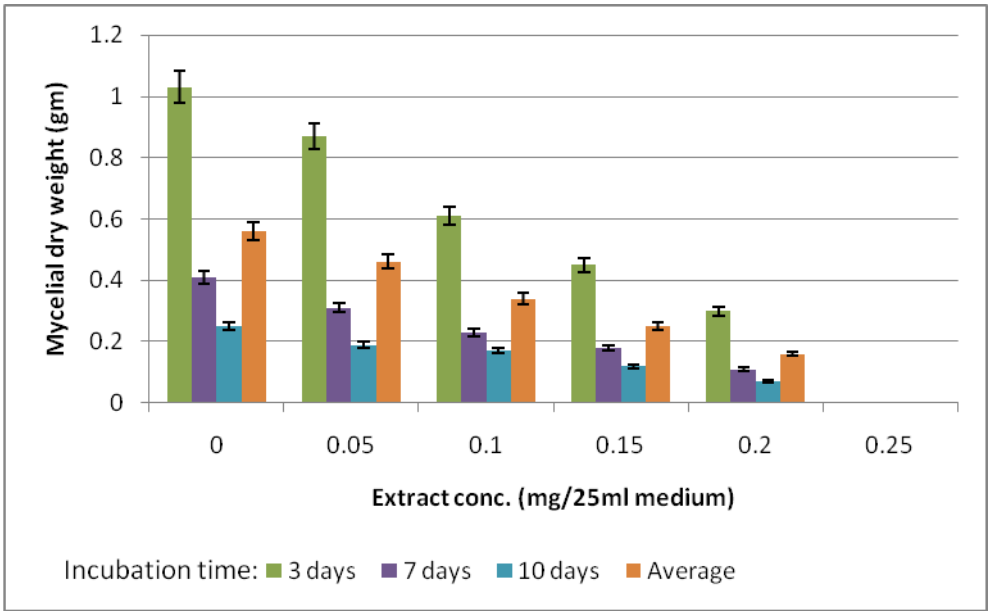

Figure 2. Change in mycelial dry weight (g/day) produced by *A. parasiticus* in 25 ml YES broth medium containing the Eos of the Egyptian orange peel incubated 28°C for up to 10 days.

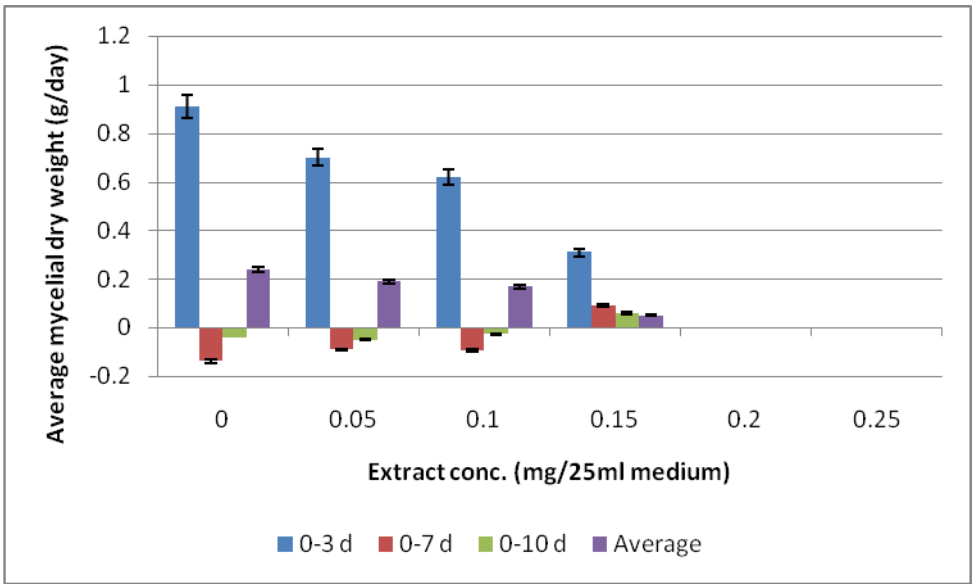

Figure 3. : Average rates of increase or decrease in mycelial dry weight (g/day) produced by *A. flavus* in 25 ml YES broth medium containing the Eos of the Egyptian orange peel incubated 28°C for up to 10 days.

