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RESEARCH ARTICLE

Isolation of pathogenic fungi from salted marine bivalve *Donax trunculus* in Egypt with particular emphasis on controlling their hazard

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Abstract

Objective: The aim of present study was to investigate fungal contamination in a salted bivalved *Donax trunculus* and controlling their hazards by some natural products. Material: A total of 70 samples were selected from two fish markets in Alexandria (Egypt). Microbiological analysis was performed to identify potential sources of fungal contamination and investigate the bioactivity potential of lemon juice, vinegar and black seed oil as antifungal agent in the preservation of *Donax trunculus.* Results: Results obtained showed that *Donax trunculus* collected were contaminated by fungi, namely: *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer*, *Fusarium oxysporum* and *Candida albicans*. Results of *in vitro* antifungal tests revealed that mixing blackseed oil and vinegar (2:1) has strong antifungal activity against all isolated fungal strains. Conclusion: The present study indicates that treatment with natural organic product like blackseed oil and vinegar can effectively reduce the fungal load. Hence, it is suggested that mollusc should be preserved and depurated before salting in both blackseed oil and vinegar (2:1) to prevent fungal contamination for human consumption.

INTRODUCTION

Marine invertebrates are known to be natural unique accumulators of specific microbial communities due to their filter feeding habit (Romanenko, *et al.*, 2008). External envelops, internal tissues and organs or liquors of these animals enriched by nutrient compounds and adhesive substances which are considered as auspicious conditions for colorization, attachment and activity of associated microorganisms. Molluscans are used for human consumption all over the world. They constitute a delicious food rich in all nutrients. On the other hand, these animals are normally found in water near the shore which subjected them to contamination of runoff water carrying soil microorganisms and sewage outfall. Since, harvesting of bivalved molluscs from contaminated areas constitutes a major public health hazard (Evison, 1985). Many pathogenic and toxicogenic microorganisms could be concentrated in the molluscs as they act as filter feeders (Apha, 1984).

Hatha, *et al*. (2005) mentioned that molluscan shellfishes, especially the biovalve elams, are considered as potentially hazardous food because of their inherant tendency to bioaccumulate pathogenic microorganisms through filter feeding. It was understood that the inappropriate disposal of raw and partially treated sewage was a principle reason for increasing incidence of shellfish-borne illness. Hence, strict guidelines are issued by the government regulatory authorties of the developed countries regarding fungal and bacteriological quality of the harvesting water of the wild caught of the shellfish (Leonard, *et al*., 1990).

In Egypt, our knowledge of the diversity of mollusc-associated fungi and their hazard is still limited. Only two attempts were carried out in this concern. The first trial was done by Abd el -massin (1989) who mentioned that molluscan shellfish which are harvested from EL- Max Coast (Alexandria) were heavily polluted by *E. coli* and this proves that there is pollution with sewage in the seawater of El Max, While molluscan shellfish harvested from Ismailia and Edco coasts contain few *E. coli*. This proves that they are harvested from areas free from sewage pollution. Mansour, *et al* (1998) investigated the bacteriological hazard of Om El -Kholoul in different localities of Egypt. They tried to investigate the inhibitory effect of lemon juice on the bacteriological load of it.

To the best of our knowledge; there is no previous research has been carried out on fungal pathogens that contaminated salted marine bivalve *Donax trunculus* and its public health hazard. Therefore, this study was conducted to provide a list of fungi commoly involved infestation and deterioration of usually available salted bivalve molluscs in Alexandria (Egypt), where the traditional salting technique of this bivalve processing is mostly practiced. In addition, praticular attention has been done to a safe prevention of such fungal contamination for human health.

Material and methods:

Sample Collection: Seventy samples of salted *Donax trunculus* were obtained (Fig 1) randomly from two fish markets in Alexandria city (Egypt) in October 2014 at an interval of one week for a period of three weeks. To avoid contamination during sampling; transportation and storage, the samples were kept in labeled polyethylene bags and taken immediately to the Laboratory of Microbiology, Department of Botany, Tanta University.

