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ORIGINAL ARTICLES

Antifungal Activity of Basil and Mustard Essential oils Against Spoilage Toxigenic Fungi in Egyptian pan Bread and its Economic Evaluation.

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ABSTRACT

The antifungal effect of tow essential oils against the most important moulds in terms of spoilage of Egyptian pan bread (*Aspergillus flavus* and *Aspergillus ochrachues*) were investigated. Basil and Mustard oils were added to yeast extract culture medium at level of 0.05, 0.075 and 0.10 %. Effect of the obtained essential oils on mycelium dray weight of *Aspergillus flavus* and *Aspergillus ochrachues* and their toxin production were studied. Aflatoxins and ochratoxin A production were determined by high performance liquid chromatography (HPLC). Basil essential oil was had a potent effect against fungal growth better than mustard oil specially in case addition of basil essential oils at level of 0.1% as well as Aflatoxin (AFs) production was reduced from 150 to 0 ng/ml and Ochratoxin A (OTA) from 135 to 98 ng/ml compared with control. Another part of the current study was to application of the studied oils in Egyptian pan bread for moderate or eliminate bread spoilage by some toxigenic fungi spp. Basil and Mustard oils were added to Egyptian pan bread at the same mentioned levels and cleared that Basil oil was more effective against *A. flavus* and *A. ochrachues* in bread better than Mustard oil during storage of pan bread for 8 days. Also the effect of essential oils additions to bread on the chemical composition of bread was studied and cleared that no significant changes were observed. These findings strengthen the possibility of using Basil and Mustard oils as preservatives agents for Egyptian pan bread.

Key words: Aflatoxins; Ochratoxin A; mycelial growth; *Aspergillus flavus*; *Aspergillus ochrachues;* essential oils.

Introduction

Fungi are the main agents of spoilage of bakery products. A part from visible growth, they also produce off-flavors and mycotoxin production can be a concern. As with other foods, bakery products which contain natural preservatives are becoming more common. However, as with bacteria, fungi are more resistant to these natural antimicrobials when challenged in foods

(Lopez-Malo *et al*. 2002). Fungi are the most common spoilers in bakery products. Commonly, a shelf-life around 3-4 days may be expected when unpreserved. The aflatoxinogenic moulds such as *Aspergillus flavus* and *Aspergillus ochrachues* are able to grow on various types of bread; these fungus can produce aflatoxin on this substrate. The highest yields of the toxin were found on whole wheat bread (Allcroft R 1963).

Aflatoxins are known to be potent hepatocarcinogens in animals and humans (Dvorackova 1990). Therefore, the presence of toxigenic fungi and mycotoxins in foods such as bread and grains stored for long periods of time presents a potential hazard to human and animal health. Considerable interest has developed on the preservation of foods by the use of essential oils to effectively retard growth and mycotoxin production. Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. For these reasons, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensified (Skandamis *et al* 2001). The exploration of naturally occurring antifungal and antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Schuenzel & Harrison, 2002). Antifungal and antimicrobial properties of herbs and spices have been

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recognized and used since ancient times for food preservation and in medicine were reports on natural antifungal and antimicrobial agents dating back more than a century (Conner, 1993). A renewed interest in natural preservation appears to be stimulated by present food safety concerns, growing problems with microbial resistance, and a rise in production of minimally processed food, together with green image policies of food industries. Numerous studies have documented the antifungal (Aligiannis *et al.* 2001; Thompson 1989) and antibacterial (Canillac & Mourey, 2001; Lachowicz *et al*. 1998).

Several essential oils of aromatic plants including, *Thymus vulgaris* (thyme) and *Ocimum basilicum* (basil), have totally inhibited fungal development on maize kernels (Montes-Belmont and Carvajal, 1998). Mustard essential oil has been used for inhibition of fungal growth on bread. (Elgayyar *et al*. 2001), examined the effectiveness basil and other essential oils for controlling the growth and survival of pathogenic and saprophytic microorganisms. The results showed inhibitory property of basil and other essential oil, which presented minimum lethal concentration (v/v) ranging between 8 and 50 ppm for *Pseudomonas aeruginosa Staphylococcus aureus* and *Yersinia enterocolitica*.

