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RESEARCH ARTICLE OPEN ACCESS

Diversity of Toxigenic Molds and Mycotoxins Isolated from Dairy Products: Antifungal Activity of Egyptian Marine Algae on *Aspergillus* and *Candida* Species

Ahmed Ezzat Ahmed[1,2], Mohammed M. AL-Kahtani[1], Eman M. El-Diasty[3], Ahmed Shaban Ahmed[4]*, Hani Saber[5], Ahmed Mahmoud Abbas[1,5], Hassan Mahmoud Diab[6], Mohammed A. Alshehri[1], Ahmed A. Elmansi[1] and Mohamed A. Hussein[5]

[1]Department of Biology, Faculty of Science, King Khalid University, Abha 61413, Saudi Arabia. [2]Department of Theriogenology, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt. [3]Department of Mycology, Animal Health Research Institute, Agricultural Research Center (ARC), Dokki, Cairo 12622, Egypt. [4]Department of Food Hygiene and Control (Milk Hygiene), Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt. [5]Department of Botany and Microbiology, Faculty of Science, South Valley University 83523, Qena, Egypt. [6]Department of Animal and Poultry Health and Environment, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt.

***Correspondence:** ahmed_shaaban@vet.svu.edu.eg; +2/01004563013

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Abbreviations: AFB1, Aflatoxin B1; AFM1, Aflatoxin M1; OTA, Ochratoxin A; TLC, thin-layer chromatography; DMSO, Dimethyl sulfoxide; GC–MS, Gas chromatography–mass spectrometry technique; CFU, Colony forming unit; aflR, Aflatoxin regulatory gene; AI, Acceptable intake; ADI, Acceptable Daily Intake; DI, Daily Intake; WI, Weekly Intake; EDI, Estimated Daily Intake; EWI, Estimated Weekly Intake; PTWI, Provisional Tolerable Weekly Intake; EI, Estimated intake; MPL, permissible limits.

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Abstract

Fungal and mycotoxin contamination of milk products constitute a potential hazard to human health and food safety. Isolation and identifications of mold and yeast out of 140 milk products samples collected from dairy shops in Qena, Egypt were done through conventional microbiological methods. Aflatoxin-M1, aflatoxin-B1 and ochratoxin-A were characterized by thin-layer chromatography; aflR regulatory gene identified by using PCR. Marine algal extracts of *Halimeda opuntia*, *Padina pavonica* and *Turbinaria decurrens* species were studied for their antimicrobial activity. Overall of 80 and 64% dairy products samples were positive for mold and yeast contamination. A total of 38 mold and 15 yeast species were isolated. *Aspergillus* and *Candida spp*. were the most abundant isolated species. Furthermore 25, 40 and 27% of cheese and 71, 78 and 73.3 of dairy desserts samples were contaminated with AFM1, AFB1 and OTA, respectively; with average estimated dietary intake level much more than the acceptable daily intake for infant and adult. PCR identified aflR gene among four selected aflatoxigenic *A. flavus*. The major constituents of *H. opuntia* extract were 2,4-Decadienal, (E,E)- (21.56%) and 9,12-Octadecadienoic acid (Z,Z)- (36.16%). Ethyl acetate extract of *Halimeda opuntia* (3mg/ml) exhibited the strongest fungicidal activity with inhibition zones of 16.5 and 22.3 mm against *A. flavus* and *A. niger*. It exhibited potent candidacidal activity against *C. tropicalis*; 11 log10 orders of killing at 750 µg/ml. The discovered antimicrobial activity of *H. opuntia* is a promising candidate for designing novel antifungal agents which can be used in food preservatives and medicine industry.

Keywords: Milk products, *Aspergillus*, *Candida*, Mycotoxins, Algae, Antimicrobial activity

INTRODUCTION

Mold and yeast can invade the dairy products during unhygienic processing and handling conditions which constitute a public health hazard[1]. Mold and yeast in milk and dairy products might act as allergen and an irritant to human health and may be the reason for the gastrointestinal disease[2,3]. Species of *Aspergillus*, *Fusarium*, *penicillium*, *Rhizopus* and *Trichderma* are common contaminants of dairy products and known as spore formers[4]. Mold species are also responsible for many serious diseases through production of toxic metabolites called mycotoxins[5]. Thus, mold and yeast counts are considered the standard test of milk hygiene[6]. *Aspergillus* and *Penicillium* species are common spore forming contaminants in the dairy derivatives[7].

Mycotoxins; aflatoxin-B1 (AFB1), aflatoxin-M1 (AFM1) and ochratoxin-A (OTA) are fungal toxic metabolites characterized by heat stable toxicity, mutagenicity, teratogenicity and carcinogenicity[8]. Aflatoxins and ochratoxins are the major mycotoxins affecting our health[9]. Aflatoxin is produced by several *Aspergillus* species including *Aspergillus flavus* and *Aspergillus parasiticus*[10]. Major aflatoxins are B1, B2, G1, G2 and plus two additional metabolites; M1 and M2[11]. AFB1 and its metabolite AFM1 in cow's milk are human carcinogenic toxins[12]. The AFB1 and AFM1 international permissible limits are 2.0 and 0.05µg/kg, respectively[13]. Ochratoxin-A is produced by *Aspergillus* and *Penicillium* on the surface of cheese during ripening[14] and it is the most common and wide-scale carcinogenic toxin in the Ochratoxins' family[15]. The international permissible limit of OTA is 5.0µg/ kg[16].

Prevention of the toxigenic molds growth and mycotoxins synthesis in raw materials and end products to control its outbreak, is achieved traditionally by using of chemical preservatives[17]. Despite of its proven efficiency, their repeated applications has resulted in side effects on human health, acquisition of microbial resistance to the applied chemicals and accumulation of chemical residues in food[18]. The growing consumer demand for high quality food, safe, preservative free with extended shelf life has focused efforts in the discovery of new natural preservatives. On light of above, efforts have been directed to developing potentially effective, safer, healthy and natural food preservatives. Marine organisms including marine macroalgae (seaweeds) are source of various natural antimicrobial compounds with pharmacological and biological activities[19]. Macroalgae contain many different secondary metabolites which have become recognized as potential sources of bioactive compounds, such as antimicrobial active compounds. The

existence of bioactive compounds with antifungal effect was recorded in crude extracts of different species of green, brown and red algae by many investigators[20]. Utilization of algal extracts as antimicrobial agents for food preservation could be a fascinating alternative to chemical and physical methods, and it has received much attention recently[21]. The algal extracts considered as natural antimicrobial agents, nutritionally safe and easily degradable[22]. The antimicrobial activity exhibited by algal extracts against mold and yeast has been demonstrated by several researchers[23, 24]. The present study aimed to investigate the mold and yeast species present in dairy products, and estimate the levels of mycotoxins; AFB1, AFM1 and OTA using thin-layer chromatography (TLC) considering their permissible limits and acceptable daily intakes (ADI) for human taking the age into consideration. Furthermore, *Aspergillus flavus* strains would be molecularly identified for the presence of *aflR* regulatory gene. Furthermore, the antimicrobial activity of some algal extracts, i.e. *Padina pavonica*, *Halimeda opuntia* and *Turbinaria decurrens* against *Aspergillus* and *Candida* species were evaluated.

MATERIALS AND METHODS

All procedures of sampling, assays and analyses were strictly carried out according to the instructions and guidelines provided with the companies brochures for lab analysis.