Figure (1): Collected samples of salted bivalve *Donax trunculus*

Sample preparation: The samples were washed with a brush and water under pressure to remove all material adhered to the shells, and then placed on a stainless-steel trays for air drying. The soft part was removed from their shell with a sterile stainless-steel instrument. The intervalvar fluid and the meat were transferred aseptically to sterile bags, then the meat was taken aseptically into a vertical laminar-flow cabinet and 25 g were transferred to a stomacher bag. 225 ml of 0.1% peptone water with salt (NaCl, 0.85%, w/v) were added and the mixture was homogenized with a stomacher for 60 seconds. Ten-fold serial dilutions were made and the samples (0.1 ml) were then spread on different Agar plates (Ortigosa, *et al*. 1994)

Fungal isolation and identification: The assayed culture media used throughout the study for the growth and maintenance of the fungal isolates were Potato Dextrose Agar (PDA), Czapek dox agar (CDA) and Sabouraud's Dextrose Agar (SDA). Each media was supplemented with 0.5 g/ L chloramphenicol. 0.1 ml volume of each dilution was separately placed on the three different media. Then PDA and CDA plates were incubated at 28 ± 2°C for 7 days to determine fungal growth, while SDA plates were incubated at 35± 2°C for 24h to determine yeast growth. All filamentous isolates growing on the plates were sub-cultured, and identified macroscopically and microscopically using colony colour, type (compact, loose, aerial hyphae), texture (velvety, cottony, coarse), shape and growth pattern according to the detailed drawing of the features and taxonomic schemes primarily based on

morphological characters, using the methods described by Singh, *et al*. (1991)., Alexopoulos (1962), Alexopoulos, *et al*. (1996), Barnet and Hunter (2010) and Blackwell (2011). *Candida albicans* isolates were re-identified by production of chlamydoconidia on Corn meal agar (Difco, USA) and green colour colonies on CHROMagar *Candida* (CHROMagar *Candida*, France). From this selective and differential medium, the isolates were identified by classical methods (Kurtzman & Fell, 1998).

Antifungal assay: Cut plug method recorded by Pridham, *et al*. (1956) was employed to determine the antifungal activity of the chosen materials as follows: Freshly prepared spore suspension of each growing fungal isolate (0.5 ml of about 10^6 cells / ml) was mixed with 9.5 ml of sterile suitable medium at 45 ºC, poured on sterile Perti dishes, and left to solidify at room temperature. Regular wells were made in the inoculated agar plates by a sterile cork borer with 0.8mm diameter. Each well was aseptically filled up with 0.2 ml of black seed oil with concentration of 50 % (v/v) that prepared in 100 ml distilled water containing 5 % Tween 80. On the other hand, in order to examine the effect of lemon and vinegar, each well was filled with 0.2 ml of each material with concentration of (50%). Three replica were made for each tested suspension. Then the average diameters of inhibition zones were recorded, and compared by all plates.

Determination the effect of different concentrations of different materials on fungal growth and minimal inhibitory concentrations:

Different concentrations (60, 70, 80, 90, 100 %) were prepared from each material separately. The effect of these concentrations on fungal growth was determined with cut plug method as described above. Three replica were made for each tested suspension. Then the average diameters of inhibition zones were recorded, and compared by all plates.

MIC was determined by mixing spore suspension of each fungus (0.5 ml of 10^6 cells /ml) with 9.5 ml of different concentrations of each material in sterile test tube and incubated for 24 hrs at 37°C for yeast and 25°C for fungus. Then 0.2 ml of each mixture was spread on the surface of previously sterile agar plates for 48 hours at a definite temp. for each one. Colony forming units were counted, represented graphically and MIC was recorded for each material. (Shadomy, *et al*., 1985).

Examination the effect of different concentrations of mixing black seed oil and vinegar on fungal growth:

Cut plug method was used as described above, but in case of mixing oil, each well was filled 0.2 ml of mixed (black seed oil + vinegar) with concentration of 100 % (v/v) that prepared in (1:1, 2:1) containing 5 % Tween 80.

Statistical analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard Deviation, student t- test and Analysis of variance [ANOVA] tests by SPSS V17, with the level of significance set at $P<0.05$. Percentage frequency of isolation was calculated by adding the number of times each fungus occurred in the colonies growing on the plates, divided by the total count of all the organisms and multiply by hundred.

$$\text{i.e. } \frac{\text{Total count of individual isolate}}{\text{Total no of all the organisms}} \times 100\ \%$$

Results:

Donax trunculus is a bivalved species in the family *Donacidae* as shown in Figure (2). It is native to the Mediterranean and Atlantic Oceans of Westren europe. It is locally known as Om El-kholoul, and is consumed as salted food in Egypt, particulary in Alexandria, Portsaid and Ismalia. It is collected from sandy shores, cleaned and salted by the addition of Rashidy salts for 48-72 hours, then consumed by pupils, children and picnickers.