The aims of the current study were to evaluation of Basil and Mustard oils as active compounds against *Aspergillus flavus* and *Aspergillus ochrachues* in Egyptian pan bread to extension the shelf life using natural safe essential oils over chemical preservatives.

Materials and Methods

Materials:

1. Yeast extract powder, was purchased from Sigma-Aldrich, France.
2. *Aspergillus flavus* and *Aspergillus ochrachues*, were isolated from Egyptian corn grains.
3. Essential oils of Basil and Mustard oils were purchased from Synthetic Chemicals Private Ltd., (Kollenchetrry, India).
4. Standards Aflatoxins (B_1-B_2- G_1-G_2) and Ochratoxin A were purchased from Sigma Chemical Company, St Louis, MO 63103, USA.

Methods:

Preparation of Yeast extract:

Yeast extract was prepared by adding twenty g yeast extract powder, 150 g sucrose adjusted to 1L by distilled water, heated to 100 ^{o}C .One hundred ml of liquid media were putted in conical flasks and sterilized at 125 ^{o}C for 15 min. The flasks were cooled and 100 µl tween 40 was added for each.

Basil and/or Mustard oils (0.05-0.075-0.10 %) and 1ml spore suspension of *Aspergillus flavus or Aspergillus ochrachues* were added for each. The inculcated flasks were incubated at 28 ^{o}C for 15 days. Dry weights of mycelial mats from liquid media were obtained by decanting off the culture and drying the mat at 65 to 70 ^{0}C.

Determination of Aflatoxins and Ochratoxin A:

1-Extraction of aflatoxins produced in the YES culture was carried out according to the method of Munimbazi and Bullerman (1998). Where, the mycelium of each flask contained YES medium was harvested by filtration through Whatman paper (No.4), and then extracted by 100 ml chloroform. Chloroform extract was dried by addition of anhydrous sodium sulfate. The residue was transferred to vial and evaporated off using a stream of nitrogen at temperature below 60 ^{o}C. The dry film was used for the detection of aflatoxins by high performance liquid chromatography (HPLC).

Determination of Aflatoxins by HPLC technique:

Derivatization:

Fifty µl trifluoracetic acid (TFA) were added to the dry film of standard and samples and the mixture was let to stand for 15 min followed by 450 µl H_2O: CH3CN (9:1 v/v) and they were mixed well by vortex for 30s and the mixture was let to stand for 5 min. (AOAC, 2000).

The detection of aflatoxins was performed using the fluorescence detector set system at 360 nm excitation and 440 nm emission wavelengths. The mobile phase system (water: methanol: acetonitrile 240:120:40 v/v/v)

2- Extraction of ochratoxin:

Fifty g samples were put into high speed blender; 25 ml phosphoric acid (0.1M) and 250 ml chloroform were added and blended for 3 min. at medium speed. Ten gram diatomaceous earth were added just before the end blending time then filtered through Whatman No.4 filter paper and 50 ml portion were collected, transferred to separator funnel, 10 ml sodium bicarbonate (3%) were added and shacked gently, then the upper phase was collected for column separation.

For clean up, a Sep-Pak C_{18} Column was placed on vacuum manifold ports, column pre-washed twice with 2 ml methanol, 2 ml water, and 2 ml sodium bicarbonate (3%). Five ml bicarbonate extract were added to the C_{18} column, followed by 2 ml phosphoric acid (0.1M) and 2 ml water, and washings were discarded. OTA was eluted with 8 ml ethyl acetate: methanol: acetic acid (95: 5: 0.5 v/v/v). The elute was collected in vial containing 2 ml water and the elute was shacked with tube shaking machine (vortex genie) to mix the two phases. Pipette OTA extract (upper phase) to 7 ml screw- cap vial. Rinse remaining upper phase from tube with 2 x l ml ethyl acetate and add to OTA. Evaporate extract just to dryness on steam bath under nitrogen for subsequent HPLC analyses (AOAC 2007). High performance liquid chromatography (HPLC) were used to both aflatoxins and ochratoxin A determinations. The system equipped with (Waters 600) delivery system. HPLC column a reverse phase analytical column packed with C_{18} material (Spherisorb 5 µm ODS2, 15cm×4.6nm). The fluorescence detector was operated at an excitation wave length of 330 nm and an emission wave length of 460 nm. The mobile phase consisted of acetonitrile: water: acetic acid (99:99:2 v/v/v). The separation was performed at ambient temperature at a flow rate of 1.0 ml/ min. Data were integrated and recorded using a Millennium Chromatography. Manger Software 2010 (Waters, Milford MA 01757). Quantitation: Calculated from chromatographic peak areas using the standard curve.