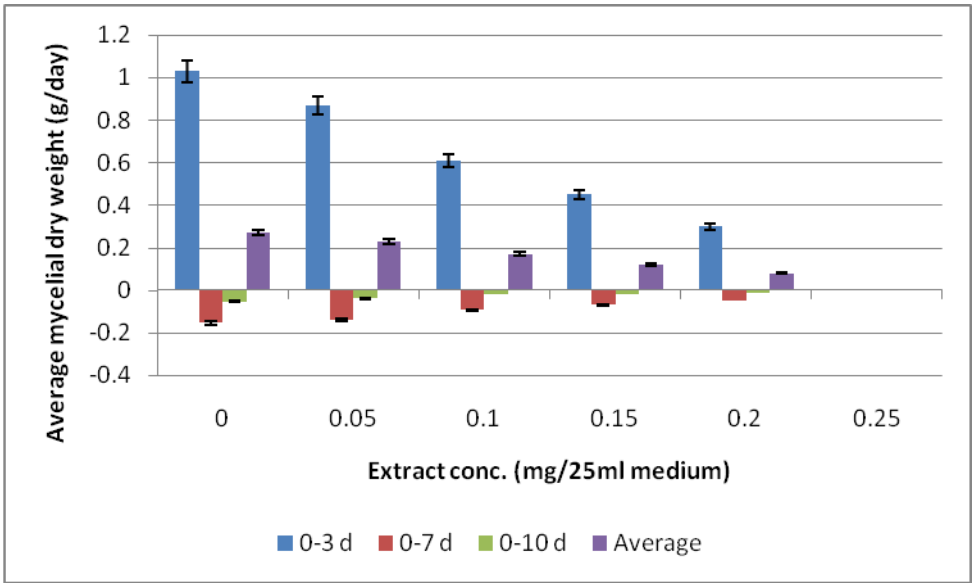

Figure 4. Average rates of icrease or decrease in mycelial dry weight (g/day) produced by *A. parasiticus* in 25 ml YES broth medium containing the Eos of the Egyptian orange peel incubated 28°C for up to 10 days.

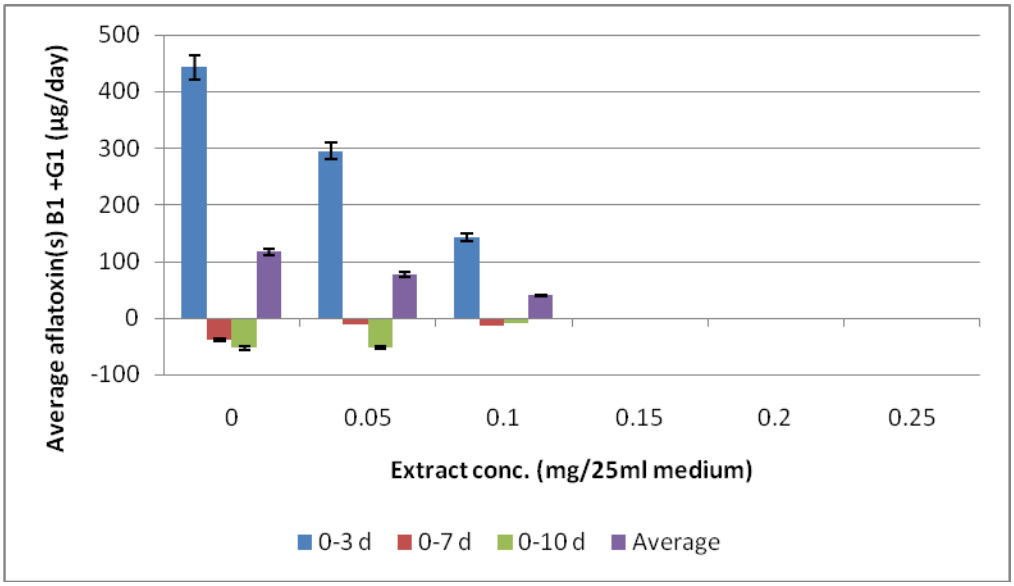

Figure 5. Average rate of accumulation or degradation of aflatoxin(s) B_1 and G_1 (µg/day) produced *by A. flavus* in 25 ml YES broth medium containing the Eos of the Egyptian orange peel incubated at 28 °C for up to 10 days .