Figure (2): *Donax trunculus* (Dorsal and ventral views of the shell)

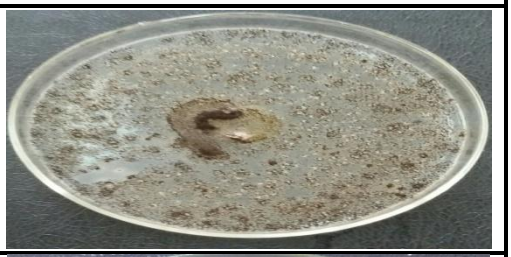
The species of fungi isolated from bivalved *Donax trunculus* and identified were *Aspergillus niger*, *Aspergillus flavus*, *Rhizopus stolonifer*, *Fusarium oxysporum* and *Candida albicans* (Table 1). *A. niger and A. flavus* were found in the samples from both markets, while *Rhizopus stolonifer* occurred in the samples of market 1 only.The samples of market 2 contained *Fusarium oxysporum* and *Candida albicans*, which were absent from the market 1 samples.
The macro features of the fungi isolated were presented in (Table 2). The difference between *A. flavus* and *A. niger* is in the form of colony as *A. flavus* has yellowish green colonial colour with scattered spores, while *A. niger* is dark in colour with ball head. *R. stolonifer* has a brownish wooly colony, *Fusarium oxysporum* colonies are initially white, becoming tinged with salmon at maturity and *Candida albicans* has a white to cream, soft, and smooth to wrinkled colonies.The frequency of occurrence of each fungus isolated is shown on figure (3), *Aspergillus niger* has the highest frequency of occurrence. This was followed by *A. flavus*, *R. stolonifer*, *Candida albicans* and *Fusarium oxysporum* in that order.

Table 1: Fungi isolated from two different markets in Alexandria obtained in October 2014

Fungal species	**Market 1**	**Market 2**
Aspergillus niger	**+**	**+**
Aspergillus flavus	**+**	**+**
Rhizopus stolonifer	**-**	**-**
Fusarium oxysporium	**+**	**+**
Candida albicans	**-**	**-**

+ = present; - = absent

Table 2: Macro features of the fungi isolated from the samples obtained from different two markets in Alexandria:

Fungal name	Colonial characteristics	Macroscopic appearance
Aspergillus niger	Dark colony	
Aspergillus flavus	Yellowish green colony.	
Rhizopus stolonifer	Brownish wooly colony	
Fusarium oxysporium	White cottony colony	
Candida albicans	White to cream colored, smooth colony	