Total fungal count of pan bread:

The various types and numbers of mould and yeast associated with pan bread were enumerated according to the method described by Harrigan and McCane (1976).

Statistical analysis:

The data were statistically analyzed using the General Linear Models Procedure of the Statistical Analysis System (SAS, 1982). The significance of the differences among treatment groups was determined by Waller-Duncan k-ratio (Waller and Duncan, 1969). All statements of significance were based on probability of $P < 0.05$.

Results and Discussion

Several essential oils of aromatic plants including, thyme and basil have totally inhibited fungal development on maize kernels (Montes and Carvajal 1998). Mustard essential oil has been used for inhibition of fungal growth on bread (Nielsen and Rios 2000).

Table 1: Effect of Basil oil on dry mycelium weight (g) of *Aspergillus flavus* and *A. ochrachues* at different concentrations.

Basil oil %	Dry mycelium weight of *A. flavus*	% of reduction	Dry mycelium weight of *A. ochrachues*	% of reduction
Control	2.78		3.15	
0.05 %	2.56	7.91	1.62	48.57
0.075 %	1.78	36.69	1.54	51.11
0.1 %	0.00	100	0.54	82.85

The results in table (1) shows the effect of Basil oil on dry mycelium weight (g) of *Aspergillus flavus* and *A. ochrachues* in yeast extract liquid medium at different concentrations (0.05, 0.075 and 0.1 %). Data cleared that the addition of basil oil at 0.1 % caused completely inhibit growth of *A. flavus*, while caused reduction of dry mycelium weight to be 7.91 and 36.69 at concentrations 0.05 and 0.075 % respectively. Also, basil oil reduced the growth of *A. ochrachues* to be 48.57, 51.11 and 82.85 % at level 0.05 %, 0.075 % and 0.1 %, respectively. It is worthy to mention that basil oil was very effective against *A. flavus* and *A. ochrachues* at high concentration 0.1 % and more effective with *A. flavus* better than *A. ochrachues.*

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Table 2: Effect of Mustard oil on dry mycelium weight (g) of *Aspergillus flavus* and *A. ochrachues* at different concentrations.

Mustard oil %	Dry mycelium weight of *A. flavus*	% of reduction	Dry mycelium weight of *A. ochrachues*	% of reduction
Control	2.78		3.15	
0.05 %	2.74	1.43	3.01	4.44
0.075 %	2.64	5.03	3.00	4.76
0.1 %	2.03	26.97	2.80	11.11

Table 2 shows the effect of Mustard oil on dry mycelium weight (g) of *A. flavus* and *A. ochrachues* at mentioned concentrations. Results proved that mustard oil had a weak to medium effect against *A. flavus* and *A. ochrachues* at three used concentrations and the reduction was 1.43 to 26.97 % for *A. flavus* and from 4.44 to 11.11 % for *A. ochrachues*. Generally basil oil had a very good potent effect against investigated fungi better than mustard oil and also basil oil had an excellent effect against *A. flavus* than *A. ochrachues.*

Basil and other herbs caused a total inhibition of A. flavus on maize kernels. The optimum dosage for protection of maize varied from 3 to 8 % (Montes and Carvajal 1998). A number of compounds and substances have been found to be effectively inhibit fungal growth and aflatoxin production, while others have stimulatory properties (Zaika and Buchanan 1987). In many instances low concentrations of test compounds stimulated fungal growth and/or toxin production, while higher concentrations completely inhibited them. Essential oils from pepper, mustard, cassia tree and clove suppressed disease development caused by F. oxysporum f. sp melonis on muskmelon and reduced the population density of F.oxysporum f. sp chrysanthemi in green house ex periments (Browers and Locke 2000).

