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Research Article
Antifungal Activity of Some Essential Oils Emulsions Against Fungi Contaminating Ras Cheese

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Abstract

Background and Objective: Ras cheese is one of the important dairy products that are consumed in great quantities. But this cheese is vulnerable to the growth of fungi during ripening and selling until consumption. Therefore, research aimed to detect fungi contaminating Ras cheese and try to resist them. **Materials and Methods:** The effect of various concentrations (0.25, 0.5, 1, 2 and 3%) of essential oils emulsions of clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*) and peppermint (*Mentha piperita*) severally on the mycelial growth of the isolated fungi as compared to the control sample was tested *in vitro*. **Results:** The results indicated that many fungal species belonging to the genera *Aspergillus*, *Mucor*, *Eurotium* and *Mortierella* were isolated from the infected Ras cheese. Ochratoxin A was found in two samples whereas recorded the highest level in sample number 1 (2.1 μg kg^{-1}). Aflatoxin M1 was found in few levels ranged between 0.012 and 0.360 μg kg^{-1} in cheese samples, while aflatoxin B1 and B2 weren't detected in all samples. Clove essential oil emulsion completely inhibited the growth of all tested fungi at the concentration of 0.5%, followed by thyme essential oil emulsion which inhibited the fungal growth of all fungi at the concentration of 1%, while peppermint essential oil emulsion was less effective. **Conclusion:** The research recommends that clove and thyme essential oils emulsions can be used to resist the fungi of Ras cheese. Also, suggests that more research could be done on these essential oils emulsions to produce safe foods free of fungi.

Key words: Ras cheese, cheese fungi, essential oil emulsions, clove, thyme and peppermint Eos, 'Turkey's X disease

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fungi are micro-organisms that cause spoilage of foods, especially products stored for a long time before selling and consuming, which makes them unfit for human consumption due to their reduced nutritional value and sometimes due to the production of mycotoxins[1]. Ras cheese is one of the most popular hard cheeses in Egypt and is similar to the Greek variety "Kefalotyri". It takes a long period to produce the full flavour and texture of the ripened cheese, which may cause the growth of fungi and produce mycotoxins[2,3].

More species of fungi belonging to the genus as following Geotrichum, Aspergillus, Emericella, Penicillium, Mucor and Rhizopus were isolated by El-Fadaly *et al.*[4] and Seddek *et al.*[5] from samples of Ras cheese. Some fungi can produce mycotoxins which may cause fatal poisoning and toxic effects. Mycotoxins have been reported several human and animal intoxications, such as 'Turkey's X disease; alimentary toxic aleukia and yellow rain. Some methods were made to control or detoxify the mycotoxins, but the effects of these methods are limited and have some restrictions in the application. So, the best way for avoiding mycotoxins in cheese is to prevent mouldgrowth[6].

Many essential oils are known to be active against a wide variety of microorganisms, including bacteria and fungi. Clove is one of the most important medicinal and aromatic plants that have been used for centuries as a food preservative and for many medicinal purposes because it contains high levels of phenolic compounds such as eugenol and eugenol acetate[7]. As well thyme is the most important medicinal plant because of its pharmacological and biological properties which are due to its main components of thymol and carvacrol. The essential oil of thyme and the compound thymol possess the strongest antimicrobial properties[8]. Also, peppermint is a medicinal plant that has received more attention from both the food and pharmaceutical industries because of its health benefits. It has antimicrobial, antioxidant and antiviral properties because of its content of menthol and polyphenolic compounds[9,10].

The study aimed to examine the inhibitory effect of essential oils as natural sources against fungi isolated from Ras cheese. Also, The effect of various concentrations of essential oils emulsions of clove, thyme and peppermint severally on the mycelial growth of the isolated fungi from Ras cheese was tested *in vitro*.

MATERIALS AND METHODS

Materials: Samples of Ras cheese were collected randomly from different supermarkets located in Giza, Egypt during September, 2020. The samples were none visually spoiled at the time of collection. The collected samples were transferred directly to the laboratory with a minimum delay under aseptic conditions. Each sample was divided into two parts. The first part was prepared for the mycological examination and the second one was kept frozen for mycotoxins estimation. Preparation of samples for mycological examination was done according to Laird *et al.*[11].

