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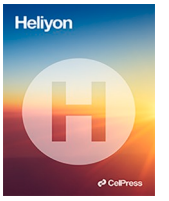

Application of lime peel oil composite nanoemulsion to prevent toxigenic fungi in nuts

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ABSTRACT

Food byproduct oils may have antimicrobial impacts when used in coating and preservation. Nuts are known to suffer from toxigenic fungi and their related mycotoxins. The present study utilized lime oil emulsion to minimize fungal infection and reduce aflatoxin B1 (AFB1). Besides, it evaluated lime oil's impact on nuts' protection against oxidation and deterioration during storage. Lime oil was extracted using hydrodistillation, and gas chromatography (GC-MS) evaluated volatile constituents. Oil was loaded into a composite emulsion of whey protein, Arabic gum, gelatin, and carboxymethyl cellulose. The antimicrobial and antifungal properties of the nut-coating emulsion were evaluated. A simulated Aspergillus flavus infection experiment evaluated composite resistance for fungal infection and AFB1 production. Oxidation and acidity changes in nuts oil composition were evaluated by proximate analysis, fatty acid composition, and induction period. The oil majority was recorded for terpenes and monoterpenes, including limonene (44.69 ± 2.11%). The emulsion was characterized by zeta potential (−21.16 ± 1.28 mV), stability (99.61 ± 0.02%), and polydispersity index (0.41 ± 0.05). Antimicrobial properties recorded a high antibacterial inhibition zone (up to 28.37 ± 0.11 mm) and anti-mycotoxigenic fungi (up to 37.61 ± 0.24 mm). For the simulated experiment, fungal growth reduction ranged between 78.02% for filmed-peanut and 84.5% for filmed-almond, while AFB1 was not detected in filmed hazelnut and almond. During the one-year storage of samples, there was a slight change in nut oil composition and oxidation progress in filmed nuts, while there was a significant change in non-filmed nuts. The result recommended lime-composite as an edible nut coating that prevents aflatoxigenic contamination, oxidation changes, and improved shelf life.

1. Introduction

Lime peels are the primary lime industry byproducts, representing about 50–65% of lime fruit weight during processing. Most of these peels are not used in proper applications in Egypt and many Mediterranean countries, and some efforts were made to use them as livestock feed [1]. However, lime peels are a potential source of valuable essential oils and plant secondary metabolites. So there is increased attention to extracting valuable products from its waste materials for use as natural food preservatives, phytomedicine, and antioxidant agents [2,3].

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Natural antimicrobial compounds, including essential oils and their constituents, can inhibit the development of various pathogenic and spoilage bacteria and can be used as effective preservatives against mycotoxin production [4]. They also have a GRAS status, indicating that regulatory authorities view them as safe [5]. It can reduce the need for synthetic preservatives that are not biodegradable, which pose severe threats to human health and the environment [6,7] and may have biological activity [8]. Concerns are warranted since these chemicals often have unintended effects and may be risky to non-target species [9,10].

Nuts include a relatively low amount of readily accessible carbs, boast an advantageous profile of fatty acids, and are an excellent source of vegetable protein, fiber, and magnesium. Also, nuts are high in oil content and rich in unsaturated fats that regulate many physiological processes [11]. Due to their high unsaturated fatty acids (>50%), they can quickly oxidize during storage, influencing their safety [12]. Nuts consumption prevents chronic diseases, regulates blood glucose, and lowers postprandial glycemic response caused by carbohydrate meals [9]. Seed-coat and cotyledons are brittle, making seeds vulnerable to mechanical damage and insect infestations and facilitating toxigenic-fungi spoilage. Consequently, mycotoxin formation during storage or handling is possible because the seed is rich in nutrients [10]. Fungi can occur during storage, including Aspergillus and Penicillium, which cause oil rancidity, decreasing the extracted oil quality and quantity [13,14].

Aspergillus flavus and A. parasiticus can produce aflatoxins on nuts. A new set of regulation limitations of 4 μg/kg for aflatoxin B1 and 20 μg/kg for total aflatoxins were recently implemented by the European Commission [15,16]. Recently, researchers have developed new risk-free strategies for managing food mycotoxins during storage, like active packaging, to stop microbial development [17,18]. Recently, multiple methods were applied for managing fungal infections and mycotoxins. Because of nanoparticles' unique features, it was utilized for creating active packaging [19], which can extend shelf life or retain the quality. Different lipid films were developed to protect food products. Essential oils, vitamins, polyphenols, antimicrobial peptides, and enzymes have been applied and investigated in edible films [20].