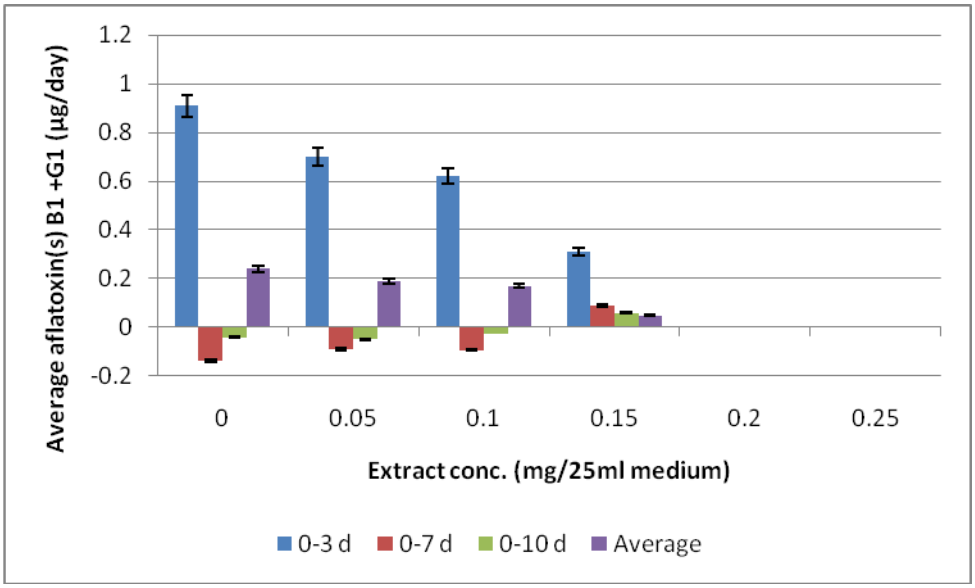

Figure 6. Average rate of accumulation or degradation of aflatoxin(s) B_1 and G_1 (µg/day) produced *by A. parasiticus* in 25 ml YES broth medium containing the Eos of the Egyptian orange peel incubated at 28 ^{o}C for up to 10 days .

Average rates of increase or decrease in mycelial dry weight were affected by the EOs added during the period of incubation. Represented data in table 2 and figures 1 & 2, clearly indicate that the average amount of mycelial dry weight produced in cultures containing the EOs of orange peel was less than in cultures free of these EOs and the EOs used at level 0.2 mg / 25ml medium completely inhibited mould growth in *A. flavus* cultures during the 0- to 3 day's interval, whereas the EOs concentration 0.25 mg / 25 ml medium completely inhibited the mould growth in all cultures at the same period. After 7 days Maximum mycelial growth occurred in the media containing none of the EOs of orange peel extract (0.0 mg/ml medium). The Eos concentration > 0.05 mg / 25 ml medium decreased the mould growth in all tested cultures during the 3 - 7 - day interval of incubation. The observed decrease in dry mycelial weight in the media supplemented with the EOs of orange peel of 0.05 mg / ml medium can be attributed to an extension of the lag phase of the mould caused by the EOs of orange peel in the medium. Results in table 2 and figures 1, 2, 3 & 4, further indicate that there was a decrease in mycelial weight during the interval from 7- to 10 days in the presence or absence of 0.05, 0.1 or 0.15 mg/ml medium of added extract, whereas in the presence of 0.2 mg/ml of the extract in the medium, the *A flavus* failed to grow. At the concentration of 0.25 mg / 25 ml, both mould strains failed to grow during the period from 7- to 10 day interval. Our results, in terms of change in mycelial growth, are similar to those observed by **Horberg (1998),** who studied the antifungal effect of the vapours of the essential oils of the caraway, spermint, thyme and garlic aganist three important postharvest pathogens of carrots. Also, our results were in the same trend of those observed by **Manal et al. (2014).**

The decrease in dry weight of mycelium observed during the final stages of the growth cycle can be explained bioautolysis, which may have occurred at that time and resulted in loss of soluble intracellular solutes which were released through hydrolysis of the mycelium during the filtration step of the analysis **(Doyle and Marth, 1978).**

3.2.2. PH of medium: Data in table (2) indicated that the initial pH (5.65) was decreased after 3 days, especialy in the media containing the EOs tested of, 0.0 or 0.05 mg /ml medium and this decrease was influenced by the EOs of orange peel added, mould strain tested and amount of mould growth that occurred. After 7 and 10 days of incubation, the pH increased in the media supplemented with the Eos from 0. 0 to 0.1 mg / 25 ml medium for both all mould tested. The rate of increase in pH values probably was influenced by the amount of mycelium that autolysed during the 7-to 10 day incubation interval. The rate of increase in pH in both tested cultures during this period was greater in the medium had Eos concentrations from 0.0 to 0.1 mg / 25 ml medium than was observed when the media had Eos concentrations from 0.15 to 0.25 mg / 25 ml medium .These results, in terms of change in pH, are similar to those observed by **Yousef and Marth (1981)** on *A. parasiticus* in the presence of sorbic acid, those obtained by **EL-Gendy and Marth (1981)** on *A .parasiticus* in the presence of *Lactobacillus casei,* and **Utpal et al. (1996)** on *Lactococcus lactis subsp.* Also, **Ahmed and Naresh (2009)** reported that pH, water activity (a_w) and temperature are important factors that have criteria for understanding the ecology of fungi that