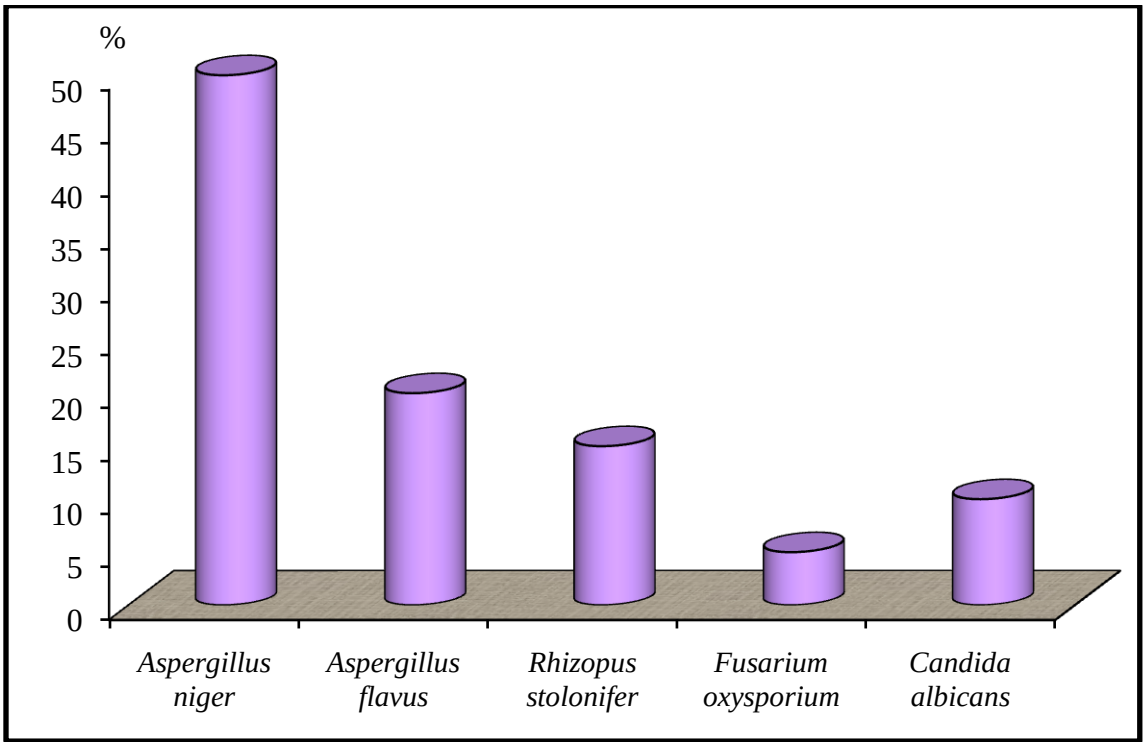


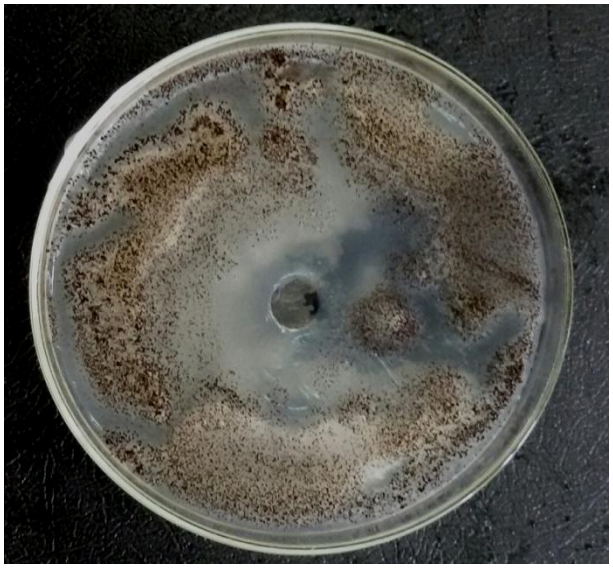
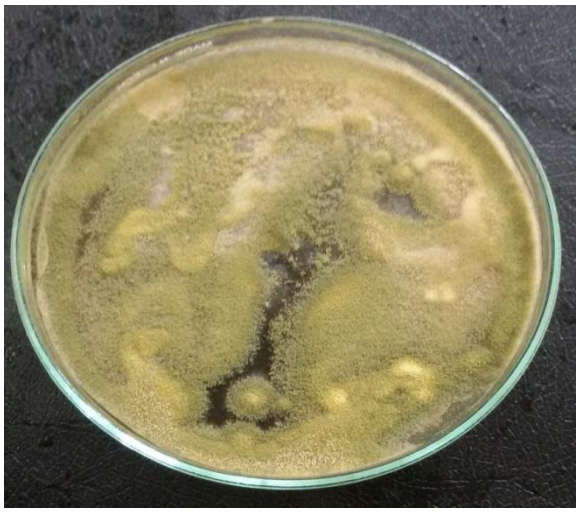
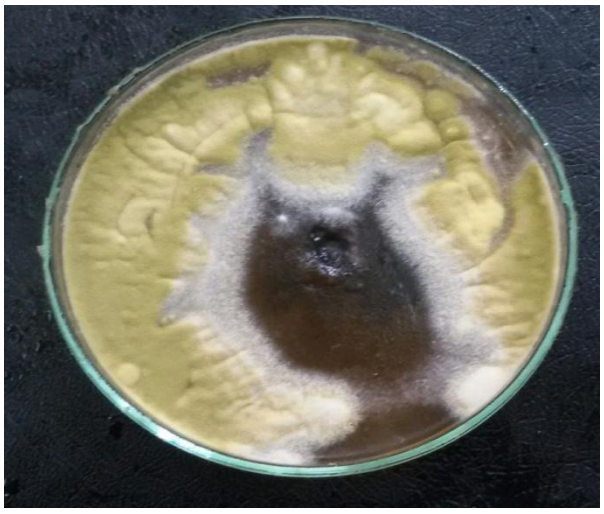
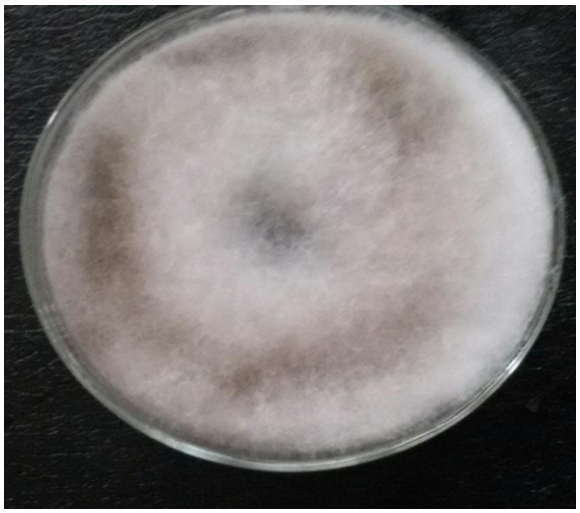
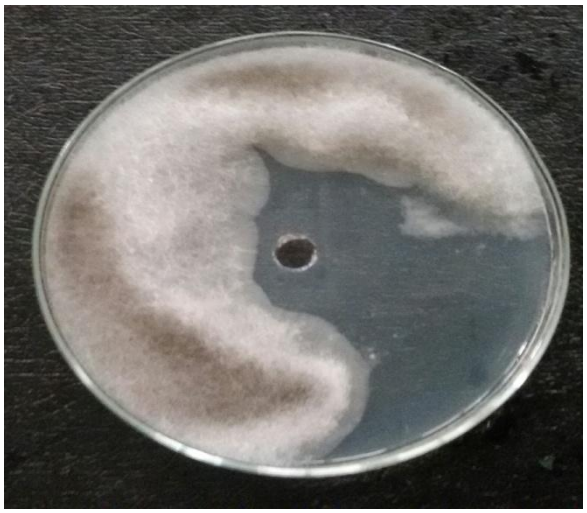
Figure 3: Occurrence percentage (%) of fungi isolates from collected samples:

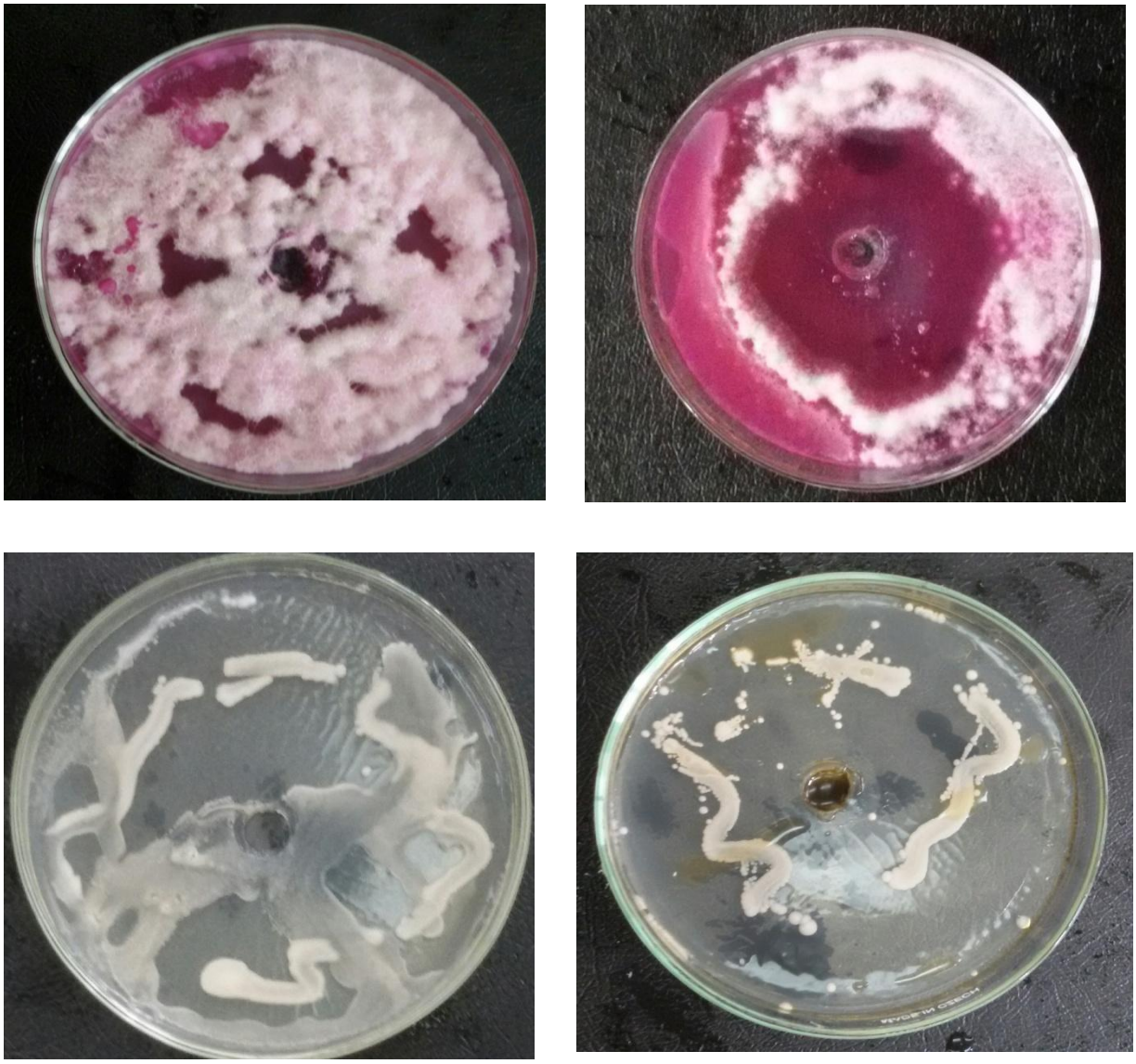
Some selected materials, that had been suggested to it's antifungal activities to prevent mollusc contamination were studied as in table (3). It showed that vinegar gave the highest inhibitory effect among the other used materials against all isolated fungi except *C. albicans* and *F. oxysporum* followed by black seed oil, which gave a high inhibition zone against *C. albicans* and *F. oxysporum* the both fungi which vinegar can't inhibit . While lemon juice had very weak antifungal activity against the tested yeast. Figure (4) represented the highest inhibition zones of vinegar and black seed oil against all isolated fungi in comparison with control.