Media and samples used for fungal isolation and identification

Sabouraud dextrose agar, Malt extract agar (MEA), Czapek yeast extract agar (CYA), 25% glycerol nitrate agar and TSB (pH 7.3) were used.

Samples

A total 140 random samples of cheese (Ras, Cheddar, Feta and Processed) and dairy desserts (Mahalabia, Custard and Rice Milk) were randomly collected from dairy markets. The samples were kept in polyethylene bags and preserved in ice box, then immediately transferred to the laboratory aseptically to be prepared for mycology.

Preparation of samples' serial dilutions

The samples were released out aseptically from their packages and thoroughly mixed in a sterile mortar. From each prepared samples, 10 grams were transferred into sterile flask containing 90 ml sterile peptone water solution as a buffer. Ten-fold serial dilutions up to 10^{-8} from each sample were prepared.

Mold and yeast counting and identification

The developing yeast and mold colonies were counted and isolated for identification according to Chay et al. (2017)[25] and Harrigan and MacCance (1966)[26]. The obtained results were compared with the maximum allowed mold or yeast counts of the Egyptian Organization for Standardization and Quality control (EQSOC). Mold isolates were inoculated in CYA, MEA and G25N (25%). Colony appearance, exudate production, reverse coloration and pigmentation were assessed. Colonies' diameters were assessed after 7 days of growth at 25°C[27]. The characteristics of yeast colonies including; the pattern and rate of growth, colony consistency, size and its surface color were studied. Vegetative reproduction was examined on corn meal agar[28]. Yeast morphology was grossly and microscopically examined[29]. Yeast growth was studied after isolates culturing on slopes of Sabouraud dextrose media and incubating it at 37°C for 3-5 days. Urease and germ tube tests were performed according to Cruickshank et al. (1975)[30] and Koneman et al. (1992)[31].

Detection of Mycotoxins Residues in the Dairy Products

Extraction of aflatoxins out of dairy products samples was done in accordance with the method mentioned by Roberts and Patterson (1975)[32]. The final samples' filtrates were combined and evaporated to dryness in a rotatory evaporator and saved for analysis. Ochratoxin-A (OTA) was detected by thin-layer chromatography (TLC) according to AOAC (1980)[33]. Mycotoxins residues were qualitatively estimated according to the method proposed by (Scott, 1965; Howell and Taylor, 1981)[34, 35]. The reference values, colors and intensities of unknown spots were compared to those standard reference values (Sigma, USA). Samples extracts which were found to contain mycotoxins by the qualitative technique were further calculated according to the standard formula:

$$\mu g/kg = (S \times Y \times V) / (X \times W)$$

S=µl of mycotoxin standard equal to unknown
Y= Concentration of mycotoxin inµg/ml

V=µl of final extract dilution.
X=µl of the extract emitting a spot intensity equal to S. W= Mass (weight) of the sample, represented by the final extract in gram.

The obtained results were compared with the maximum permissible limits (MPL) of the local Egyptian Organization for Standardization and Quality control (EQSOC) and International organizations latest guidelines standards.

Health risk assessment: estimated daily intake and estimated weekly intake

Calculations of the estimated daily intake (EDI) and estimated weekly intake (EWI) of mycotoxins in the examined milk products samples were performed according to previous accredited equations. The obtained results were compared to the acceptable daily intake (ADI) according to the international standards organization.

Detection of *Aspergillus flavus* ability to produce aflatoxins using PCR

Genomic DNA was extracted from ground frozen *Aspergillus flavus* mycelium/spores using Spin Column DNeasy plant minikit (Geneaid, USA). The target fragment of regulatory aflatoxin gene fragments of aflatoxigenic DNA was amplified by PCR. The forward-reverse primers *aflR1* regulatory gene was; 5¯ AACCGCATCCACAATCTCAT 3¯, and 5¯AGTGCAGTTCGCTCAGAACA 3¯, involved within 800-base pairs (bp) gene size[36]. The reaction mixtures consisted of extracted *A. flavus* target DNA, forward primer (F), reverse primer (R) and PCR Master Mix: DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, USA, Cat., No. K1081). Amplification of DNA was performed via optimized PCR reactions; denaturation, primer annealing and extension. Subsequently, 35 amplification cycles were done in a programmable thermocycler (Gradient Thermal cycler; 1000 S Thermal cycler BioRAD USA). The PCR-product was analysed by electrophoresis on agarose gel (1.5%) stained by ethidium bromide. The gel image was visualized by trans-illuminator. A 100 bp-size ladder is the marker for amplicons size.

Algal collection and extraction preparation

Three marine algae; *Halimeda opuntia* (Chlorophyta), *Padina pavonica,* and *Turbinaria decurrens* (Phaeophyta) were collected during October, 2018 from Hurghada coast, Red Sea Coast, Egypt. Seaweeds were collected in sterilized polyethylene bags, kept in ice box till reach the laboratory for identification, preparation and analysis[37]. Algal extraction was performed by using different solvents; ethyl acetate for *Padina pavonica,* and petroleum ether for *Halimeda opuntia* and *Turbinaria decurrens*. The extracts were suspended in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml, and then stored in airtight bottles at 4 °C till used[38].

Gas chromatography-mass spectrometry (GC-MS) analysis of algal extract

Algal extracts were identified by using GC-MS (Thermo Scientific Technologies, Trace-1310, capillary column TG-5; 30m×250µm×0.25µm). Split mode-mass detector and helium gas with flow rate carrier 1.5 ml/minutes (min) were used. Injector was operated at 230°C, and initial oven temperature 60°C for 2 min and ramp 10/min to 300°C for 8 min. Mass spectra were taken at 70 eV and the total GC running time was 35 min.

Microorganisms

Strains of *Aspergillus flavus*, *Aspergillus niger* and *Candida tropicalis* as identified by Mycology Department, Animal Health Research Institute, Cairo, Egypt were used to study the antifungal activities of algal extract. All isolates were cultured onto Sabouraud dextrose agar, 24 hrs prior to assay.

Antifungal assay

Inoculums of *Aspergillus flavus* and *Aspergillus niger* cultures were suspended in a sterile saline solution (0.85 %). Suspension turbidity was modified by the spectrophotometry at 530 nm till obtain a final concentration of 0.5 McFarland standards (0.5-2.5×10^3). The antifungal activity was evaluated by using well diffusion method on the agar plates of Sabouraud dextrose media[39], where the inhibition zones were detected after 48 hours of incubation at 27°C. The assay was done in triplicates (n = 3).

Candidacidal assay

Candida tropicalis blastoconidia grown for 24 hrs in 1% TSB (pH 7.3) were rinsed, resuspended to 10^6 - 10^7 CFU/ml in Sabouraud dextrose broth, and then mixed with DMSO, in equal volume, containing series of the algal extracts; 3 mg/ml, at 2-fold serial dilutions. The mixture was plated on Sabouraud dextrose media and incubated at 28°C for 24 hrs followed by further incubation at 30°C for another 24 hrs. The algal colonies were finally counted in log CFU/ml in the assay media[40].

Statistical analysis

The inhibition zones in response to treatment of the fungi with algae were analysed by using *two-way* analysis of variance (ANOVA) and Bonferroni was the *post-hoc* test. All data were set as mean ± standard error (SEM) for three replicate-assays. Graph-Pad Prism software, San-Diego, USA, v. 5 was used. The difference among groups was considered significant at $P<0.05$.