Encapsulation of essential oil is a modern strategy to prevent the active ingredient and achieve antibacterial and preservation [21, 22]. Encapsulation is one of the practical applications of protecting biologically active substances, as it allows a controlled releasing, increasing the effect's effectiveness and efficiency [23]. It also can reduce mycotoxigenic fungi growth and increases the safety of the food system [24,25]. To our knowledge, however, there need to be more overviews on active coating materials and techniques paired with developing technology for detecting fungus and mycotoxins in food items.

This research aimed to create an edible coating shielding nuts against mycotoxin contamination or establishing a detoxifying mechanism. Also, this coating may lessen mycotoxin's harmful effects that contaminate nut seeds. This strategy could be achieved by utilizing lime peel byproduct as a natural source of essential oils with high antimicrobial potency. The bioactive film contains lime peel oil nano-emulsion used as a natural antimicrobial agent to reduce mold growth and mycotoxins in food while it is being stored.

2. Materials and methods

2.1. Materials

Egyptian lime fruit peels (Citrus aurantifolia) were collected as fresh under high sanitary conditions, identified in the herbarium of the National Research, Cairo, Egypt. The raw fruits were purchased from an identical farm, Sadat City, Alex-Cairo desert road, latitude 30° 21', and longitude 30° 30', Middle Delta, Egypt. Peels were carefully peeled using a peeler of stainless-steel toolkit, dried using a hot air oven (35 °C/1 h), and then prepared for the extraction step.

2.2. Solvent, chemical, and microorganisms

The solvents applied in this experiment were of analytical grade. All media growth of microorganisms was purchased from Fluka, 46 Rupert-Mayer-Str., Munich D-81379, Germany. The peels utilized as a source of essential oil were collected without the squeezing process of fruits (as it was hand-made peeled).

The pathogen strains applied for antibacterial evaluation were Listeria monocytogenes, Clostridium butyricum NRRL B-41122, *Pseudomonas aeruginosa* ATCC 10145, and *Klebsiella pneumoniae* NRRL B199. These isolates came from the DSMZ collection of microorganisms (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). They were kept on nutrient agar slants (24 h/37 °C) and then at 4 °C until used. Strains were reactivated in nutrient broth three times before the evaluation step.

Four different strains of toxic fungi were used in the experiment, all of which came from the agro-food microbial culture collection (ITEM), ISPA, CNR, Italy. *Aspergillus flavus* ITEM 698, *Aspergillus carbonarius* NRRL 369, *Penicillium verrucosum* NRRL 5571, and *Fusarium oxysporum* NRRL 32931 are some of the fungal strains that have been isolated. Fungal strains were stored on Czapek-dox medium until the experiment. Solvents and chemicals were of analytical chromatographic grade, and they were all purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, US).

2.3. Hydrodistillation of the extracted essential oil

The oil from peels was hydrodistilled (HD), and the experiment was carried out three times following the method described by Farouk et al. (2016). Samples (100 g) were hydrodistilled for 3 h using Clevenger-type equipment. The essential oil was extracted, dried with anhydrous sodium sulfate, and kept in sealed glass vials with aluminum foil until analysis at −20 °C.

2.4. Gas chromatography-mass spectrometry

Recovered HD-essential oil was put through a GC-MS to analyze its constituents. Separation was achieved using a 60 m 0.25 mm 0.25 m thick TG-5MS capillary column and a Trace GC Ultra Chromatography system (Thermo Scientific, USA) coupled to an ISQ-mass spectrometer (Thermo Scientific, USA). Separation column temperature was held at 50 °C for 3 min [26]. At a rate of 4 °C/min, the temperature was raised to 140 °C and maintained there for 5 min.

The temperature continued to rise at a rate of 6 °C per minute up to 260 °C while maintaining an isothermal holding time of 5 min. The temperatures were 180 °C for the injector, 200 °C for the ion source, and 250 °C for the transition line. The mass spectrometer's scan range extended from *m/z* 40–450, and the ionization energy was calibrated to be 70 Ev. Helium served as the transport medium at a 1 mL/min rate throughout the experiment. The matching of profile fragments with those included in the MS computer library (version 2005 of the NIST library) and subsequent comparisons with actual compounds and published data were the methods that were used to determine the identities of the compounds. The GC peak regions were used to determine the specified elements' proportions. For each substance, the Kovats index was calculated by looking at how long a homologous series of C6–C26 n-alkanes stayed in the water and comparing these times to the values in the relevant research.