Table 3: Effect of Basil and Mustard oils on production of Aflatoxins.

Con. of Basil oil	Con. of total AFs (ng/ml)	% of reduction
Control	150	
0.05 %	120	20.00
0.075 %	50	66.66
0.1 %	0	100
Con. of Mustard oil		
Control	150	
0.05 %	135	10.00
0.075 %	127	15.33
0.1 %	98	34.66

Table 3 showed the effect of Mustard and Basil oils on Aflatoxins production and from the obtained results Basil oil reduced AFs production to be 20, 66.66 and 100 % at experimented concentrations (0.05, 0.075 and 0.1 %) respectively, while Mustard oil reduced AFs production by 10, 15.33 and 34.66 % at the same experimented basil oil concentrations (0.05, 0.075 and 0.1 %). In general, Basil oil had a potent effect against AFs production better than Basil oil. (Neilsen and Rios, 2000) reported that the Mustard essential oil, which primarily contains allyl isothiocyanate, showed a very significant antifungal effect against all test fungi for more than two weeks.

Table 4: Effect of Basil and Mustard essential oils on production of OTA.

Basil oil %	Concentration of OTA(ng/ml)	% of reduction
Control	370	
0.05 %	179	51.62
0.075 %	161	56.48
0.1 %	94	74.59
Mustard oil %		
Control	370	
0.05 %	344	7.02
0.075 %	339	8.37
0.1 %	317	14.32

Table 4 shows the effect of Mustard and Basil oils on production of OTA. Data cleared that Basil oil had a good effect against OTA production and the reduction was ranged from 51.62 to 74.49 %, while Mustard oil had a weak effect to reduce OTA which ranged from 7.02 to 14.32 %. Generally Basil oil was very good potent effect against growth for both *A. flavus and A. ochrachues* than Mustard oil, moreover Basil oil reduced AFs and OTA productions to reach 100 % reduction better than Mustard oil. The most important feature of moulds from a food safety perspective is their ability to produce mycotoxins, such as aflatoxins, which are toxigenic secondary metabolites. *Aspergillus ochraceus* produces OTA which is responsible for nephropathies in pigs and humans. Fortunately, essential oils derived from plants are also known to possess antifungal activity (Soliman and Badeaa, 2002).

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Table 5: Effect of Basil and Mustard essential oils on chemical composition of produced pan bread.

Basil oil %	Protein	Ash	Fiber	Lipid	Carbohydrate by difference
Control	11.00	0.50	0.60	0.30	87.60
0.05 %	10.99	0.51	0.59	0.30	87.61
0.075 %	10.99	0.50	0.60	0.31	87.61
0.1 %	10.99	0.50	0.59	0.33	87.59
Standard Error	±0.00014	±0.0033	±0.0088	±0.0057	±0.0088
Mustard oil %					
Control	11.00	0.50	0.60	0.30	87.60
0.05 %	10.98	0.50	0.60	0.31	87.61
0.075 %	10.98	0.52	0.59	0.33	87.58
0.1 %	10.97	0.53	0.58	0.34	87.58
Standard Error	±0.00014	±0.0001	±0.0033	±0.0033	±0.0088

Table 5 shows the effect of Basil and Mustard oils on chemical composition of produced pan bread. Results proved that addition both Basil and Mustard oils had not significant effect on chemical composition of produced pan bread just slightly changes in chemical composition as a normal result to add the experimented oils. The protein, ash, fiber, lipid and total carbohydrate were ranged from 10.97 to 11 %, 0.50 to 0.53%, 0.58 to 0.60%, 0.30 to 0.34 % and 87.58 to 87.61 % respectively.

Table 6: Effect of Basil and Mustard oils on total fungal count of produced pan bread.