RESULTS

The examined dairy samples were variably contaminated with mold and yeast, ranged 50 - 100 % above the allowed limits; 10 and 400 CFU/g, respectively proposed by EOSQC (2005)[41]. The mean values of mold detected ranged from 1.84×10^3 up to 7.25×10^4 CFU/g (Table 1), and that of yeast ranged from 2.14×10^3 to 1.73×10^5 CFU/g (Table 1). *Aspergillus* spp. were mostly the dominant species isolated from cheese and milk desserts with 42 and 25 %, respectively, i.e. *A. flavus*, *A. niger*, *A. ochraceus*, *A. fumigatus* and *A. parasiticus* (Table 2). Also, the most predominant yeast species were those of *Candida*, i.e. *C. tropicalis*, *C. krusei* and *C. parapsilosis* (Table 3).

Dairy products showed heterogeneous mycotoxins that were chromatographically detected as shown in Table 4. The concentrations of aflatoxin-M1 (AFM1), aflatoxin-B1 (AFB1) and ochratoxin-A (OTA) in the examined samples ranged; 0.0-13.9, 11.1-13.8 and 4.5-13.9µg/kg, in 40-85, 15-85 and 15-80 %, respectively. Mostly all values of AFM1 and AFB1 estimated were exceeding their permissible limits according to Egyptian regulation permissible limits and EU regulation[13,42,43]. However, 100, 100, 67, 88, 92, 6 and 94% of the examined ras, cheddar, feta, processed cheese, mahalabia, custard and rice milk had Ochratoxin A above the allowed limit (> 5µg/g) according to Creppy (2002)[16] (Table 4 A). The acceptable intakes (AI) compared to those estimated mycotoxins (EI) for children and adult human, either per day or week, were presented in Table 4 (B). The estimated daily intake (EDI) of mycotoxins from milk products was evaluated by using the consumed amount of the milk products and the average concentrations of mycotoxin estimated in each product type, considering the body weight average of the different groups. The current study indicated that all EDI levels of AFM1, AFB1 and OTA for infant and adult much exceeded over their acceptable daily intakes (ADI); 0.002, 0.0 and 0.014µg/kg b.w., respectively, according to the international regulation limits proposed by

Table 1. Mold and yeast counts, presented as colony forming unit (CFU)/g, in different dairy products' samples (n = 20), showing the safety from mold and yeast according to EOSQC[41]

A	Milk products	+ve Mold (CFU/g)					
		No.	%	Min.	Max.	Mean±S.E	> 10 (%)
Cheese	Ras cheese	17	85	1.00×10^1	1.60×10^4	$3.54\times10^3\pm1.14\times10^3$	80
	Cheddar	10	50	3.00×10^1	1.10×10^4	$1.84\times10^3\pm5.43\times10^2$	50
	Feta	17	85	1.00×10^1	3.00×10^4	$4.85\times10^3\pm1.58\times10^3$	85
	Processed	12	60	7.00×10^2	2.50×10^5	$3.33\times10^4\pm1.29\times10^2$	60
Dairy	Mahalabia	18	90	3.30×10^2	5.50×10^4	$1.31\times10^4\pm3.94\times10^3$	90
desserts	Custard	20	100	3.00×10^2	1.34×10^4	$6.84\times10^3\pm9.40\times10^2$	100
	Rice Milk	19	95	1.30×10^2	3.30×10^4	$7.25\times10^4\pm2.43\times10^3$	95
B	Milk products	+ve Yeast (CFU/g)					
		No.	%	Min.	Max.	Mean±S.E	>400 (%)
Cheese	Ras cheese	15	75	1.00×10^1	8.70×10^4	$1.39\times10^4\pm5.96\times10^3$	60
	Cheddar	11	55	2.40×10^2	6.00×10^5	$1.73\times10^5\pm3.72\times10^4$	50
	Feta	10	50	1.00×10^2	4.00×10^4	$6.66\times10^3\pm1.98\times10^3$	40
	Processed	12	60	1.20×10^2	1.00×10^5	$1.78\times10^4\pm5.74\times10^3$	55
Dairy	Mahalabia	12	60	2.00×10^2	5.00×10^4	$9.58\times10^3\pm3.19\times10^3$	35
desserts	Custard	15	75	3.00×10^2	4.40×10^4	$5.75\times10^3\pm2.32\times10^3$	60
	Rice Milk	15	75	2.00×10^2	1.20×10^4	$2.14\times10^3\pm6.82\times10^2$	45

Table 2. Percent of contamination of mold isolates in different dairy products samples (n = 20)

Isolates of molds	Percent of contamination (%)						
	Cheese samples				Dairy desserts		
	Ras	Cheddar	Feta	Processed	Mahalabia	Custard	Rice Milk
Acremonium strictum	0.0	0.0	0.0	0.0	3.7	8.3	0.0
Arthrinium phaeospermum	0.0	0.0	0.0	0.0	3.7	0.0	0.0
Aspergillus flavus Link	17.1	9.1	16.2	6.3	3.7	12.5	6.5
Aspergillus fumigatus	5.7	0.0	0.0	0.0	0.0	0.0	0.0
Aspergillus niger Tiegh. nom. cons.	11.4	9.1	18.9	43.7	14.8	14.6	12.9
Aspergillus ochraceus	5.7	0.0	0.0	0.0	0.0	0.0	0.0
Aspergillus parasiticus	2.9	0.0	0.0	0.0	0.0	0.0	0.0
Aspergillus sydowii	5.7	0.0	0.0	0.0	0.0	0.0	0.0
Aspergillus terreus Thom	0.0	0.0	5.4	0.0	0.0	4.2	0.0
Aureobasidium pullulans	0.0	0.0	0.0	0.0	3.7	8.3	3.2
Botrytis cinerea	0.0	0.0	0.0	0.0	0.0	0.0	6.5
Cladosporium cladosporioides G.A. de Vries	17.1	9.1	24.4	18.7	22.2	8.3	32.3
Cladosporium herbarum Link	0.0	0.0	5.4	12.5	0.0	0.0	0.0
Emericella nidulans	0.0	0.0	0.0	0.0	3.7	0.0	0.0
Eurotium chevalieri	0.0	0.0	0.0	0.0	7.4	4.2	3.2
Eupenicillium spp.	0.0	0.0	0.0	0.0	0.0	18.8	0.0
Fusarium chlamydosporum	0.0	0.0	0.0	0.0	0.0	4.2	0.0
Fusarium poae Wollenw	0.0	18.2	0.0	0.0	0.0	0.0	0.0
Mucor plumbeus	0.0	0.0	0.0	0.0	0.0	0.0	6.5
Paecilomyces variotii	0.0	0.0	0.0	0.0	0.0	0.0	6.5
Penicillium aurantiogriseum Dierckx	0.0	18.2	0.0	0.0	0.0	0.0	0.0
Penicillium caseifulvum Lund, Filt. & Frisvad	8.6	0.0	0.0	0.0	0.0	0.0	0.0
Penicillium chrysogenum	14.3	0.0	0.0	0.0	0.0	0.0	0.0
Penicillium citreonigrum Dierckx	0.0	0.0	2.7	0.0	3.7	0.0	0.0
Penicillium citrinum Thom	2.9	0.0	5.4	0.0	7.4	8.3	9.7
Penicillium corylophilum Dierckx	5.7	18.2	8.1	6.3	0.0	0.0	0.0
Penicillium digitatum Sacc.	2.9	0.0	0.0	0.0	0.0	0.0	0.0
Penicillium implicatum Biourge	0.0	9.1	0.0	0.0	0.0	0.0	0.0
Penicillium paneum Frisvad	0.0	0.0	0.0	12.5	0.0	0.0	0.0
Penicillium paxilli Bainier	0.0	9.1	0.0	0.0	0.0	0.0	0.0
Penicillium purpurogenum Stoll	0.0	0.0	2.7	0.0	0.0	0.0	0.0
Penicillium raistrickii G. Sm	0.0	0.0	2.7	0.0	0.0	0.0	0.0
Penicillium restrictum	0.0	0.0	0.0	0.0	18.5	6.3	12.9
Penicillium simplicissimum Thom	0.0	0.0	5.4	0.0	0.0	0.0	0.0
Penicillium variabile Sopp	0.0	0.0	2.7	0.0	0.0	0.0	0.0
Penicillium variable	0.0	0.0	0.0	0.0	0.0	2.1	0.0
Rhizopus microsporus	0.0	0.0	0.0	0.0	3.7	0.0	0.0
Scopulariopsis brevicaulis	0.0	0.0	0.0	0.0	3.7	0.0	0.0
Total	100	100	100	100	100	100	100