2.5. Preparation of the oil coarse used to form nanoemulsion

The coarse emulsion was established following the technique outlined by Saliva et al. [27], using the magnetic stirrer (30 min) to dissolve the oil in polysorbate 80. Briefly, the oil was added to the polysorbate (T80) to form a combination (2:1; v/v), which will be utilized to support the emulsion. The finished solution was agitated for 2 h to achieve complete homogeneity using a magnetic stirrer.

2.6. Applied film composite prepared for coated nuts

A unique edible film was created using components of the highest food grade to improve the safety features of food products. The composites and film base were made, and they consisted of solutions. The solutions were prepared as whey protein (5% w/v), Arabic gum (3% w/v), carboxymethyl cellulose (CMC; 3% w/v), and gelatin (Gt; 3% w/v). The GT solution was first acidified using a 1% formic acid solution before the solution was stirred. The solutions were mixed in equal quantities, then glycerol (5%) (v/v) was added to plasticize the film, and sorbitol (3%) (v/v) was added as a surfactant agent. The formed emulsion was stirred (1200 rpm; at 50 °C/1 h). Before loading, the resultant composite was left standing for 2 h/40 °C.

After the composite solution was mixed well, it was loaded with the coarse oil (at a ratio equal to 1:2 of the base dry matter) using a microsyringe (0.1 mL/min) during the stirring (1200 rpm; up to 30 min). After altogether loading coarse oil into the composite, it was stirred for 6 h/25 °C to achieve complete stability of the loaded composite.

2.7. Nanoemulsion characteristics

Using the same methods as Malik et al. [28], a nanoemulsion was assessed for its characterization in terms of particle size (PSz), zeta potential (ZP), and polydispersion index (PDI). The PSz, ZP, and PDI values were calculated using the Malvern equipment (Nano-S90, Zetasizer, Malvern P Analytical Ltd., Enigma Business Park, Grove Wood Road, United Kingdom). The emulsion was put into a 20 ml cylinder, sealed, and kept at 25 °C for 24, 48, 72, 96, and 120 h [29]. The emulsion stability index is determined to evaluate how easily the serum separates from the emulsion. The following equation (eq. (1)) is used to compute the height of the separated serum from the emulsion:

$$\%\mathbf{ES} = (\mathbf{H1} / \mathbf{H0}) \times \mathbf{100} \quad (1)$$

ES: stability of the emulsion.
H1: the height of upper phase.
H0: the initial height of the emulsion.

2.8. Evaluation of the pH, titratable acidity, and viscosity of the emulsion

The viscosity of the emulsion was measured following the methodology described by Maskanet al. [30]. A viscometer from Brookfield Engineering Laboratories (DV-E Model, Middleboro, MA, USA) with a spindle-type measurement device (CPA-40Z) determined the emulsions' viscosity. The samples were brought to the device and allowed to acclimate to the measuring temperature (25 °C/5 min) before being measured, where experiments were done in triplicate. A pH meter determined emulsion pH (GenWay, pH 2001—Genway Instruments, United Kingdom). A quantity of 10 mL extract was titrated with NaOH (0.1 N) solution to a pH of 8.1; the titrable acidity was determined as a percentage of citric acid).

2.9. Assessment of nanoemulsion antibacterial and antifungal properties

Emulsions were used in the disc and well diffusion experiments to test their antifungal efficacy against fungi grown in a Czapek-dox agar medium. Bacterial strains used in applications were reconstituted from lyophilized stocks by incubating the reconstituted spores

in nutrient agar media. After spreading the activated strains throughout the nutrient agar plates, 100 μL of the nanoemulsion was put into the discs or wells. Diffusion tests were used to quantify the nanoemulsions' antibacterial activity, as described by Abdel Razek et al. [31]. Each strain's apparent zone diameter (in millimeters) of inhibition after applying the emulsion was recorded; a larger diameter indicates a more potent dose.

Fungal strains used in the study were first cultured and inoculated on a potato dextrose agar media. Each fungal strain's spore suspension (10^5 CFU/mL) was placed into about 100 μL of well-diffusion-filled film composites on mounted plates. Using the same circumstances and technique as Abu-Sree et al. [32] the inhibitory effect was measured as the diameter of a clear zone surrounding each well.

3. Evaluation of coated nuts

3.1. Extraction and chemical characterization of seed oils

Using a lab mixer, the seeds from various samples were ground into a powder. Sonication for 1 h was used to extract the oil twice from seed samples. To plant 50 g of seeds, use 250 mL of petroleum ether. The solvent was removed using a Heidolph rotary evaporator, and the oils were stored in the amber bottle in the fridge at 4 °C until analysis.