Table (3): Screening for inhibitory effect of different materials (conc. =50 %) on growth of isolated fungi from collected samples:

Tested materials		**Diameter of inhibition zone (cm)**				
		Fungal isolates				
		A. niger	*A. flavus*	*F. oxysporum*	*R. stolonifer*	*C. albicans*
Citrus limon (lemon juice)		0.0±0.0	0.0±0.0	0.8±0.06	1.2±0.15	0.0±0.0
Dilute Acetic acid (Vinegar)		4.1±1.6	3.0±1.4	0.5±0.09	5.3±1.2	0.0±0.0
Nigella sativa (Black seed oil)		0.0±0.0	0.0±0.0	4.5±1.3	1.0±0.2	5.0±1.54
ANOVA	F	65.639	45.872	34.977	25.390	75.684
	P-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

* P value is statistically highly significant at<0.001



Control **Treated**

Figure (4): The most inhibitory effects of different tested materials against isolated fungi:

Significant differences were observed on the effect of different concentrations of vinegar and black seed oil on the growth of isolated fungi by Oneway Anova test as shown in table (4). To detect the suitable concentration of vinegar and black seed oil, minimal inhibitory concentration (MIC) was found to be the lowest concentration of material giving the highest inhibitory effect on fungi as in figure (5). Figure (6) showed high inhibitory effects of mixed materials as blackseed oil + vinegar (2:1) give the best effect followed by blackseed oil + vinegar (1:1), as it is clear from this figure that blackseed oil + vinegar (2:1) has the most observable inhibition activity among all tested material. This means that mixing oils increase their antifungal activity on the inhibition of isolated fungi.