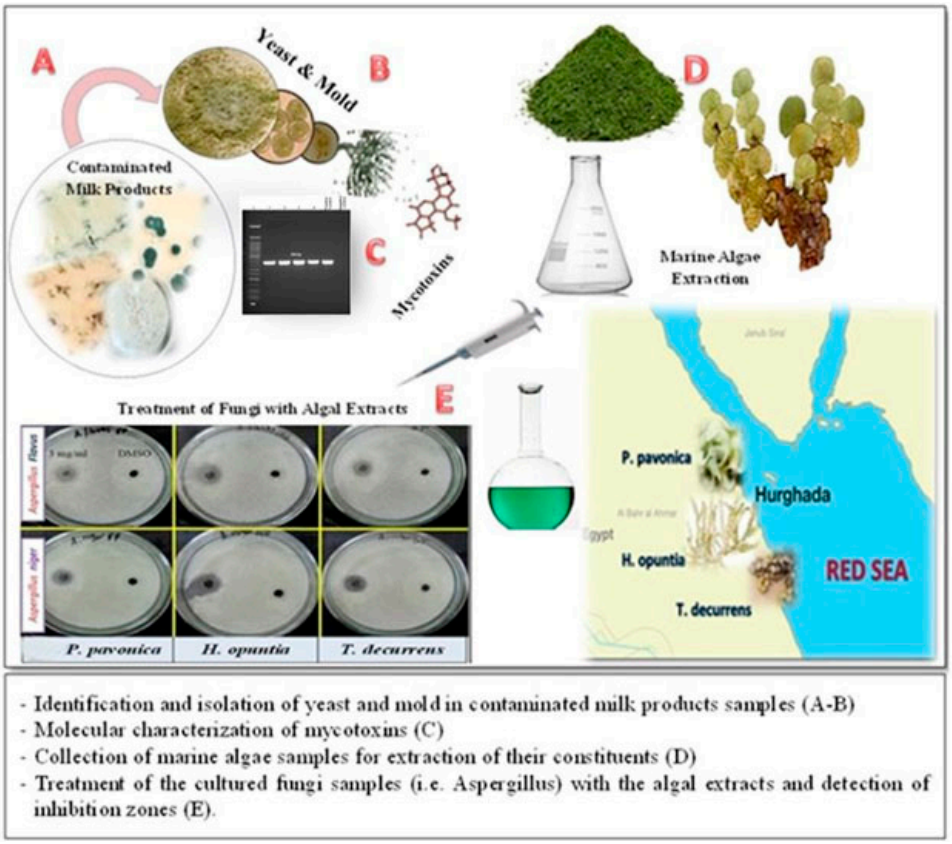

Fig. 1. Graphical abstract of the study

Table 3. Percent of contamination of yeast isolates in different dairy products samples (n = 20)

Isolates of Yeast	Percent of contamination (%)						
	Cheese samples				Dairy desserts		
	Ras	Cheddar	Feta	Processed	Mahalabia	Custard	Rice Milk
Candida famata	0.0	0.0	0.0	0.0	11.8	0.0	5.0
Candida guilliermondii	0.0	0.0	7.7	6.7	5.9	0.0	5.0
Candida holmii	6.7	0.0	0.0	0.0	0.0	0.0	0.0
Candida krusei	6.7	0.0	15.4	0.0	5.9	4.3	0.0
Candida parapsilosis	0.0	0.0	0.0	0.0	0.0	4.3	0.0
Candida tropicalis	13.3	10.0	23.1	13.3	17.6	17.4	20.0
Cryptococcus albidus	20.0	0.0	0.0	6.7	11.8	34.8	20.0
Debaryomyces hansenii	13.3	30.0	15.4	20.0	5.9	8.7	10.0
Geotrichum candidum Link	20.0	0.0	0.0	0.0	0.0	0.0	5.0
Pichia anomala	6.7	0.0	7.7	0.0	0.0	0.0	0.0
Pichia membranaefaciens	0.0	10.0	0.0	0.0	0.0	0.0	0.0
Rhodotorula glutinis	0.0	20.0	0.0	0.0	29.4	8.7	20.0
Rhodotorula spp.	6.7	20.0	15.4	6.7	0.0	0.0	5.0
Saccharomyces cerevisiae	6.7	10.0	15.4	40.0	5.9	17.4	10.0
Trichosporon spp.	0.0	0.0	0.0	6.7	5.9	4.3	0.0
Total	100	100	100	100	100	100	100

Table 4A. Maximum permissible limits (MPL; µg/kg) of mycotoxins in dairy products (n = 20); aflatoxin-M1 (AFM1), aflatoxin-B1 (AFB1) and ochratoxin-A (OTA) by the thin-layer chromatography (TLC). Letters (a, b, c, d) denote the international standards for MPLs. E.R.: Egyptian Regulation; I.S.: International Standards - a: EOSQC[43], b, c: European commission regulation[13], d: Creppy[16]. (B) Acceptable and estimated intakes (µg/kg b.w) of mycotoxins in the dairy products consumed by children and adult (n = 20). Letters (a, b, c) denote the international standards for the acceptable daily intake (ADI). STD = Standard references - a: Kuiper-Goodman[44], b: Brera et al.[46], c: JECFA[45] - ADI, acceptable daily intake; EDI, estimated daily intake; EWI, estimated weekly intake; PTWI, provisional tolerable weekly intake