3.2. Proximate analysis

Extracting oil from a 100 g seed sample with petroleum ether 40–60 in a soxhlet device [33] allowed us to calculate the oil content. The acid value (AV) was computed using the AOCS standard protocol Cd 3a-63 [34]. Using the AOCS-approved technique Cd 8b-90 [34], we calculated the peroxide value (PV). The *p*-anisidine value (p-AV) was calculated using the methods previously reported [35]. The total oxidation Value (TOTOX value) is calculated by adding the PV and the p-AV to provide an idea of the oil's overall oxidative degradation. The following equation was used in the calculation:

$$\text{TOTOX value} = p\text{-AV} + 2\ \text{PV} \tag{2}$$

3.3. Identification of fatty acids

To make fatty acid methyl esters (FAME), we followed the procedure outlined in our prior work [36] and documented in the AOCS Official Method Ce 1k07. Using the formula published by Fatemi et al. [37], the oxidizability (COX) value of the oil was determined (eq. (3)).

$$\boldsymbol{Cox\ value} = \{\mathbf{1}\ \boldsymbol{x}\ [\boldsymbol{C}\mathbf{18}\text{: }\mathbf{1}(\%)] + \mathbf{10.3}\ \boldsymbol{x}\ [\boldsymbol{C}\mathbf{18}\text{: }\mathbf{2}(\%)] + \mathbf{21.6}\ \boldsymbol{x}\ [\boldsymbol{C}\mathbf{18}\text{: }\mathbf{3}(\%)]/\mathbf{100} \tag{3}$$

3.4. Estimation of induction period by rancimat method

Metrohm Rancimat apparatus model 892 (Metrohm, Switzerland) was used to calculate the induction duration of the oil samples in accordance with Kowalski et al. [38]. A 5 g oil sample weight was put in the glassware of the reaction vessel, and deionized water up to a volume of 60 ml was added to the conductometry cells. At 110 °C, air flowed through the oil as the samples were heated at a rate of 20 L' per hour.

3.5. Anti-aflatoxin simulation experiment using edible film application

This section evaluated the prepared film for its anti-aflatoxigenic effect using a simulated experiment using sealed polyethylene bags (20 g) of peanut, hazelnut, or almond; each type of nut seeds was packed in two groups (before and after coating). Each group was treated with a strain-producing toxin of aflatoxin B1 (Aspergillus flavus ITEM 698) using spore suspension at a concentration of (1.7 × 105 CFU/mL/bag). Samples of the two groups were incubated (24 °C/4 days for fungal growth evaluation; on 28 °C/12 days for toxin production evaluation). At the end of the incubation period, samples of fungal growth evaluation were washed on filter paper (Whatman No.1); the filter papers were dried in a hot air oven until the staple weight and the mycelia growth weight was calculated against the control.

Samples of toxin-production evaluation were crushed at the end of incubation, extracted using methanol (80%), and the collected solvent was prepared for aflatoxin evaluation. The solvent was evaporated, and the extracted toxin was kept in a sealed amber vial as a dry film until the measuring step.

3.6. Aflatoxin reduction evaluation

In the prior extraction, it was found that the fungal strain reduced the concentration of aflatoxin. HPLC-grade methanol was used to

Table 1
Essential volatile components of lime oil involved in nut-seeds filming.

Compound	Byproduct content	KI $_D$	KI $_R$	Identification
α - Pinene	5.48 ± 0.84	940	939	MS, KI
Sabinene	2.08 ± 0.31	965	968	MS &KI
β - Pinene	11.66 ± 0.16	970	971	MS &KI
β - Myrcene	2.05 ± 0.55	981	983	MS, KI&ST
α - Terpinene	0.87 ± 0.02	1006	1008	MS, KI&ST
O- Cymene	1.02 ± 0.34	1010	1012	MS &KI
Limonene	44.69 ± 2.11	1033	1033	MS, KI&ST
Linalool	1.08 ± 0.02	1036	1037	MS & KI
γ - Terpinene	16.22 ± 1.05	1047	1050	MS, KI &ST
α - Terpinolene	2.21 ± 0.07	1092	1094	MS &KI
1-Terpineol	0.11 ± 0.02	1125	1125	MS &KI
β- Citronellal	0.14 ± 0.05	1131	1130	MS, KI &ST
Terpene-4-alcohol	0.44 ± 0.06	1165	1165	MS &KI
α-Terpineol	1.67 ± 0.11	1176	1175	MS &KI
α-Citral	3.56 ± 0.21	1195	1196	MS, KI &ST
Nerol	0.69 ± 0.24	1205	1205	MS & RI
β-Citral	0.74 ± 0.08	1235	1235	MS & RI
Geraniol	0.48 ± 0.02	1310	1310	MS & RI
δ-elemene	0.18 ± 0.02	1331	1330	MS & RI
Neryl-acetate	1.81 ± 0.37	1343	1344	MS & RI
Trans-geraniol acetate	0.37 ± 0.02	1355	1356	MS & RI
α-Farnesene	0.22 ± 0.11	1374	1374	MS & RI
β-elemene	0.16 ± 0.02	1381	1380	MS & RI
Trans-α-bergamtene	2.24 ± 0.67	1430	1430	MS& RI