Cultivation media: Malt Extract Agar (MEA) medium was described by Beuchat and Deak[12] was used for cultivation, isolation, purification and identification of fungi from examined Ras cheese. Also, this medium was used when testing the effect of essential oils emulsions on isolated fungi *in vitro*. Bacteria may be suppressed by the addition of lactic acid.

Methods of analysis: Fungal Count and Isolation: For Fungal isolation and identification, 1 g from each sample was cultivated onto a plate of (MEA) medium by using a hockey stick. The inoculated plates were incubated at 25°C for 4-5 days and examined daily[13]. Mould count in each plate was recorded after 48 hrs of incubation. Colonies were transferred to MEA plates for a subculture to obtain pure fungal isolates[14]. The fungal isolates were maintained on MEA slants at 5°C until use. Before use, the fungal isolates were subcultured on new slants of MEA and incubated at 25°C for 5 days.

Identification of fungi isolates: Identification of fungi isolates was done at the Agricultural Research Centre, Giza[15,16]. The pure fungal isolates were identified by morphological characteristics of colonies in the MEA medium. In addition, the vegetative and reproduction strictness was observed using a light microscope (Olympus CX31 Binocular Halogen Microscope made in Japan) with different magnification power and morphological features according to standard identification keys and the most documented atlas keys in fungal identification.

Determination of mycotoxins: Aflatoxin M1 estimation in Ras cheese samples including, sample preparation, toxin extraction, clean-up via immunoaffinity column IAC was done. Preparation of Ras cheese was done according to the preparation of roomy cheese was done[17], 25 g of Ras cheese samples were mixed with 5g of NaCl and blended with 125 mL methanol: water (70:30) at high speed for 2 min. The extract was filtered through fluted filter paper. A total of 15 mL of the filtered extract was diluted with 30 mL distilled water and then filtered through a 1.5 µm glass micro fiber filter. About 15 mL

of the filtered extract (15 mL = 1.0 g sample equivalent) was used for analysis. The final extract of each sample was passed through IAC (Vicam M1 FL+Column, Vicam, Watertown, MA, USA) completely at a rate of 1-2 drops sec^{-1} until air came through the column. The column was washed twice using 10 mL of washing solution. The toxin was eluted using 1 mL methanol (HPLC grade) at a rate of 1 drop sec^{-1} until air came through the column and all of t he samples were eluted (1.0 mL) and collected in a glass cuvette. One mL diluted Afla. Test developer was added to the elute in the cuvette and mixed well, then placed in a calibrated fluorometer (VICAM Series 4,4EX, MA, USA).

For Ochratoxin A estimation in Ras cheese, preparation of cheese samples was done according to Scott[18], 25 g of the sample was mixed with extraction solvent (methanol) and aqueous sodium bicarbonate followed by high-speed blending. The extract was filtered through fluted filter paper. The diluted extract was filtered through a 1.5 µm glass microfiber filter then directly passed through IAC (Vicam Ochratest affinity column, Vicam, Watertown, MA, USA).

LC-MS/MS methods: For AFB1, a validated liquid chrom atography-tandem mass spectrometry (LC-MS/MS) method[19] was used with slightly modified. Methanol was used for mobile phase A, 5 mM ammonium acetate and 0.1% formic acid for mobile phase B. Elution was set as follows: 20% A at Ethyl acetate (1 mL) Supernatant (0.5 mL) Add Vortex vibration/5 min Mix Ultrasonic vibration/10 min Centrifugal 7000×g/5 min/4°C Supernatant Merge supernatant Repeat ×3 Dry by N2/50°C Be re-dissolved with 1 mL of 30% methanol solution Micro membrane filtration (1 mL) Detection by LC-MS/MS 22 0 min, increased to 90% A from 0-4 min, held at 90% A from 4-7 min, decreased to 20% A from 7-7.1 min and then held at 20% A for 10 min. The flow rate was set at 250 µL min^{-1} and a maximal retention time of 6.0 min. The samples and standards were infused directly into the LCMS/MS, a 10 µL aliquot injected into the Hypersil GOLD column and operated in the positive ion mode using electrospray ionization. IN the same manner, LC-MS/MS was used to measure AFB1 concentration. The maximal retention time was set at 6.0 min. The most intense transition ion products (m/z) of AFB1 used for quantification on the Selected Reaction-Monitoring (SRM) mode of a mass spectrometer were 304.95 and 285.10 m/z.