Days	Control	Basil oil %			Mustard oil %		
		0.050	0.075	0.100	0.050	0.075	0.100
1	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0
4	9	0	0	0	5	3	1
5	50	6	0	0	13	9	5
6	77	9	5	0	18	12	9
7	124	21	13	0	32	13	17
8	158	52	23	0	71	44	41

Table 6 shows the effect of Basil and Mustard oils levels on total fungal count (TFC) of produced pan bread. The produced natural and treated pan bread were stored for 8 days at room temperature 22-30 ^{o}C and daily total fungal count was carried out. From obtained results data cleared that the control had 9 colonies at forth day to reach 158 colonies at eighth day, while pan bread treated with 0.05 % basil oil had 6 colonies at fifth day and increased at eighth day to 52 colonies, but second treatment 0.075 inhibited fungi growth to sixth day and TFC was 5 to 23 at final time, however the third treatment completely inhibited growth of fungi. Concerning the Mustard oil had a medium inhibition of TFC on pan bread and the growth appeared after three days to be from 5 to 71, 3 to 44 and 1 to 41 colonies at mentioned three concentrations respectively.

It is worthy to report that Basil oil was better than Mustard oil for pan bread shelf life keeping quality as well as in all tested parameters in our current study and kept the shelf life of pan bread eight days at high concentration and therefore utilization of Basil oil for bread keeping quality has a good ecnomic value and will help to increase the value of all out puts.

Economic evaluation:

The produced quantity of Basil oil:

The produced quantity of Basil oil was estimated at about 94.2 tons and that was from the Basil leaves which were estimated about 25.1 tons for each Fedan for total planted area at about 4.7 thousand fedan, and the cost for each Kg from Basil oil at about 25 pounds.

The following equation shows:

The produced quantity of Basil= the planted area from Basil (fedan)Xthe oil quantity extracted from one fedan (Kg)

= 20X4709= 94.2 thousand Kg=94.2 tons oil

The produced quantity of pan bread using the quantity produced from oil:

The oil quantity which is extracted from one fedan of leaves was estimated at about 20 Kg oil and the Basil oil quantity which is added to one loaf of pan bread, which weighs about 100g flour was estimated at about 0.1

mg of Basil oil, that leads to produce about 200 thousand of pan bread from only one fedan of Basil oil. That mean that we can get about 18.8 million loaves of pan bread per year, which mean 51.5 thousand loaves of pan bread per day.

The following equation shows:

1- The quantity of pan bread that can be produced by adding Basil oil extracted from one fedan = the oil extracted from one fedan ÷ the oil quantity required to be added to one loaf = 20 Kg ÷ 0.1 mg = 200 thousand loaves produced from the oil extracted from one fedan.

2- The pan bread quantity which can be produced using the produced quantity of Basil oil = the bread quantity which can be produced from one fedan X the oil quantity which is extracted from the planted area = 200 thousand loaves X 94.2 tons of oil = 18.8 million loaves per year.

3- The pan bread quantity which can be produced per day = the bread quantity produced per year ÷ the number of years days = 18.8 million loaves ÷ 365 days = 51.5 thousand per day.

The cost of the Basil oil which is added to one loaf to the total cost of producing a loaf of pan bread:

The cost of the Basil oil which is added to a loaf of pan bread is estimated at about 2.5 piaster for each 0.1 mg of oil and as the total cost of producing a loaf of pan bread is estimated at about 47.5 piaster, the total cost of producing a loaf of pan bread is estimated at about 50 piaster after adding the Basil oil, that is why cost of the Basil oil added to one loaf is estimated at about 5 % from the total cost of producing one loaf of pan bread.

The following equation shows:

The rate of the Basil oil cost in pan bread to the total cost of one loaf = the cost of the Basil oil in one loaf ÷ the cost of producing one loaf = (2.5 piaster ÷ 50 piaster) X 100 = 5%.,and by raising the price of the pan bread loaf after adding the Basil oil to 5 % the benefit of one loaf of pan bread can be estimated at about 5% from the price of the loaf of the producer with increasing the benefit for the consumer as a result of lengthening the period of shelf life of the loaf from 4 days to be 8 days. Also the lengthening of shelf life can lead to increase the demand of this loaf. The economic evaluation was carried out according to (UN 1972).

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