KID: the Kovats Retention Indices were calculated from our analysis concerning a series of n-alkenes; KIR: the Kovats Retention Indices that referenced from the literature concerning a series of n-alkenes.
%: Percentage composition of a compound in the analysis materials.

Table 2
Emulsion characterization for the oil-composite used in nut-seed coating.

Sample	Particle size (nm)	Zeta potential (mV)	PDI	Emulsion stability (%)	Viscosity (mPa/sec)	pH	Acidity (g citric/L)
Oil composite	184.52 ± 1.74	- 21.16 ± 1.28	0.41 ± 0.05	99.61 ± 0.02	1.41 ± 0.06	6.21 ± 0.54	0.41 ± 0.11

The data were expressed as means ± SEM (where n = 3).

dissolve the aflatoxin dry film. Afla-test immunological affinity column was loaded with 1 mL of solution and washed twice with 10 mL of distilled water (flow rate: 6 mL/min). Aflatoxin was eluted from the column using 2 mL of methanol at a flow rate of 0.3 mL/min. The fluorometer used for the quantitative analysis was already calibrated (VICAM Series 4EX Fluorometer, Watertown, MA, USA; LOD 1.0 ng/L).

3.7. Data analysis statistics

The data was presented as the mean standard deviation (n = 3) of three independent samples. Microsoft Excel was used to analyze the statistical data. One-way ANOVA was compared to the least significant difference test (P = 0.05). Individual mean significant differences at the 0.05% level were determined by analyzing the statistical significance of differences across extract concentrations.

4. Results

4.1. Essential components as bioactive source

The essential volatile components extracted by the HD assay from lime applied in nut seeds filming were evaluated using the GC-MS (Table 1). The results represent limonene as the major component of the applied oil (44.69 ± 2.11%). Other components, including *β* –pinene, γ–terpinene, and α –pinene, which possess antimicrobial effects, exist in considerable content. Terpenes and mono-terpenes were the significant groups of bioactive components in this oil. This feature could recommend its application for antimicrobial potency in food production.

4.2. Characterization of oil-emulsion

Results in Table 2 indicate the high acidity and other parameters related to the emulsion stability. The prepared emulsion of lime oil

Table 3
The antibacterial and antifungal effects of lime essential oil, base emulsion, and oil-loaded emulsion.

Microbial Strains	Lime Oil (HD)	Emulsion	Oil-emulsion (disk diffusion)	Oil-emulsion (well diffusion)	Standard antimicrobial
Antibacterial effect IZD (mm)					Zithrocin
***Listeria monocytogenes* ATCC 19111**	10.05 ± 1.71 [b]	3.51 ± 0.94[a]	24.11 ± 2.05[c]	23.41 ± 1.56[c]	27.25 ± 0.31[d]
***Clostridium butyricum* NRRL B-41122**	9.47 ± 1.25 [b]	4.12 ± 1.05[a]	28.14 ± 2.27[c]	26.44 ± 1.61[c]	28.37 ± 0.11[d]
***Pseudomonas aeruginosa* ATCC 10145**	10.69 ± 1.81 [b]	4.07 ± 0.54[a]	27.31 ± 1.67[c]	26.74 ± 1.54[c]	27.62 ± 0.21[d]
***Klebsiella pneumoniae* NRRL B199**	10.31 ± 2.02 [b]	4.13 ± 0.87[a]	23.51 ± 1.39[c]	23.02 ± 2.37[c]	27.34 ± 0.44[d]
Antifungal effect IZD (mm)					Nystatin
***Aspergillus flavus* ITEM 698**	14.51 ± 1.78 [b]	7.81 ± 1.61[a]	32.12 ± 2.55[c]	35.21 ± 1.66[c]	36.18 ± 0.34[d]
***Aspergillus carbonarius* NRRL 369**	16.31 ± 2.41 [b]	9.64 ± 1.78[a]	34.51 ± 2.47[c]	36.05 ± 2.41	36.55 ± 0.22[d]
Penicillium verrucosum* *NRRL 5571	10.28 ± 2.16 [b]	7.96 ± 1.05[a]	29.31 ± 2.18[c]	28.89 ± 3.05[c]	37.14 ± 0.15[d]
***Fusarium oxysporum* NRRL 32931**	18.81 ± 1.74 [b]	9.21 ± 1.11[a]	36.34 ± 1.21[c]	37.54 ± 2.37[c]	37.61 ± 0.24[d]