causing spoilage, especially mycotoxigenic species. Results of previous studies have demonstrated that growth of fungi could be affected by 'Hydrogen ion concentration' - which known as pH value - in a medium in which it grows, either directly by the effect of its action on the cell surfaces or indirectly by its ability to made changes on the availability of nutrients. However, acid/alkaline requirement for growth of fungi is quite broad, ranging from pH 3.0 to up to pH 8.0, with optimum around pH 5.0 if nutrient requirements are satisfied **(Pardo et al, 2006).** Also, **David et al. (2005)** recorded that growth of *Aspergillus carbonarius*, which had been isolated from wine products and grapes was influenced better at pH 4.0 and 7.0 than at pH 2.6, regardless of water activity (a_w) level. As general results, *Aspergillus* species are more tolerant to media with alkaline pH, otherwise; *Penicillium* species appear to be more tolerant to acidic pH medium **(Wheeler et al., 1991).** Many studies on pH explain and demonstrated that fungi grow at pH 3.0 - 8.0, with maximum production of dry mycelial weight and sporulation at pH 5.5 and pH 6.5 respectively, by using of liquid media **(Saha et al., 2007; Deshmukh et al., 2012)**. In general, a neutral to weak acidic environment was suitable for mycelial growth, with optimum pH 5.0 –7.0 and pH 5.0 –8.0 for conidial production (**Zhao et al., 2012**).

3.2.3. Aflatoxin production. The data presented in table (2 and figures 5, 6) indicate that aflatoxin(s) production by the all tested fungal strains and a complete inhibition was occurred at concentrations of the EOs of the tested orange peel above 0.1 mg / 25 ml medium. The amount of aflatoxin B_1 produced by both mould strains (Table 2) after 3, 7 or 10 days at 28°C was less than that of aflatoxin G_1. These data further indicate that the extend of inhibition by the EOs of orange peel of aflatoxins B1 plus G_1 during the entire period of incubation (10 days) increased as the concentration of the EOs of orange peel increased from 0.05- to 0.25 mg/ml medium. Also, the Eos of sweet orange peel at concentration 0.2 mg/ml meduim completely inhibited growth by *A. flavus* up to 10 days of incubation at 28°C and at concentration 0.25 mg/ml medium completely inhibited mould growth and aflatoxin(s) production for all mould strains tested, up to 10 days at 28°C. These results, in terms of change of aflatoxin(s) production, are agree with those obtained by **Shimoyamada et al. (1996),** who studied the inhibitory effect of steroid saponin isolated from the bottom part of white asparagus (*Asparagus officinalis* L.) and similar to those obtained by **Omer *at el.* (1997) and Manal et al. (2014).**

In comparison between the fungal growth and mycotoxin production during 10 days of this study (table 2), there is an opposite relation between mycelial growth and mycotoxin production. These results, in this pat of change, are in the same trend with those observed by other investigators. According to **Huynh and Lloyd (1984),** they were examined a strains of *Aspergillus parasiticus* for its toxin production comparing to mycelial weight, and they found an opposite relation between mycelial growth and mycotoxin production. According to **Ritter et al. (2011),** there were many evidences that explained shared regulatory elements between aflatoxin biosynthesis and fungal development; that means, fungi may take one pathway on computation of the other pathway in the same time, either fungal growth or toxin production, which means one of them, was weaken the other. Not with standing, **Bennett et al. (1986)** found that, the amount of aflatoxin which excreted by four strains of *A. parasiticus* was affected by mycelial growth rate and sclerotial formation, by increased in sclerotial formation and /or mycelium weight, aflatoxin formation ratio was decreased. **Cleveland and Bhatnagar (1990)** found that Aflatoxin Bl levels were seen to increase sharply in liquid shake Cultures of A. parasiticus about 2 days after inoculation, at which time mycelial growth rate declines.

From the previous studies it is concluded that the conditions that promote mycotoxin production are usually more restricted than those for mould growth. **Guitakou et al. (2006**) and **Bragulat et al. (2001)** mentioned that mycotoxin production depends on fungal species, substrate, and temperature of the media, pH, relative humidity, and storage or incubation time. **Malgorzata et al. (2013)** reported that; mycotoxin production is determined by genetic capability related to strain and environmental factors including the substrate and its nutritious content.