Resistant of isolated fungi: Various concentrations of essential oils emulsions of clove, thyme and peppermint severally on the mycelial growth of the isolated fungi were tested *in vitro.* Each oil emulsion was added to a sterilized Malt Extract Agar (MEA) flask before solidifying to obtain the proposed concentration of (0.25, 0.5, 1, 2 and 3%). The bactericide (lactic acid) was added to the medium to avoid bacterial contamination. Three plates for each treatment were inoculated with discs (5 mm-diam) of isolated fungal strains from Ras cheese. These Petri dishes were then incubated in a digital incubator at 25°C for 5 days according to Lee *et al.*[20].

Preparation of essential oils emulsions: Stable oil emulsions (Oil-in-water) were prepared using natural oils (clove, thyme and peppermint) severally in water. The composition of emulsion droplets is small spherical particles consisting of oil and surfactant molecular dispersed in the water phase as reported[21].Essential oils were obtained from Sakkara Essential Oils Company, Giza, Egypt. They were extracted by using the steam distillation method[22]. About 10mL of each essential oil and 5 mL of non-ionic surfactant (Tween 80) were added slowly with gentle stirring until a homogeneous mixture formed. Then, water (85 mL) was added to reach the final mixture of each oil to 100 mL according to Hassan *et al.*[23] and subjected to High-Pressure Homogenizer (HPH) cycles for 10 min at 500 rpm, to be stable and ready for testing as vehicles of natural hydrophilic extracts against fungi.

Essential oil extraction and analysis: The essential oils were extracted by hydrodistillation using a Clevenger apparatus for 3 hrs, then dried with anhydrous sodium sulfate and stored at 4°C until use. The Gas chromatography analysis of the samples of essential oils was carried out using Ds Chrom 6200 Gas Chromatograph apparatus, fitted with capillary column BPX-5, 5 phenyls (Equiv.) polysillphenylene-siloxane 30×0.25 mm ID×0.25 µ film. The temperature program varied in the range of 70-200°C, at a rate of 10°C min^{-1}. Gases flow rates were nitrogen at 1 mL min^{-1}, hydrogen at 30 and 330 mL min^{-1} for air. Detector and injector temperatures were 300 and 250°C, respectively as mentioned by Hassanin *et al.*[24].

RESULTS AND DISCUSSION

Fungal Counts, Isolation and Identification: The growth of fungi is a common problem in cheese making during ripening or storage, as well as for the retailer and the consumer. These fungi cause spoilage for cheese and most of them produce mycotoxins, in addition to causing economic losses.

The results of the fungal analysis of three samples of Ras cheese obtained from Giza Governorate showed that they were contaminated with different strains of fungi. The data of Table 1 shows that the fungal strains isolated from these

Table 1: Frequency percentages of the isolated fungi from Ras cheese samples

Isolated fungi	Sample 1	Sample 2	Sample 3	TC	Frequency (TC) (%)
Aspergillus ochraceus	12	4	7	23	20.18
Aspergillus flavus	7	6	8	21	18.42
Aspergillus sydowii	4	0	0	4	3.51
Aspergillus glaucus	9	3	5	17	14.91
Eurotium herbariorum	2	5	7	14	12.28
Mortierella spp.	5	4	2	11	9.65
Mucor circinelloides	11	0	3	14	12.28
Mucor spp.	8	2	0	10	8.77
Total	58	24	32	114	100