Dairy Products		(A) Maximum Permissible Limits											(B)Acceptable vs. Estimated Intakes							
		+ve Cases		Mean ± SE (µg/kg)	E.R.	>MPL		I.S.	>MPL		STD		Children				Adults			
													DI		WI		DI		WI	
		No.	%			No.	%		No.	%	ADI	PTWI	EDI	>ADI %	EWI	>PTWI %	EDI	>ADI %	EWI	>PTWI %
AFM1	Ras Ch.	8	40	8.9±1.1	0.0[a]	8	100	0.05[b]	8	100	0.002[a]	0.014	15.9	40	111.8	40	6.1	40	43.0	40
	Ched. Ch.	0	0	--		-	-		-	-			0.0	0	0.0	0	0.0	0	0.0	0
	Feta Ch.	3	15	9.4±1.0		3	100		3	100			16.9	15	118.4	15	6.5	15	45.6	15
	Proc. Ch.	9	45	13.9±1.8		9	100		9	100			25.0	45	175.1	45	9.6	45	67.4	45
	Mahalabia	12	60	10.3±1.5		12	100		9	75			43.5	60	304.3	60	16.7	60	117.0	60
	Custard	14	70	8.6±1.1		14	100		10	71			34.7	70	242.6	70	13.3	70	93.3	70
	Rice Milk	17	85	10.6±1.2		17	100		16	94			45.1	85	315.8	85	17.4	85	121.5	85
AFB1	Ras Ch.	11	55	11.5±1.8	0.0[a]	11	100	2.0[c]	11	100	0.0[b]	0.0	20.6	55	144.3	55	7.9	55	55.5	55
	Ched. Ch.	3	15	13.2±1.1		3	100		3	100			23.7	15	166.1	15	9.1	15	63.9	15
	Feta Ch.	12	60	13.4±1.8		12	100		11	92			24.2	60	169.5	60	9.3	60	65.2	60
	Proc. Ch.	6	30	13.7±1.6		6	100		6	100			24.6	30	172.3	30	9.5	30	66.3	30
	Mahalabia	13	65	12.7±1.7		13	100		11	85			53.9	65	377.2	65	20.7	65	145.1	65
	Custard	17	85	9.9±1.2		17	100		16	94			42.1	85	295.1	85	16.2	85	113.4	85
	Rice Milk	17	85	11.1±1.2		17	100		16	94			46.9	85	328.2	85	18.0	85	126.2	85
OTA	Ras Ch.	7	35	13.9±1.5	0.0[a]	7	100	5.0[d]	7	100	0.014[c]	0.098	25.0	35	175.2	35	9.6	35	67.4	35
	Ched. Ch.	4	20	9.9±0.9		4	100		4	100			17.8	20	124.6	20	6.9	20	47.9	20
	Feta Ch.	3	15	6.2±0.6		3	100		2	67			11.2	15	78.4	15	4.3	15	30.1	15
	Proc. Ch.	8	40	8.0±1.0		8	100		7	88			14.4	40	100.8	40	5.5	40	38.8	40
	Mahalabia	12	60	7.5±1.0		12	100		11	92			31.9	60	223.2	60	12.3	60	85.8	60
	Custard	16	80	4.5±0.42		16	100		1	6			18.9	80	132.5	80	7.3	80	51.0	80
	Rice Milk	16	80	8.1±0.9		16	100		15	94			34.3	80	240.4	80	13.2	80	92.5	80

Kuiper-Goodman (1990); JECFA (2007); Brera *et al.* (2008)[44-46].

Electrophoretic banding of *aflR1* set was detected from four morphologically identified *Aspergillus flavus* as demonstration in connection with aflatoxin production which makes its detection is easily in compared to conventional plating techniques. The sizes of DNA fragments (800 bp) were estimated in accordance to commercial DNA ladder 100 bp (Fig. 2).

The antifungal activity of the algal extracts was reported in Fig. 3 and 4. The results revealed that *Padina pavonica* (petroleum ether extract), *Halimeda opuntia* (ethyl acetate extract) and *Turbinaria decurrens* (petroleum ether extract) were effective in suppressing the growth of *A.*

Table 5. Major bioactive chemical constituents identified in the algal extracts according to the gas chromatography-mass spectrometry (GC-MS) chromatogram analysis

Algae	Extract	Sample No.	RT (min.)	Compound Name	Molecular Formula	Molecular Weight	%
		1	7.30	4H-Pyran-4-0ne,2,3-dihydro- 3,5-dihydroxy -6-methyl-	C6H8O4	144	1.66
Padina pavonica	Petroleum ether	2	12.21	cis-a-Farnesene	C15H24	204	1.24
		3	15.62	Santalol	C15H24O	220	1.01
		4	16.00	Bisabolone oxide	C15H24O2	236	1.77
		5	17.50	Bisabolol oxide A	C15H26O2	238	48.62
		6	20.23	En-in-dicycloether	C13H12O2	200	27.63
		7	22.04	Hexadecanoic acid, ethyl ester	C18H36O2	284	5.22
		8	24.98	Ethyl linoleate	C20H36O2	308	2.21
		9	25.08	8, 11, 14-Eicosatrienoic acid	C20H34O2	306	1.53
Halimeda opuntia	Ethyl acetate	1	14.46	2,4-Decadienal, (E,E)-	C10H16O	152	21.56
		2	15.07	Eugenol	C10H12O2	164	1.91
		3	20.62	cis-Asarone	C12H16O3	208	3.32
		4	21.73	9,12,15-Octadecatrienal	C18H30O	262	1.44
		5	26.91	Alantolactone	C15H20O2	232	2.97
		6	27.71	Eudesma-5,11(13)-dien-8,12-olide	C15H20O2	232	3.39
		7	28.16	Hexadecanoic acid	C16H32O2	256	11.46
		8	28.65	Eremanthin	C15H18O2	230	2.76
		9	31.60	9,12-Octadecadienoic acid(Z,Z)-	C18H32O2	280	36.16
		10	31.71	Oleic Acid	C18H34O2	282	6.39
		11	31.95	Octadecanoic acid	C18H36O2	284	3.23
Turbinaria decurrens	Petroleum ether	1	15.57	5-Hydroxymethylfurfural	C6H6O3	126	1.51
		2	20.23	2-Allyl-5-t-butyl-hydroquinone	C13H18O2	206	2.61
		3	23.53	Ar-tumerone	C15H20O	216	8.26
		4	24.34	Curlone	C15H22O	218	1.68
		5	28.73	Hexadecanoic acid, methylester	C17H34O2	270	13.47
		6	29.93	Hexadecanoic acid	C16H32O2	256	13.42
		7	32.54	Octadecanoic acid, methylester	C19H38O2	298	5.31
		8	33.22	Oleic acid	C18H34O2	282	25.62
		9	33.4	Isochiapin B	C19H22O6	346	7.57
		10	38.82	Dotriacontane	C32H66	450	11.63
		11	46.56	Lucenin 2	C27H30O16	610	3.67

flavus, *A. niger* and *C. tropicalis* with variable potency. Ethyl acetate extract of *Halimeda opuntia* at the concentration of 3 mg/ml was the most effective retarding the growth of all tested pathogenic fungi with inhibition zones of 16.5 ± 0.6 and 22.3 ± 0.73 mm against *A. flavus* and *A. niger*, respectively. It is followed by petroleum ether extracts of *Padina pavonica* and *Turbinaria decurrens* with inhibition zones of 9.2 ± 0.6 and 6.8 ± 0.44 mm, against *A. flavus* and 10.0 ± 0.58 and 9.7 ± 0.33 mm against *A. niger,* respectively (*P*<0.5), and the later showed higher sensitivity response to all algal extracts rather than the former, *A. flavus* (Fig. 3).