It is important to consider the average rate of net aflatoxin(s) produced during the period of incubation (10 days). The data in table (3) indicate that for the 0- to 3 day incubation interval, the average rate of aflatoxin(s) production was greater in control media than in the treatment with the Eos of 0.05 to 0.1 mg. No aflatoxin(s), B_1 or G_1, were produced when the media contained 0.15, 0.2 or 0.25 mg / 25 ml medium of the Eos added. A decrease in the average amount of aflatoxin(s) produced by both mould strains, occurred after 10 days in the presence or absence of 0.05 to 0.1 mg/ml medium of the Eos added.

Table (3): The average rates of net mycelial dry weight, and aflatoxins B1 and G1 (µg) produced by one gram of mycelial dry weight of *A. parasiticus* and *A. flavus* in one day at 28 °C during the period of incubation (10 days).

Average rate during 10 days incubation period	*A.Parasiticus*			*A.Flavus*		
	Extract added (mg /25 m l medium)			Extract added (mg /25 ml medium)		
	0.0	0.05	0.1	0.0	0.05	0.1
Mycelial dry weight, gm / day	0.25	0.191	0.123	0.236	0.185	0.165

Aflatoxin B1, µg	38.3	14.5	5.5	21.7	13.5	7.2
Aflatoxin G1, µg	112	61.5	34.5	98	65.5	34.8
Aflatoxins B1+ G1	150.3	77.6	40	119.7	79	42

Aflatoxin production per unit of growth (Tables 4) is a more precise measure of the mold's ability to produce aflatoxin than are the absolute values for aflatoxin accumulation. Examination of aflatoxin(s) production per unit growth indicated that the decrease in aflatoxin(s) production during incubation period (10 days) was primarily due to inhibition of growth. The extent of stimulation by the EOs of orange peel on production of aflatoxin(s) B_1, G_1 (Table 2) or B_1 plus G_1 during the entire period of incubation (10 days) decreased from 0.0 to 0.25 mg/ ml meduim. From the foregoing seems that this calculation was useful to make relative comparisons to demonstrate the effect of tested additive on aflatoxin product. It has been reported that a compound is considered as a positive inhibitor if it reduces aflatoxin formation to 50% of that of control **(Masimango et al., 1978).**

Table (4): Effect of the Eos of Egyptian orange peel on the average rate of net aflatoxins B1 and G1 produced by one gram of mycelial dry weight of *A. parasiticus* and *A. flavus* in one day at 28 °C during the period of incubation (10 days).

Item	*A.Parasiticus*			*A.Flavus*		
	Extract added (mg /25 m l medium)			Extract added (mg /25 ml medium)		
	0.0	0.05	0.1	0.0	0.05	0.1
*Average rate net aflatoxin produced by 1 gm of mycelial dry wt. in 1 day	153.2	75.92	44.72	91.95	72.97	43.64
Aflatoxin B1	448	321.99	209.09	415.25	354.05	210.91
Aflatoxin G1	601.2	397.9150.	253.81	507.2	427.03	254.55
Aflatoxins B1+ G1						
*Net amount of inhibition or stimulation (%)		50.44b	70.81b		35.05b	52.54b
Aflatoxin B1		28.13b	53.33b		14.74b	49.18b
Aflatoxin G1		33.81b	57.79b		15.81b	49.81b
Aflatoxins B1 + G1						

a: % stimulation
b: % inhibition

In conclusion,

results in this the study (Tables 1,2,3, 4 and Figures 1,2,3,4,,5,6) indicate that the biosynthesis and accumulation of aflatoxin(s) were influenced by concentration of the Eos of the Egyptian sweet orange peel in the media, mycelial growth and length of incubation period. It is also evident from our results that, if possible, a sufficient amount of the Eos of the Egyptian sweet orange peel to prevent mould growth needs to be used if one wishes to prevent aflatoxin(s) production.

Acknowledgements

We would like to appreciate Mr. Mohammed Ramadan El-Gohary and Mr. Gomaa Nour-Eldeen of the Food Toxins and Contaminants Department, for excellent technical support and excellent assistance in the preparation of this manuscript.

References

A.O.A.C. (1980): Official methods of analysis "13th ed. Association of analytical chenmists. Washington D. C., U. S. A.

Adams, R.P. (1995): Identification of essential oil components by gas GC-MS spectroscopy. Allured Publishing Corporation, Carol Stream. IL.USA

Adam, R. P. (2001): Identification of essential oils components by gas chromatography quadruple mass spectroscopy. p 456.