The data were expressed as means ± SD (where n = 3, p = 0.05); IZD: inhibition zone diameter measured in millimeters (mm); HD: hydro distillation. Standard antimicrobial agents were applied as Zithrocin for antibacterial and Nystatin for antifungal.
Means with different superscript letters are significantly different for each raw.

Table 4
Chemical composition of nut seeds at zero-time and after one year of storage condition (22 °C, Rh 65%) of coated and non-coated seeds.

Composition (%)	Peanut seeds		
	Zero time	Non-coated	Coated
Moisture	7.61 ± 2.05[a]	14.21 ± 1.26 [b]	6.27 ± 1.05[a]
Carbohydrate	18.27 ± 1.24[a]	15.66 ± 2.51 [b]	18.01 ± 1.88[a]
Protein	24.37 ± 1.02[a]	22.15 ± 2.41 [b]	26.74 ± 1.34[c]
Fat	34.82 ± 1.54[a]	33.71 ± 2.05 [b]	36.01 ± 1.56[a]
Ash	5.47 ± 1.05[a]	4.82 ± 1.02[a]	4.71 ± 1.04[a]
Fiber	9.27 ± 0.89[a]	9.34 ± 2.08 [b]	9.41 ± 1.82[a]
	Hazelnut seeds		
	Zero time	**Non-coated**	**Coated**
Moisture	9.18 ± 1.37[a]	14.91 ± 2.54 [b]	10.36 ± 1.31[a]
Carbohydrate	5.28 ± 1.08[a]	4.66 ± 2.37 [b]	5.34 ± 1.02[a]
Protein	16.94 ± 1.81[a]	15.21 ± 2.51 [b]	18.2 ± 2.17[a]
Fat	47.12 ± 1.16[a]	41.34 ± 2.77 [b]	46.21 ± 1.43[a]
Ash	6.55 ± 1.27[a]	7.51 ± 1.11[a]	6.77 ± 1.41[a]
Fiber	13.27 ± 1.02[a]	14.18 ± 1.37 [b]	13.21 ± 0.96[a]
	Almond seeds		
	Zero time	**Non-coated**	**Coated**
Moisture	10.06 ± 1.21[a]	14.29 ± 2.05 [b]	9.02 ± 0.78[a]
Carbohydrate	9.14 ± 1.54	7.26 ± 1.43 [b]	9.33 ± 1.42[a]
Protein	19.64 ± 0.24[a]	18.05 ± 1.37 [b]	20.56 ± 1.12[a]
Fat	43.02 ± 1.14[a]	42.57 ± 0.94 [b]	43.27 ± 1.09[a]
Ash	5.21 ± 0.56[a]	6.41 ± 0.28 [b]	5.77 ± 0.74[a,b]
Fiber	12.23 ± 0.41[a]	13.31 ± 1.37 [b]	12.4 ± 0.73[a]

The data were expressed as means ± SD (where n = 3, $p = 0.05$).
Means with different superscript letters are significantly different for each raw.

particle size, zeta-potential, and poly dispersing index was evaluate for the quality and stability. The results in **Table (2)** point out the distinctive properties of the lime emulsion. as the zeta value (- 21.16 ± 1.28 mV) indicates high stability of the formed emulsion, which is emphasized by the calculation of (%EE = 99.61 ± 0.02%). Also, the measured acidity value reflected the higher acidity of the formed emulsion (0.41 ± 0.11 g of citric acid equivalents/L). Other parameters of the created emulsion's stability were evaluated, including pH (6.21 ± 0.54) and viscosity (1.41 ± 0.06 mPa/s). This result of viscosity is a value reflect the internal friction of moving fluids layers, which is joining to the stability and oil releasing characteristics from the emulsion.

4.3. Antimicrobial effect of lime-oil emulsion

To evaluate their antimicrobial efficiency, a comparative antimicrobial evaluation has been done between the oil and pure

Table 5
Oil parameters of control, stored-coated, and stored non-coated nut seeds (lime-emulsion coating).