Table (4): Effect of different concentrations of vinegar and black seed oil on growth of isolated fungi:

Concentration (%)		Diameter of inhibition zone (cm)				
		Vinegar			Black seed oil	
		A. niger	*A. flavus*	*F. oxysporum*	*R. stolonifer*	*C. albicans*
60		4.8±1.2	3.5±1.3	6.0±1.34	5.5±1.4	5.9±1.6
70		6.5±1.52	5.32±1.6	6.3±1.7	5.7±1.6	6.5±1.72
80		6.8±1.6	6.0±1.37	6.5±1.6	5.8±1.9	7±1.64
90		8.1±2.5	6.2±1.64	8.3±2.8	7.0±1.8	7.2±2.4
100		8.0±2.4	8.0±1.32	8.0±2.1	7.2±2.2	8±1.56
ANOVA	F	49.207	125.017	28.546	19.525	18.809
	P-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

*P value is statistically highly significant at the <0.001

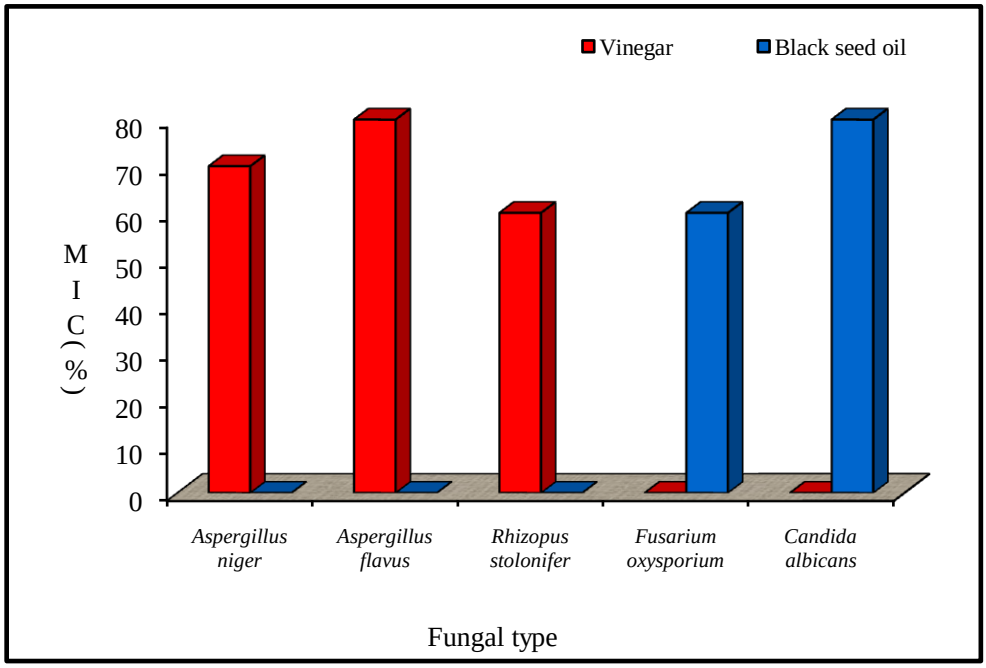


Figure (5): Minimal inhibitory concentration of vinegar and black seed oil aganist isolated fungi:

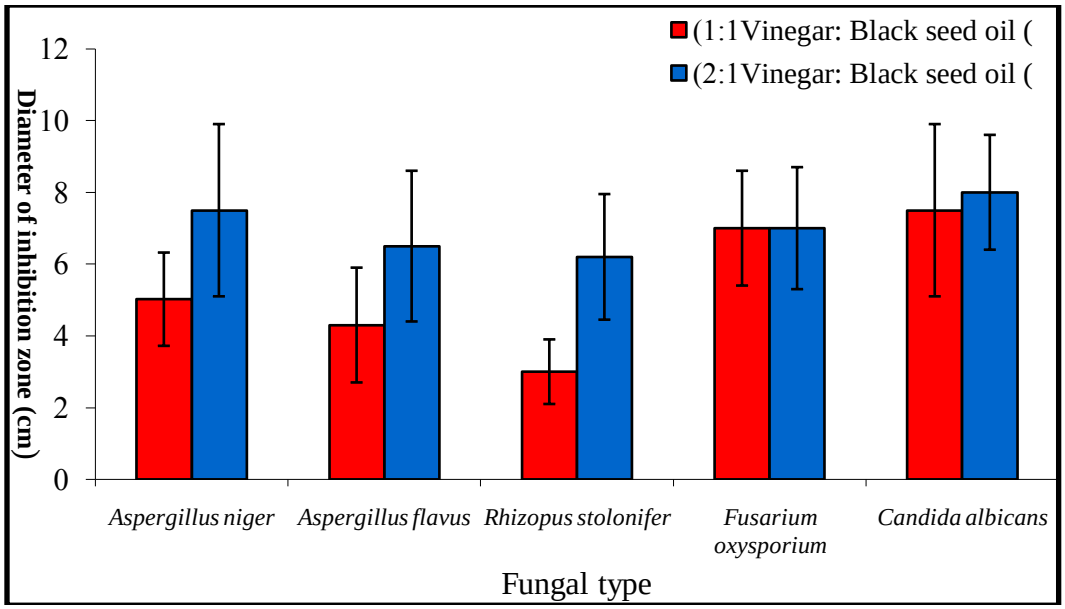


Figure (6): Inhibition of isolated fungi growth by combinations of the most efficient tested materials (conc. 100%):