Table 2: Mycotoxins concentrations in examined cheese samples

Samples	Type of mycotoxins	Concentration (μg kg^{-1})
1	Ochratoxin A	2.100
	Aflatoxin M1	0.012
	Aflatoxin B1	ND
2	Ochratoxin A	ND
	Aflatoxin M1	0.360
	Aflatoxin B1	ND
3	Ochratoxin A	0.257
	Aflatoxin M1	0.047
	Aflatoxin B1	ND

samples, which the results showed that the presence of eight species belonging to four genera, namely Aspergillus, Eurotium, Mortierella, Mucor. The strains were identified as *Aspergillus ochraceus*, *A. flavus*, *A. sydowii*, *A. glaucus*, *Eurotium herbariorum*, *Mortierella* sp., *Mucor circinelloides* and *Mucor* spp. Count and percentage frequency of fungi species in Ras cheese samples on malt extract agar medium were 23 (20.18%), 21 (18.42%), 4 (3.51%), 17 (14.91%), 14 (12.28%), 11 (9.65%), 14 (12.28%) and 10 (8.77%) for *Aspergillus ochraceus, A. flavus, A. sydowii, A. glaucus, Eurotium herbariorum*, *Mortierella* sp., *Mucor circinelloides* and *Mucor* spp., respectively. The highest of these strains were *Aspergillus ochraceus*, where it counted frequency percentages of 20.18% of the total fungi in samples. It was followed by *A. flavus* strain, where it was found in a ratio of frequency 18.42% of the total fungi in samples. *Aspergillus sydowii* strain was the lowest strain of frequency in samples (3.51%).

For the results of *A. flavus* and *Mucor* sp., nearly similar results were reported previously[4,25,26] isolated *Aspergillus ochraceus, A. glaucus* and *Mucor* spp. From Ras cheese. For *Mucor circinelloides* and *Eurotium herbariorum*, nearly similar results were reported previously[27] and they reported that *Aspergillus sydowii* isolated from Yoghurt. The presence of both *Mucor circinelloides* and *Mucor* sp. Indicates that the tested Ras cheese was produced from raw milk[28].

The presence of *A. flavus* correlates with the possibility of producing aflatoxin B1, B2 and G2[13,25]. *Aspergillus ochraceus* was found that it has the potential to produce Ochratoxin A, which is one of the most mycotoxins contaminating foods like soil, agricultural commodities, farmed animals and marine organisms. It is also a causative agent for asthma[29,30]. *Mortierella* spp. has potential as lipid producers and a significant portion of the fungal lipid contains Essential Fatty Acids (EFAs)[31]. *Aspergillus glaucus* is one of the strains which produce Ochratoxins that may result in acute renal failure for humans. This toxin is nephrotoxic, hepatotoxic, teratogenic and immunotoxic[32,33]. *Aspergillus sydowii* is a species of fungus that is typically found in soil, on seeds, on wheat and decomposing organic matter. It is occasionally pathogenic to humans[34,35].

Analysis of Ras cheese samples for mycotoxins contamination: Mycotoxins produced by certain moulds as toxic metabolic substances. They can be found in dairy products when dairy cows ingest feed that contains mycotoxins that pass into the milk, or because of the intentional or accidental growth of moulds. There are many mycotoxins of worldwide public health importance including, aflatoxins, ochratoxins, fumonisins, zearalenone and trichothecenes[36]. Some moulds are related to a range of pathologies ranging from gastroenteritis to cancer, as these mycotoxins are highly toxic, mutagenic, teratogenic and carcinogenic substances[37]. The potential mycotoxins were tested for detection in samples of Ras cheese. Ochratoxin A, Aflatoxin B1 and M1 were analyzed in all samples and the concentration of mycotoxins in the studied Ras cheese samples is shown in Table 2.

The presence of Ochratoxin A was observed in two of the three samples: in sample No. (1), it was at a concentration of 2.1 μg kg^{-1}, while it was in sample No. 3 at a concentration of 0.257 μg kg^{-1}. This is inevitably due to the presence of *A. ochraceus* or *A. glaucus* which was present in cheese samples. Ochratoxin A (OTA) is a mycotoxin produced by different species of *Aspergillus* (*A. ochraceus*, *A. glaucus*, *A. melleus*, *A. sulphureus*, *A. Niger*, *A. carbonarius*, *A. awamori*) and Penicillium (*P. verrucosum*, *P. chrysogenum* and *P. nordicuzm*) as mentioned by[29,38-40,33]. Ochratoxin A has hepatotoxic, nephrotoxic and teratogenic effects[41].