	PV (mEqO_2/kg oil)	*P*-Av	TOTOX value	FFA%	IP (h)
Peanut seeds					
control zero time	4.25 ± 0.54[a]	1.79 ± 0.05 [b]	10.29 ± 0.41[a]	0.23 ± 0.02[a]	6.74 ± 1.05 [b]
Stored non-coated	18.21 ± 1.67 [b]	2.94 ± 0.28[c]	39.36 ± 1.54 [b]	0.55 ± 0.14[c]	4.81 ± 1.47[c]
Stored-coated	5.04 ± 0.74[a]	1.25 ± 0.08[a]	11.33 ± 1.18[a]	0.24 ± 0.01 [b]	6.55 ± 0.64[a]
Hazelnut seeds					
control zero time	2.17 ± 0.31[a]	1.84 ± 0.02 [b]	6.18 ± 0.21[a]	0.91 ± 0.05[a]	22.31 ± 0.45[a]
Stored non-coated	14.42 ± 2.02[c]	3.02 ± 0.74[c]	31.86 ± 1.05[c]	1.29 ± 0.41[c]	15.41 ± 1.26 [b]
Stored-coated	3.25 ± 0.21 [b]	1.18 ± 0.05[a]	7.68 ± 0.37 [b]	0.96 ± 0.02 [b]	21.87 ± 0.77[a]
Almond seeds					
control zero time	3.71 ± 1.02[a]	1.57 ± 0.14 [b]	8.99 ± 0.67[a]	0.26 ± 0.01[a]	7.27 ± 0.94[a]
Stored non-coated	21.05 ± 2.51[c]	2.62 ± 0.88[c]	44.72 ± 0.88[c]	0.39 ± 0.11[c]	5.08 ± 1.41[b]
Stored-coated	7.04 ± 1.08 [b]	1.39 ± 0.12[a]	15.47 ± 1.37 [b]	0.27 ± 0.02 [b]	7.02 ± 0.77[a]

The data were expressed as means ± SD (where n = 3).
TOTOX = 2PV + AV; IP = induction period (Rancimat method @110 °C).
Means with different superscript letters are significantly different for each column of nut seed type individually.

Table 6
Fatty acid profile of control, stored-filmed, and stored non-filmed nut seeds (lime-emulsion coating).

Fatty acid	Peanut			Hazelnut			Almond		
	Control	Non-filmed	filmed	Control	Non-filmed	filmed	Control	Non-filmed	filmed
Palmitic acid (C16:0)	12.91 ± 0.54	11.28 ± 1.24	12.74 ± 0.34	6.72 ± 1.02	7.92 ± 1.14	6.46 ± 0.27	7.95 ± 0.74	6.01 ± 1.18	9.33 ± 0.34
Palmitoleic acid (C16:1)	0.17 ± 0.05	0.08 ± 0.01	0.17 ± 0.03	0.34 ± 0.05	0.18 ± 0.11	0.28 ± 0.02	0.81 ± 0.08	0.59 ± 0.12	0.63 ± 0.04
Stearic acid (C18:0)	3.16 ± 0.27	2.08 ± 0.31	3.14 ± 0.29	3.07 ± 0.33	2.69 ± 0.18	3.04 ± 0.25	1.55 ± 0.37	2.21 ± 0.41	1.93 ± 0.21
Oleic acid (C18:1 (ω9))	45.64 ± 1.05	49.91 ± 1.24	44.55 ± 1.21	81.67 ± 0.44	83.22 ± 1.01	82.01 ± 0.56	63.77 ± 1.37	69.4 ± 1.26	65.89 ± 1.05
Linoleic acid (C18:2 (ω6))	36.60 ± 1.02	31.34 ± 1.66	37.24 ± 1.11	8.24 ± 0.67	6.91 ± 0.44	8.21 ± 0.27	25.92 ± 0.81	21.74 ± 1.02	26.22 ± 0.88
Arachidic acid (C20:0)	1.39 ± 0.27	1.31 ± 0.31	1.37 ± 0.18	ND	ND	ND	ND	ND	ND
Gondoinic acid (C20:1)	1.14 ± 0.14	1.16 ± 0.08	1.03 ± 0.05	ND	ND	ND	ND	ND	ND
SFA	17.45 ± 1.07	14.67 ± 1.56	17.25 ± 1.05	9.79 ± 0.27	10.61 ± 0.67	9.50 ± 0.37	9.50 ± 0.42	8.22 ± 0.97	11.26 ± 0.36
MUFA	46.95 ± 1.86	50.99 ± 2.14	45.75 ± 1.54	81.97 ± 1.67	82.48 ± 2.37	82.29 ± 1.89	64.58 ± 1.71	70.04 ± 2.33	66.52 ± 1.46
PUFA	35.60 ± 1.11	34.34 ± 0.94	37.21 ± 0.69	8.24 ± 0.77	6.91 ± 0.56	8.21 ± 0.89	25.92 ± 1.05	21.74 ± 1.31	22.22 ± 1.07
Total UFA	82.55 ± 1.55	85.33 ± 2.01	82.75 ± 1.74	90.21 ± 1.47	89.39 ± 2.05	90.5 ± 1.64	90.5 ± 1.54	91.78 ± 1.88	88.74 ± 1.21
PUFA/SFA	2.04 ± 0.1	2.34 ± 0.08	2.14 ± 0.05	0.84 ± 0.02	0.65 ± 0.01	0.86 ± 0.01	2.73 ± 0.12	2.73 ± 0.09	1.97 ± 0.05
COX Value	4.14	4.05	4.27	1.67	1.54	1.67	3.32	2.24	2.95