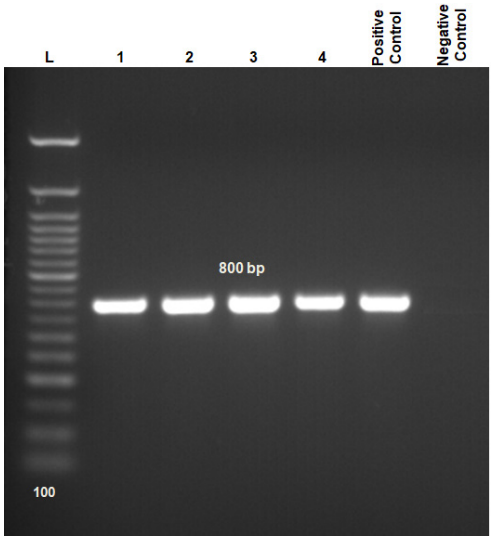

Fig. 2. Agarose gel electrophoresis (1.5%) of PCR amplification for DNA showing 800bp of aflatoxin regulatory gene-1 (*aflR1*). Lane 1-4 = samples; compared with control positive and control negative; Lane (L) DNA ladder 100 bp.

The candidacidal activity of algal extracts by using the dilution method was reported in Fig. 4 (A and B). The algal extracts incubated with *C. tropicalis* for 24 hr exhibited strong candidacidal activity in dose-dependent manner (Fig. 4 A). Ethyl acetate extract of *Halimeda opuntia* was very effective against *C. tropicalis* resulting in severe reduction in CFU of yeast (11 log10 order of killing). Also, the extracts of *P. pavonica* and *T. decurrens* showed effective candidacidal activity (5-6 log10 order of killing power) (Fig. 4 B). The constituents of algal extracts detected by GC-MS were shown in Table 5 and Fig. 4. It showed presence of nine compounds in the petroleum ether extract of *P. pavonica* (Fig. 5 A), eleven compounds in the ethyl acetate extract of *H. opuntia* (Fig. 5 B), and eleven compounds in petroleum ether extract of *T. decurrens*. The major constituents of *H. opuntia* extract were 2, 4-Decadienal, (E, E; 21.56 %), hexadecanoic acid (11.46 %) and 9, 12-octadecadienoic acid (Z, Z; 36.16 %) (Fig. 5 C).

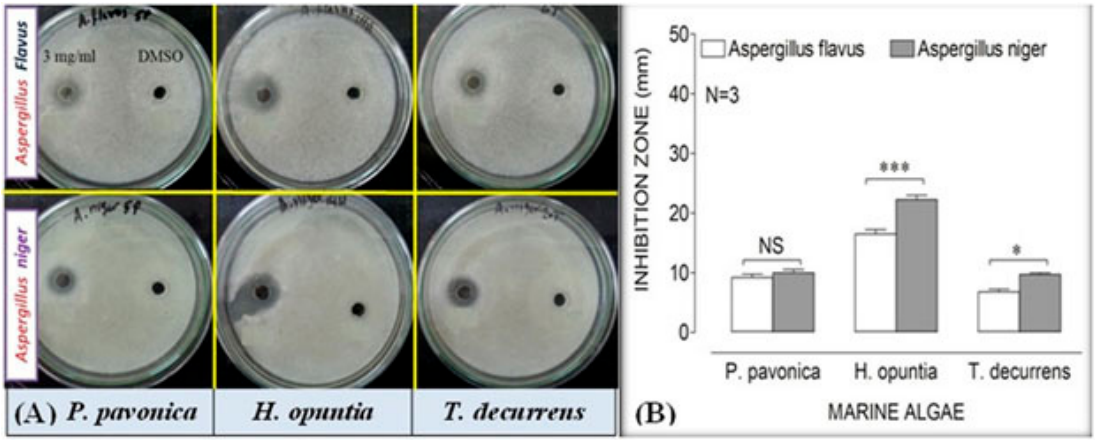

Fig. 3. Antifungal screening of algal extracts (3 mg/ml); *Padina pavonica, Halimeda opuntia* and *Turbinaria decurrens*, and dimethyl sulfoxide (DMSO), as negative control, against *Aspergillus flavus* and *Aspergillus niger*. The activity was determined by zone of inhibition using Sabouraud agar well diffusion method. Only +ve values of the examined algal extracts were recorded (Fig. 3A). DMSO showed no inhibition. Effect of each algal extract on *Aspergillus species* was shown in Fig. 3B. All data were set as mean ± SEM of three replicates (n = 3). The difference between groups were considered significant at *P<0.05 and ***P<0.001.

DISCUSSION

Unhygienic handling of milk products may lead to contamination by different fungi and propagation of mycotoxins. However, our results declared that milk products were contaminated with mold and yeast more than the permissible limit according to EOSQC (2005)[41]. A total of 80.7 and 64.3% of all samples were contaminated with mold and yeast, respectively. These results agreed with former findings reported by researchers in Egypt. They stated that milk samples are contaminated with mold and yeast in majority of the examined regions and most of samples didn't comply with the permissible limit predetermined by National and International Standards Organizations[47, 48]. Furthermore, Italian researches found mold in 54 of the 122 analysed cheese samples (44.3%), stated that the potentially toxigenic fungal species were mainly detected in cheese samples[49]. Mold and yeast are widely distributed as environmental contaminants which can grow at variable temperature, so their presence in milk products could be attributed to unsanitary measures during manufacturing, processing and storage or using of bad quality raw ingredients. They induce undesirable changes such as off-flavor, color defects, rancidity and changes in texture[50]. Mold and yeast counts in dairy products are used as an index of the proper hygienic quality[51]. The mycotoxins-producing molds are potential hazard to food safety and human health[51].

On the other hand, a total of 38 and 15 different species of mold and yeast were isolated and identified from examined milk products samples. *Aspergillus* and *Candida* spp. were the most dominant species of mold and yeast isolated from 32.2 and 29.2% of the milk products, respectively, which coincide with Khalifa et al. (2013)[52] and ELbagory et al. (2014)[53].

Candida species; *C. tropicalis*, *C. krusei* and *C. parapsilosis* are the most common human-specific fungi responsible for systemic and superficial infections originated from food or the environment[54]. Moreover, *C. tropicalis* is implicated in the higher mortality rates than other *Candida* spp. particularly in neutropenic and oncogenic patients. Several cases of *C. tropicalis* cross-resistance to antifungal agents have been reported in clinical isolates[55]. *Aspergillus* species including; *A. flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus*, *A. fumigatus* have the potential to contaminate food and environment and

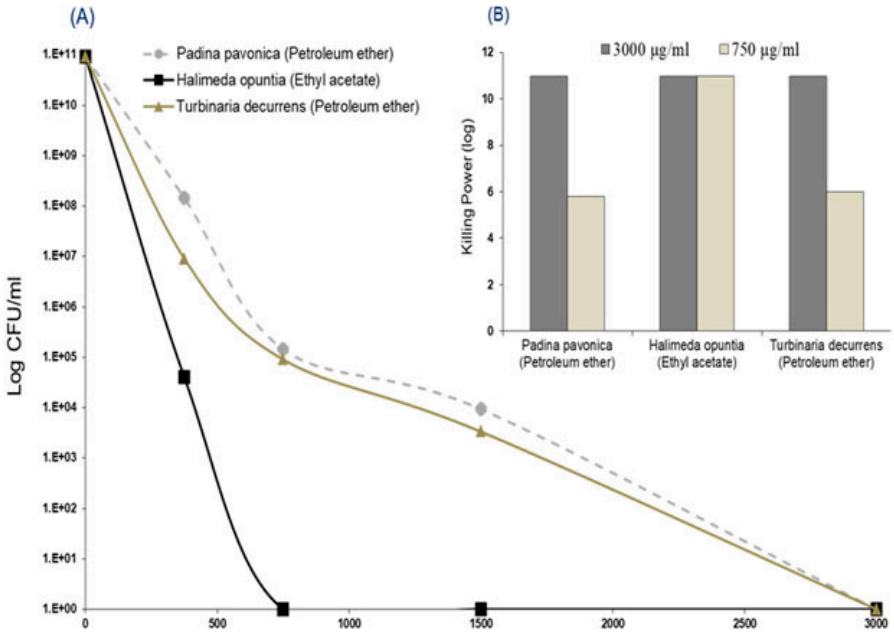

Fig. 4. Anticandidal activity of three algae extracts (log CFU/ml); *P. pavonica, H. opuntia* and *T. decurrens*, against *Candida tropicalis* were shown in Fig. 4A. The Killing powers of two concentrations (3000 and 750 µg/ml) per each extract against the C. tropicalis were shown in Fig. 4B. The assays were performed in triplicate (n = 3).