Table 3: Effect of essential oils emulsions on the isolated fungi from Ras cheese

		Concentration of essential oil emulsions in media (%)					
Isolated fungi	Essential oil emulsions	0	0.25	0.5	1	2	3
Aspergillus ochraceus	a	+++	+++	++	+	-	-
	b		++	+	-	-	-
	c		+	-	-	-	-
Aspergillus terreus	a	+++	++	++	+	-	-
	b		++	+	-	-	-
	c		+	-	-	-	-
Aspergillus sydowii	a	+++	++	+	-	-	-
	b		+	-	-	-	-
	c		+	-	-	-	-
Aspergillus glaucus	a	+++	+++	++	+	-	-
	b		+	-	-	-	-
	c		+	-	-	-	-
Mortierella sp.	a	+++	++	+	-	-	-
	b		+	+	-	-	-
	c		+	-	-	-	-
Mucor sp.	a	+++	+++	++	+	-	-
	b		+	-	-	-	-
	c		+	-	-	-	-
Mucor circinelloides	a	+++	+++	++	+	+	-
	b		+	-	-	-	-
	c		+	-	-	-	-
Eurotium herbariorum	a	+++	+	-	-	-	-
	b		+	-	-	-	-
	c		+	-	-	-	-

a: Peppermint essential oils emulsion, b: Thyme essential oil emulsion c: Clove essential oil emulsion. -: Mean: the plate is free of fungal growth. +: Mean: only a few of fungal growth that didn't exceed 5%. ++: Mean: many of fungal growth that didn't exceed 50%. +++: Mean: most of the plate is full of fungal growth

OTA has been classified by the International Agency of Research in Cancer (IARC) as a carcinogenetic of 2B class[42].Also, the presence of *A. flavus* in cheese samples sparked interest in the detection of aflatoxin. *A. flavus* is the main producer of Aflatoxins that produce aflatoxin B1 and B2[13]. Results in Table 2 show that the analysis of the three samples for Aflatoxins proved to be free of Aflatoxin B1even though they were contaminated with *A. flavus*. In this regard, previous studies[43] clarified that mycotoxins can spread in the environment and can be found in food or forage areas, which show no sign of fungal growth. Therefore, the absence of mould does not guarantee the absence of mycotoxins. Conversely, the presence of toxin-producing mould does not automatically imply the presence of mycotoxins in food and feed.

Aflatoxin M1(AFM1) was found in few levels in the three samples of cheese that ranged between 0.012 and 0.360 μg kg^{-1} as shown in Table 2. The presence of AFM1 in the milk referred to the feeding of animals on AFB1containing feed which transformed in the liver by hepatic microsomal cytochrome P450-AFM1 and descend in milk[44]. Also, Churchill[45] mentioned that the amount of AFM1 found in milk represents normally 1-2% of the ingested AFB1. However, it can be as high as 6% in high-producing cows.

The Egyptian regulation recommended that raw milk, heat-treated milk and milk for the manufacture of milk-based products must be free from aflatoxin (B1 and B2) and for the level of aflatoxin M1, the maximum permissible limit is 0.05 μg kg^{-1} [46]. While, Yongning[47] regulation recommended that the maximum permissible limit for the presence of AFM1 in milk be 0.5 μg kg^{-1}.

FAO/WHO[47] explained the toxicological guidance value for aflatoxin M1 that cancer potency estimates at specified residue levels using worst-case assumptions, the additional risks for liver cancer predicted with the use of proposed maximum levels of aflatoxin M1 of 0.05 and 0.5 μg kg^{-1} are very small.

Effect of essential oil emulsions on fungal growth: The result of Table 3 showed essential oils emulsions of three types of medicinal and aromatic plants (clove, peppermint and thyme) with concentrations of 0.25, 0.5, 1, 2 and 3% from the media to resist fungi isolated from the studied Ras cheese. The results showed the superiority of the effect of clove oil emulsion on the emulsions of thyme oil and peppermint oil on the growth of all fungi, where the clove oil emulsion prevented the growth of all fungi at a concentration of 0.5%, followed by the thyme oil emulsion, which inhibited the growth of all fungi at

Table 4: Chemical composition of tested essential oils (%)