Ahmed, A. and Naresh, M. (2009): Influence of physiological factors on growth, sporulation and ochratoxin A/B production of new Aspergillus ochraceus grouping. World Mycotoxin Journal. 2(4): 429 – 434.

Akhilesh, K.; Raghvendra, P. S.; Gupta, V. and Singh, M. (2012): Antimicrobial properties of peels of citrus fruits. International Journal of Universal Pharmacy and Life Sciences 2(2): 24-38.

Andrea,V.; Nadia, N.; Teresa, R. M. and Andrea, A. (2003): Analysis of some Italian lemon liquors (Limoncello). Journal of Agricultural Food Chemistry 51(17): 4978- 4983.

Bauer, J.; Montgelas, A.V. and Gedek, B. (1983): Aflatoxin B1 production in the presence of preservatives antimicrobial agents. Proc. Int. Symp. Mycotoxins, pp. 249 – 255.

Bennett, J.W.; Leong, P.M.; Kruger, S. and Keyes, D. (1986): Sclerotial and low aflatoxigenic morphological variants from haploid and diploid Aspergillus parasiticus. Experientia 42:841–51

Bhatnagar D; Ehrlich, K.C. and Cleveland, T.E. (2003): Molecular genetic analysis and regulation of aflatoxin biosynthesis. Appl. Microbiol. Biotechnol. 61: 233-93.

Bragulat, M.R.; Abarca, M.L.; Cabanes, F.J. (2001): An easy screening method for fungi producing ochratoxin A in pure culture. Int. J. Food Microbiol. 71:139-144.

Choi, H. S.; Song, H. S.; Ukeda, H. and Sawamura, M. (2000): Radical-Scavenging Activities of Citrus Essential Oils and Their Components: Detection Using l,l-Diphenyl-2-picrylhydrazyl. Journal of Agricultural Food Chemistry 48: 4156-4161.

Cleveland T.E, and Bhatnagar D. (1990): Evidence for de novo synthesis of an aflatoxin pathway methyl transferase near the cessation of active growth and the onset of aflatoxin biosynthesis in Aspergillus parasiticus mycelia. Can J Microbiol. 36:1–5.

David, M.; Neus, B.;Sonia, M.; David, A.; Vicente, S. and Naresh, M. (2005): Water relations of germination, growth and ochratoxin 'A' production by Aspergillus carbonarius isolates from wine and table grapes from the northern Mediterranean basin. Ecophysiology of ochratoxigenic moulds. Journal of Applied Microbiology 98: 839-844.

Davis, N. D.; Diner, U.L. and Eldridge, D.W. (1966): Production of aflatorxins B and G by Aspergillus flavus in semi synthetic medium. Appl. Microbiol. 14: 378.

Deshmukh, S.A.; Chavan, S.R. and Gaikwad, D.K. (2012): Morphometric studies in the genus ClerodendrumL. Current Botany 3 (5): 41-44.

Dharmawan, J.; Kasapis, S.; Curran, P., and Johnson, J. R. (2007): Characterization of volatile compounds in selected citrus fruits from Asia. Part I: freshly-squeezed juice. Flavour and Fragrance Journal 22: 228–232.

Doyle, M. P. and E.H. Marth (1978): Aflatoxin is degraded at different pH values by mycelia of Aspergillus parasiticus. European J. Applied Microbiol. Biochem. 6: 95-100.

Duda-Chodak A. and Tarko T. 92007): Antioxidant properties of different fruit seeds and peels. Acta scientiarum Polonorum. Technologia alimentaria 6(3):29-36.

EL-Gendy, SM and Marth, G.H (1981): Growth and aflatoxin production by Aspargillus parasiticus in the presence of Lactobasillus casei. J. food Prot. 44: 211-213.

Guitakou, S. et al. (2006): Study of aflatoxin B1 and ochratoxin A production by natural microflora and Aspergillus parasiticus in black and green olives of Greek origin. Food Microbiology 23: 612-621.

Horberg, H. (1998): Influecnce of volatile plant extracts on storge pathogens of carrots in vitro.Vaxtskyddsnotiser 62: 4, 87-89.

Hussain, A.I.; Anwar, F.; Sherazi, S.T.H and Przybylski, R. (2008): Chemical composition, antioxidant and antimicrobial activities of basil (Ocimum basilicum) essential oils depend on seasonal variations. Food Chemistry 108: 986-995

Huynh V.L. and Lioyd A.B. (1984): Synthesis and degradation of aflatoxin by aspergillus parasiticus: I. Synthesis of aflatoxin B1 by young mycelium and its subsequent degradation in aging mycelium. Aust. J. Biol. Sci.; 37:37 – 43.