linked to the life-threatening disorders, i.e. aspergillosis and mycotoxicosis[56]. Aspergillosis causes wide scaled clinical manifestations, i.e. allergy, pulmonary, ocular infections, otomycosis, endocarditis, osteomyelitis, and skin infections, and so the invasive aspergillosis could lead to high mortalities[57]. Moreover, *Aspergillus* is an important genus in foods, causing more spoilage and biodeterioration than other fungi[58]. Almost all food kinds including milk and its products are vulnerable to contamination by *Aspergillus* species especially in tropical and subtropical climates resulted in huge agro-economic losses in the world[59].

Aspergillus is the most significant genera of mycotoxigenic fungi, over 40 species of *Aspergillus* have been known their ability to produce a wide range of mycotoxins having

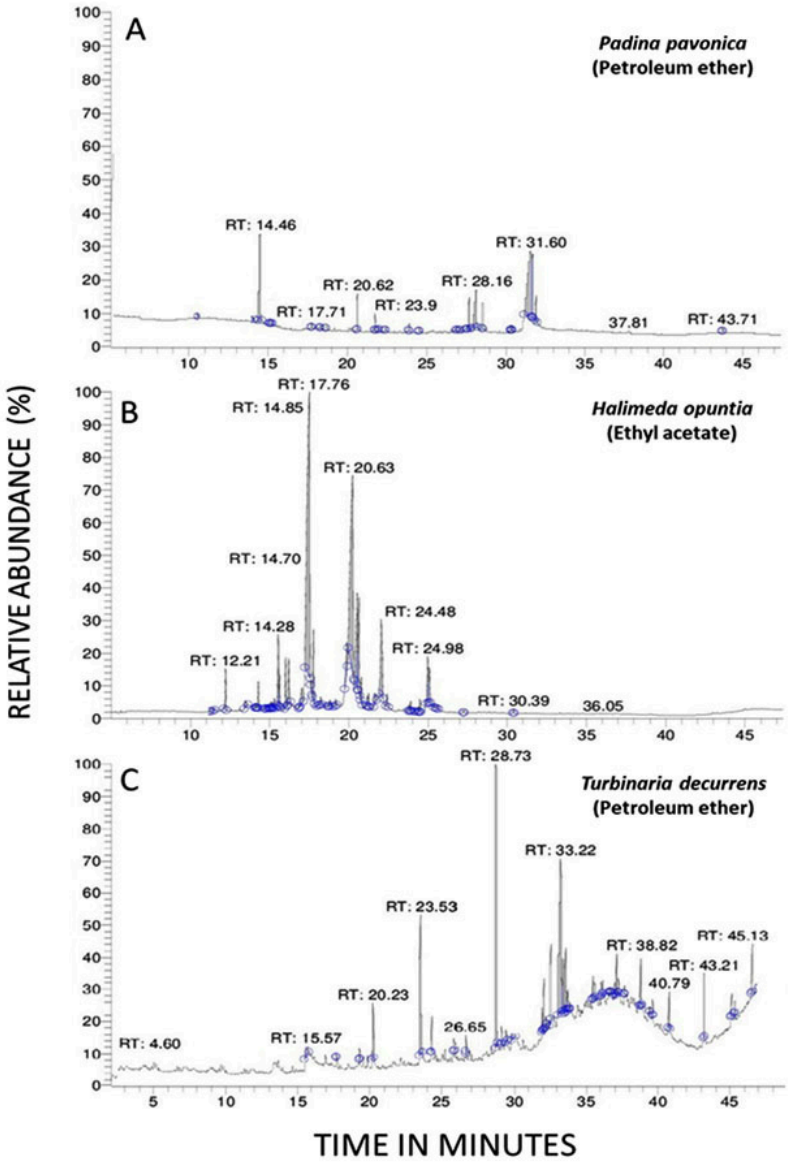

Fig. 5. GC-MS chromatogram analysis of the most effective extract constituents of algae; A) *P. pavonica* (extracted by petroleum ether), B) *H. opuntia* (extracted by ethyl acetate) and C) *T. decurrens* (extracted by petroleum ether). RT = Retention time.

adverse effect on health of humans and animals consuming it. Aflatoxins are products of *A. flavus* and *A. parasiticus* and ochratoxin-A is produced by *A. niger, A. ochraceus* and *A. carbonarius*[60]. Furthermore, Drusch and Aumann (2005)[61] declared that Mycotoxins can diffuse into the food without any sign of mycelium growth. Consequently, the absence of mold does not guarantee mycotoxins free food.

The results showed heterogeneous mycotoxins mixtures; 25, 40 and 27.5% of cheese and 71.7, 78.3 and 73.3 % of dairy desserts were contaminated with mycotoxins; AFM1, AFB1 and OTA, respectively (Table 4). The concentrations of mycotoxins detected were exceeding more than the permissible limits declared by National/ International Standards Organizations[13,16,42,43]. These values are in accordance with the results reported by other Egyptian researchers who stated that, mycotoxins residues were contaminated milk products samples in various levels more than the permissible limit set by Egyptian and European regulation limits[48,62]. They stated that the highest incidence of mycotoxins recorded in milk products may refer to unhygienic conditions in processing, package or storage which provide favourable conditions for mold growth and subsequently toxin production. Moreover, Turkish and Iranian researchers studied mycotoxins concentration in milk products samples, stated that mycotoxins concentrations were more than the maximum residue limits recommended by National and International standard Organizations[63,64]. They concluded that the content of mycotoxins remains relatively stable during the different steps of dairy products production and storage and thermal processing used in dairy industry cannot inactivate it.

For what the data reported in this paper, the amounts of AFM1, AFB1 and OTA detected seem to be dangerous. In fact, the average consumption amount of cheese and dairy desserts for the adult and children assumed 45 and 106 g/ day, respectively (data referred to Egypt - Cairo Nutrition Institute, 1996; 2007)[65,66], and the EDI levels of mycotoxins for infant and adult were much more than the ADI proposed by international regulation standards[44-46]. The AFB1, the most dangerous mycotoxin, should be absent or the lowest recorded according to ALARA (as low as reasonably achievable) for food safety[46]. Our data is agreed well with El-Badry, (2016)[67] and Milicevic et al. (2017)[68] whom found that most of analysed milk and milk products samples collected from different localities in Egypt and Serbia were above ADI of mycotoxins declared by International Standards Organizations. In contrast to our results, former international researchers concluded that collected milk samples containing mycotoxins level that were accepted for human as compared with recommended limits[69].