Components (%)	Essential oil of clove	Essential oil of peppermint	Essential oil of thyme
Eugenol	75.8	-	-
Thymol	-	-	23.62
Menthol	-	30.51	-
β-caryophyllene	4.2	-	2.65
Eugenyl acetate	18.9	-	-
α-humulene	0.1	-	-
α-pinene	-	0.29	1.76
Camphene	-	-	1.83
β-pinene	-	1.80	1.87
Caryophyllene oxide	0.3	-	-
β-myrcene	-	-	1.75
ρ-cymene	-	-	52.36
Methyl salicylate	0.1	-	-
Limonene	-	0.66	0.96
γ-cadinene	0.3	-	-
1,8-cineole	-	0.71	-
β-ocimene	-	-	1.10
α–terpinolene	-	-	2.09
Menthone	-	14.45	-
Iso menthone	-	5.44	-
Menthofuran	-	6.26	-
Borneol	-	-	5.80
Menthyl acetate	-	23.06	-

a concentration of 1%. Peppermint oil emulsifier had the least effect on the growth of fungi, where it prevented the growth of all fungi at a concentration of 2% except for *Mucor circinelloides*, while it prevented the growth of all fungi at a concentration of 3%. This inhibitory effect of these oils against fungi may be due to their component which is shown in Table 4, the importance of them eugenol, menthol, menthone thymol and carvacrol.

Eugenol is the main compound of clove oil and it comprises 83-95% of the oil[48,49]. Eugenol is also found in the shoots of the thyme plant[50]. The antifungal activity of eugenol is linked with the altered cell membrane and cell wall structure resulting in the release of cell content[51]. Eugenol has also demonstrated potential as an anti-cancer agent[52,53].

Peppermint oil contains many compounds, the most important of which are menthol and menthone, in addition to several other minor constituents, including pulegone, menthofuran and limonene. Menthol has been reported to having antibacterial and antifungal properties[9,54]. Furthermore, menthone exhibited strong antifungal activity as reported previously[55].

Paster *et al*.[56] also determined that thyme essential oil has a strong effect on the growth of fungi. Nieto,[57]mentioned that thyme contains phenolic compounds (thymol and carvacrol) which are antioxidants, antimicrobial and antifungal compounds.

The mode of action of the active substances in the oils of medicinal and aromatic plants as an antimicrobial agent is depending on multi-target mechanisms such as; damage bacterial cells due to disruption of the cytoplasmic membrane, electron flow, active transport and coagulation of cell contents.

Kumar *et al*.[58] stated that these antifungal materials have high capacities to damage cell structure and function of enzymatic biomechanical activity. The effect of essential oils on fungal growth may be due to their ability to penetrate fungal cells. It may be due to the increased permeability of the fungal cell, medicinal oils can also affect the fungal cell respiration (oxygen uptake) and contain toxic substances that act as anti-spore compounds[59,23]. The importance of this study is because Ras cheese is stored for several months to complete the ripening of this type, which exposes it to the growth of fungi in abundance. The use of essential oils, which are naturally antimicrobial and antifungal, is an important alternative to the use of chemical compounds that have an undesirable health impact on the general health of consumers of this type of cheese. This study recommends expanding the use of essential oils as a healthy and safe alternative to the use of chemical antifungals.

CONCLUSION

Through the results of this research, it was observed the broad of antifungal activity for essential oils emulsions of clove, followed by thyme, then peppermint and their ability to

resist the fungi isolated from the studied Ras cheese samples. This observation encourages the using these oils in cheese manufacture and further studies of them. So, these essential oils can be used as antifungal agents in cheese manufacture, being the main reason for their suitability and their natural origin. Thus, the application of these essential oils could be considered as good alternatives to inhibit fungal growth and to reduce the use of synthetic fungicides in dairy product manufacture, especially ripened cheese.

SIGNIFICANCE STATEMENT

This study discovers the inhibitory effect of essential oils emulsions as natural sources against fungi isolated from Ras cheese that can be beneficial for the production of safe cheese-free of fungi. This study will help the researcher to uncover the best percentages of essential oil emulsions which affect fungal growth that many researchers were not able to explore. Thus, new methods can arrive at the production of safe Ras cheese, free of fungi and their toxins, using natural sources that don't affect cheese properties.

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