Jayaprakasha, G.K.; Girennavar, B. and Patil, B. S. (2008): Radical scavenging activities of RioRed grapefruits and Sour orange fruit extracts in different in vitro model systems. Bioresource Technology 99 (10): 4484-4494.

Kuate, J.; Foko, J.; Ndindeng, S. A.; Dongmo, P. M. J.; Foure, E. and Damesse, F., et al. (2006): Effect of essential oils from citrus varieties on in vitro growth and sporulation of Phaeoramularia angolensis causing citrus leaf and fruit spot disease. European Journal of Plant Pathology 114: 151–161.

Malgorzata, P.; Slizewska, K. and Biernasiak, J. (2013): Mycotoxins in Cereal and Soybean-Based Food and Feed. Editors; M Piotrowska, J Biernasiak, K Śliżewska. Soybean-Pest Resistance Book. Intech–open publisher, http://dx.doi.org/10.5772/54470

Manal M. Ramadan; H.A. Yehia; Mohamed S. Shaheen and Abed EL-Fattah, Sh.M. (2014): Aroma Volatiles, Antibacterial, Antifungal and Antioxidant Properties of Essential Oils Obtained from Some Spices Widely Consumed in Egypt American-Eurasian J. Agric. & Environ. Sci.14 (6): 486-494.

Masimango N; Ramaut, J.L. and Remacele,J. (1978): Contribution an l'etude du role de additives chemiques dans la lutle contre l'aflatoxin. Rev. Ferment. Ind Aliment. 33: 166.

Minh Tu, N. T.; Thanh, L. X.; Une, A.; Ukeda, H. and Sawamura, M. (2002): Volatile constituents of Vietnamese pummelo, orange, tangerine and lime peel oils. Flavour Fragrance Journal 17: 169–174.

Mondello, L.; Casilli, A.; Tranchida, P. Q.; Dugo, P. and Dugo, G. (2005): Comprehensive two-dimensional GC for the analysis of Citrus essential oils. Flavour and Fragrance Journal 20: 136-140.

Nissen,O. (1990): MSTAT-C: A Microcomputer Program for the Design Management and Analysis of Agronomic Research Experiments.

Omer E. A; El-Bazza, E. and Eggbo, G.E. (1997): Constituents and biological activities of volatile oil of some Lamiacae plants as affected by irradiation. Egypt. J. Hort. 24 (2): 175 – 196.

Pardo, E.; Marín, S.; Ramos, A. J. and Sanchis, V. (2006): Ecophysiology of ochratoxigenic Aspergillus ochraceus and Penicillium verrucosum isolates. Predictive models for fungal spoilage prevention - a review. 23(4):398-410.

Omran, S.M.; Moodi, M.A.; Amiri, S.M.B.N.; Mosavi,S.J.; Saeed,S.M.G.M.S; Shiade,S.M.J.; Kheradi,E. and Salehi, M. (2011): The effects of limonene and orange peel extracts on some spoilage fungi. Int. J. of Molicular and Clin. Microbiol., 1: 82-86.

Ormancey, X.; Sisalli S., and Coutiere, P. (2001). Formulation of essential oils in functional perfumery. Parfums, Cosmetiques, Actualites, 157: 30-40.

Quoc Le, P. T.; My, V.O. A. and Nguyet, T. T. M. (2011): Volatile constituents of essential oil from Citrus sinensis grown in Tien Giang Province, Vietnam. As. J. Food Ag-Ind. 4(03): 183-186.

Raper, K. B. and Fennel, D.L. (1955): The Genus Aspergilla S, "p.41.

Ritter A.C; Hoeltz M. and Noll, I.B. (2011): Toxigenic potential of Aspergillus flavus tested in different culture conditions. Ciencia e Tecnologia de Alimentos Campinas 31(3): 623-628.

Sacchetti, G.; Maietti, S.; Muzzoli, M.; Scaglianti, M.; Manfredini, S.; Radice, M.; et al. (2005): Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. Food Chemistry 91: 621–632.

Saha, D.; Acharya, D.; Roy, D.; Shrestha, D. and Dhar, T. (2007): Simultaneous enzyme immunoassay for the screening of aflatoxin B1 and ochratoxin A in chili samples. Analytica Chimica 584(2); 343–349.

Saidani, M.; Dhifi, W. and Marzouk, B. (2004): Lipid evaluation of some Tunisian Citrus seeds. Journal of Food Lipids 11: 242–250.