The carcinogenic properties of aflatoxins motivate us to develop rapid, sensitive and specific approach for the identification and detection of aflatoxin producing *A. flavus* from food samples. Aflatoxin regulatory *aflR1* gene forms the accurate and specific marker for aflatoxigenic strains of *A. flavus* in foods[70]. In agreement with ELbagory et al. (2014)[53], the aflR-specific primer designed for aflatoxigenic *A. flavus* was approved as an easily detecting method, so the detection of aflatoxigenic *A. flavus* from food using polymerase chain reaction (PCR) shows no false results. The PCR technique allows screening of many suspected samples in high sensitivity and accuracy, with the capacity to proceed a high number of samples in a short time.

Preventing food spoilage and protecting human health from the harmful effects of mold or yeast pathogens has become extremely challenging. The limited application of chemical preservatives, susceptibility, toxicity, microbial resistance and its adverse effects on human health increase the demand to search for natural, healthy, safer and potentially effective antifungal agents. Thus, antimicrobial activity of algal extracts can provides a key aspect of treatment of fungal infections and to be used as natural preservatives to ensure healthy and safe food. In the present study, the crude extracts of the three algae species including, *Halimeda opuntia*, *Turbinaria decurrens* and *Padina pavonica*, showed fungicidal activities against recovered three fungal pathogens, *A. flavus*, *A. niger* and *C. tropicalis* with various inhibitory actions depending on the seaweed species and the solvent used.

Various studies evaluated the antimicrobial activities of the marine algae viz., *H. opuntia*, *P. pavonica* and *Turbinaria* Species. Different solvents viz., petroleum ether, diethyl

ether, ethyl acetate, ethanol, chloroform, hexane and water were used for algae extraction to investigate the antimicrobial activity against mold and yeast including *A. flavus*, *A. niger*, *A. fumigatus*, *Fusarium moniliforme*, *Penicillium herquei*, *Candida tropicalis*, *Candida albicans*, *Candida kefir*[71,72]. Some Egyptian researches reported the fungicidal activities of *H. opuntia*, *P. pavonica* and *Turbinaria* Species which showed the inhibitory effect were 12 to 32 mm of *H. opuntia* and *Padina pavonica* extracts[73] and 10-25 mm of methanolic *H. opuntia* extract against *Candida* spp.[74]. Globally, Indira et al. (2013)[75] stated that methanolic extracts of *Halimeda* species exhibit the highest antifungal activity against several fungal strains including *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, *Rhizopus* Spp. and *Pencillium* Spp. in vitro using minimum fungicidal concentration and well diffusion method with the potential use of algal extracts as antimicrobial candidate. In addition, the ethanolic extract of *Padina pavonica* generated maximum inhibition zone against *Candida* spp.[76]. Moreover, it was reported that *Turbinaria* Species exhibited significant inhibition effect against *Candida* spp. (7.0±0.0 mm) and *Aspergillus flavus* (7.0±0.0 mm)[77].

The current article identified the phytochemical algal constituents against the mycotic pathogens by using the GC-MS analysis, showing their active principles with retention times (RT), molecular formulae, molecular weight and relative concentration (%) in the different algal extracts. The GC-MS analysis reported the presence of 9 compounds in the extracted *P. pavonica*, eleven compounds in *H. opuntia* and eleven compounds in *T. decurrens*. The most predominant compounds in the *P. pavonica* extract were bisabolol oxide A (48.62%) and En-in-dicycloether (27.63%), whereas those in *H. opuntia* extract were 2, 4-decadienal, (E, E)- (21.56%), Hexadecanoic acid (11.46%) and 9, 12-Octadecadienoic acid (Z,Z) (36.16%). However, the *T. decurrens* mostly contained hexadecanoic acid, methylester (13.47%), hexadecanoic acid (13.42%), oleic acid (25.62%) and dotriacontane (11.63%).

The great efficacy of the seaweeds extracts against the pathogenic mold and yeast could be attributed to the active phytochemicals and metabolites compounds in addition to the fatty acids and their derivatives[78,79]. Those abundant compounds had been previously identified and characterized from various herbal and algal sources. The in vitro data presented intensively in previous literatures showed that Octadecadienoic acid and Bisabolol oxide A poses potent fungicidal activities against wide range of fungal species. There are several reports regarding that bisabolol has strong fungicidal and bactericidal proprieties[80]. Bisabolol produced nearly 98% loss in the viability of the germinating conidia of *A. niger*, *A. flavus*, *A. fumigatus*[81]. In addition, bisabolol showed antimicrobial activity against *A. niger* at concentrations above 125µg/ml with hyphal growth and conidial production inhibition[82]. Many of the studies in the scientific literature highlighted the antifungal activity Octadecadienoic acid (Z,Z)-. Furthermore, Ali et al. (2017)[83] identified 9-octadecenoic acid (Z)-, methyl ester (10.27%) and 9-octadecenoic acid (Z)-, methyl ester (12.75%), that were found to be responsible for antifungal activity against various fungal genera. In addition to another study, their result indicated the abundances of methyl ester (Z,Z)-9,12-octadecadienoic acid, (E)- 9-octadecanoic acid, 9,12-octadecadienoic acid, which showed great antifungal agent against various fungus isolates[84]. Bisabolol can be used to prevent microbial growth in wide ranging applications such as food, cosmetics and topical antifungal owing to its excellent nontoxic properties[81]. Finally, it could be concluded that those identified molecules have potential antifungal activities and, notably, that the extracts of the algae where they are abundant also showed potent antifungal activity. The explored algal species could be more effective against the fungal infection rather than those traditional fungicidal agents. Therefore, it can be considered as natural preservatives providing healthy and safe food without the unpleasant effects of chemical one. The current study presented the ability of algae to promote as an antifungal agent via stable its biologically active compounds. Also, provides insights into designing of novel antifungal drugs for the clinical use or in food preservation.

CONCLUSION AND FUTURE TRENDS

Broad fungal diversity and heterogeneous mycotoxins mixture were detected in the locally

produced dairy products which denote the unhygienic measures of either processing or preservation in dairy shops. Presence of mycotoxins in the dairy products despite of low levels represents public health hazards. *Aspergillus* and *Candida* spp. were mostly detected in unhygienic preserved milk products. Interestingly, the current study for the first time presented the antifungal activity of Egyptian marine algae; *P. pavonica H. opuntia* and *T. Decurrens* which exhibited effective fungicidal activity in vitro. The GC-MS revealed presence of 9 to 11 compounds in the algal extracts, including bisabolol oxide A, 2, 4-decadienal and oleic acid. Implementation of regulatory and monitoring measures for mitigation of mycotoxin production and inhibit of toxigenic molds growth in food and feed are very crucial to overcome the higher level AFB1 and AFM1 in dairy products. Future pharmacological studies are required for studying the comparative effects of the algal constituents on mold and yeast growth in vitro, and the possibility for using the marine macro-algal extracts as safe natural preservatives instead of the currently used chemical preservatives. Using the discovered novel antifungal agent of marine algae, as novel natural preservatives, could have positive impact on the safety and quality of dairy product and medicine industry as potent fungicidal agents. Moreover, toxicological studies are needed to be performed for the drug discovery; and comparative in vitro studies should be performed on the IC_{50} of those algal constituents on different species of mycotoxins-producing fungi.

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None.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS' CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY

All datasets generated or analysed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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