Discussion:

The obtained results revealed that the hazards of fungal contamination of salted *Donax trunculus* in Egypt increase because of the high number of detected pathogenic fungi. The hazards are expected from the investigated samples due to the higher PH value of the salted samples. This come in accordance with *Mansour, et al*., 1998 who investigated the bacteriological hazard of salted Om El-Kholoul. In addition, shell of the investigated samples as hard calcareus material represent a suitable habitat for many fungi to sporulate. This explanation agrees to some extent with noticeable report on the role of fungi in marine ecosystems proposed by Kevin, *et al*., 1998; besides the examined samples were harvested from polluted area as well as its nature as filter feeders. This conclusion agreed to certain extent with those reported by Tamarapu, *et al*., 2001, and El- Olemy, *et al*., 2014; who reported that the high incidence of microbial infection in marine animals could be the resulty of improper handling, improper storage and cross contamination. Czeczug (2000) also strengthes this conclusion by proposing that the increased incidence of aquatic fungi on mollusc can be explained by the character of water which promote mycoflora species diversity. It is important to declare that bivalve molluscs accumulate contaminants within the water column they inhabit. The important ingredients of these contaminants, including microorganisms, can cause acute disease in humans who eat mollscs raw, salted or undercooked (Fatma, *et al*., 2014). From above, it can be safely concluded that depuration process is important to be performed before salting the investigating samples to reduce the level of contamination with toxic fungi.

The present study revealed a varied number of aquatic fungus species in the particular mollusc species examined. This may be associated with several factors among which, as demonstrated on fish (Wessler and werner, 1957), a significant role can be ascribed to mucous cover, which protects the organism against microbe invasion. Also stressogenic factors, such as water chemist, temperature or nutrition had an effect, reducing the organism resistance to microbe infections (Snieszko, 1974). So, our investigation was looking into sources of alternative, more natural and environmentally friendly antimicrobials, antioxidants and food protection agents. Therefore, some natural materials were tested for their antifungal activities aganist food contamination for human health.

The present study indicated that black seed oil has antifungal activity, this result comes accordance with Salman, 2009 who showed the antimicrobial activity of this oil. In contrary, another study done by Salman, *et al*., 2004 who showed less significant effect against *P. aeruginosa* isolated from different sources. This variation in antimicrobial activities might be due to that black seed oil obtained from different commercial sources or isolated by different methods from the same seeds have been shown to vary significantly in their content of Thymoquinone, which has

antimicrobial activity and various storage conditions are expected to make a difference in the amounts of the quinone constituents of the oil, especially if the seed oil samples are exposed to heat and light. Apple cider vinegar is a commonly prescribed antifungal agent in folk medicine. In our present study, apple cider vinegar showed significant antifungal effect against several isolates. Such results agreed with Shahidi- Bonjar, 2004; who found that topical application of apple cider vinegar is effective treatment of fungal infections. The antifungal activity of apple cider vinegar might be attributed to its mallic acid, acetic acid contents or to other non identified ingredients.
In this study, the *lemon* juice showed very weak antifungal activities against isolated fungi. Similar results were obtained by Cvetnic (2004) who found that no inhibiting effect of *C. paradisi* on the growth of gram negative microorgansims. This was in accordance with Winniczuk and Parish, 1997 who found that citric and lactic acids and d-limonene were less effective as anti microbial compounds.

Finally, the present investigation showed high antifungal activity of mixing blackseed oil with vinegar against all fungal isolates. This was similar to Bansod and Rai (2008) who represented the high antimicrobial activity of some mixed oils of (*Eucalyptus globulus*, *Cymbopogon citratus* and *Cinnamomum zylanicum*) against different pathogenic bacteria and *Trichophyton rubrum* . Also, Fu, *et al*. (2007) showed that mixed oils (clove + rosemary) gave high antimicrobial activity against pathogenic yeast and bacteria.

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