Sawamura, M. (2000). Aroma and functional properties of Japanese yuzu (Citrus junos Tanaka) esential oil. Aroma Research, 1 (1), 14-19.

Sawamura, M.; Tu, N. T. M.; Yu, X. and Xu, B. (2005): Volatile constituents of the peel oils of several sweet oranges in China. Journal of Essential Oil Research 17(3): 2–6.

Sawalha, S.M.S. Arráez,R. D; Segura, C. A.; and Fernández, G. A. (2009): Quantification of main phenolic compounds in sweet and bitter orange peel using CE– MS/MS. Food Chemistry 116:567-574.

Silalahi, J. (2002): Anticancer and health protective properties of Citrus fruit components. Asia Pacific Journal of Clinical Nutrition 11: 79–84.

Shimoyamada - M; M-Suzuki; M. Maruyama and K. watanabe (1996). An antifugal saponin from white asparagus (Asparagus officinalis l.). J. of Sci. of Food and Agric., 72 (4): 430-434.

Sokovic, M. and Griensven, L. J. L. D. (2006): Antimicrobial activity of essential oils and their components against the three major pathogens of cultivated button mushroom Agaricus bisporus. European Journal of Plant Pathology 116: 211–224.

Sonia, P. R.; Sharma, H., and Garg, M. (2014): Antimicrobial and Antioxidant activities of fruits and vegetable peels: A review. Journal of Pharmacognosy and Phytochemistry 2014, 3 (1): 160 -164.

Sultana, H. S.; Ali, M. and Panda, B.P. (2012): Influence of volatile constituents of fruit peels of Citrus reticulata Blanco on clinically isolated pathogenic microorganisms under In-vitro. Asian Pacific Journal of Tropical Biomedicine, pp: 1299-S1302.

Tepe, B.; Akpulat, H.A.; Sokmen, M., Daferera, D.; Yumrutas, O.; Aydin, E.; Polissiou, M. and Sokmen, A. (2006): Screening of the antioxidant and antimicrobial properties of the essential oils of Pimpinella anisetum and Pimpinella flabellifolia from Turkey. Food Chemistry 97: 719-724.

Tu, M.N.T.; Onishi, Y.; Choi, H.S.; Kondo, Y.; Bassore, S.M.; Ukeda, H. and Sawamura, M. (2002): Characteristic odor components of Citrus sphaerocarpa Tanaka (Kabosu) cold-pressed peel oil. Journal of Agricultural Food Chemistry 50: 2908-2913.

Utpal - Roy; Batish V.K.; Grover, S. and Neelakantants, S. (1996): Production of antifungaal substances by Lactococcus lactis subsp., lactis CHD-28.3 Int. J of food microb. 32 (1/2): 27-34.

Velazquez, N. M. J.; Avila,S. R.; Palou, E., and López, A. (2013): Antifungal activity of orange (Citrus sinensis var. Valencia) peel essential oil applied by direct addition or vapor contact. Food Control 2013; 31:1-4

Verzera, A.; Trozzi, A.; Dugo, G.; Di Bella, G. and Cotroneo, A. (2004): Biological lemon and sweet orange essential oil composition. Flavour and Fragrance Journal, 19(6), pp 544–548.

Viuda-Martos, M.; Ruiz-Navajas, Y.; Ferna´ndez-Lo´pez, J. and Pe´rez-A´lvarez, J. (2008): Antifungal activity of lemon (Citrus lemon L.), mandarin (Citrus reticulate L.), grapefruit (Citrus paradisi L.) and orange (Citrussinensis L.) essential oils. Food Control 19 (12): 1130-1138.

Wheeler, K. A.; Hurdman, B. F. and Pitt, J. I. (1991): Influence of pH on the growth of some toxigenic species of Aspergillus, Penicillium and Fusarium. International Journal of Food Microbiology 12: 141-150.

Yaghoub, A; Ashrafi, M.; and Fatemeh, M. (2006): New Elaborated Technique for Isolation and Purification of Limonene from Orange Oil. Iranian Journal of Pharmaceutical Sciences, pp 87-90.

Yousef, A. E. and Marth, E.H. (1981): Growth and synthesis of aflatoxin by Aspergillus parasitius in the presence of ascorbic acid. J. Food Prot. 44: 736 – 741.

Zhao, H.; Huang,L., Xiao, C. L., Liu, J., Wei, J. and Gao, X. (2012): Influence of Culture Media and Environmental Factors on Mycelial Growth and Conidial Production of Diplocarpon mali. The Society for Applied Microbiology, Letters in Applied Microbiology 50: 639–644.