Results were expressed as means ± SD (standard deviations; n = 3, $p = 0.05$).
SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; Total UFA: total MUFA and PUFA.
Filmed: nuts have a coat of lime oil emulsion solution; non-filmed: nuts that do not have the lime oil emulsion on them.

emulsion as control samples and oil-loaded emulsion. The results in Table (3) reflected more efficiency for oil-loaded emulsion against pathogen strains of bacteria, followed by lime oil, where crude emulsion comes later compared to the standard antibacterial of Zithrocin. These results about the enhancement in activity of encapsulated bioactive components against bacterial pathogens were referred to before [39]. The antifungal impact of lime oil emulsion was recorded as an effective material against applied strains of toxigenic *fungi*. Moreover, its activity was found to be close to the activity of standard antifungal, particularly for the strains of *Aspergillus* fungi. This result leads us to think about the oil-emulsion utility as a filming material of seeds suffering from *Aspergillus* infection. These results could be linked to the storage stability that provided by the encapsulation process for the lime oil components [40].

4.4. Determination of proximate changes

The results of the proximate composition of almond, hazelnut, and peanut seeds at zero time and after storage for a year are shown

in Table 4 (coated and non-coated seeds). Results in Table 4 showed an increase in moisture, ash, and fiber content in non-coated samples after one-year storage than in zero-time samples, where the coated samples showed a lower increase in some samples. The nutrients, carbohydrates, proteins, and fat content in the non-coated samples showed a decrease in these content after storage, and these nutrients were found to be increased in most of the coated nut samples after storage.

4.5. Effect of coating film on the protection of the nuts oil composition during storage

Table 5 indicates different nut oil samples' proximate analysis and oxidation stability. The primary and secondary oxidation of oils were determined by PV and P-Av, which shows a more significant increase in non-coated seeds after one year of storage than that the control samples. It was noticeable that the PV was slightly higher in the coated seed samples (3.25–7.04 m. eq O_2/Kg) than that in the control samples (2.17–4.25 m. eq O_2/Kg) but still within the permitted value (less than ten m. eq O_2/Kg). On the other hand, there was a high increase in peroxide values for the non-coated samples (14.42–21.05 m. eq O_2/Kg) compared with the coated samples after storage for 12 months. It was found that the P-Av of non-coated samples (2.94, 3.02 and 2.62 for peanut, hazelnut and almond seeds respectively), were higher than that in case of coated samples (1.25, 1.18 and 1.39). The total oxidation of oils (TOTOX value) was higher in non-coated samples (Table 5). The results recorded in Table 5 showed that the FFA% values increased significantly in non-coated samples and approximately stayed constant in coated models of three types of nuts after the storage time. The IP results, which indicate the stability of nut oils, in Table 5 showed a decrease in the IP of non-coated samples after storage than in the control samples at zero time, where the IP of coated samples was almost unchanged from the control samples.

4.6. Fatty acid profile changes of stored filmed and non-filmed nut seeds

Table 6 shows the fatty acid profiles of the three nuts at different treatments, as measured by GC. Each nut had a distinct fatty acid pattern. Oleic acid (C18:1) was the predominant monounsaturated fatty acid MUFA in all three nuts, with palmitoleic acid (C16:1) being present in small amounts. Linoleic acid (C18:2) was the predominant polyunsaturated fatty acid (PUFA) present, while linolenic acid (C18:3) was absent. The two primary saturated fatty acids (SFA) detected in all samples were palmitic (C16:0) and stearic (C18:0) acids. All nut samples had comparable levels of total UFA, respectively, which ranged from 82.55 to 91.78%.

The major fatty acid was oleic in non-filmed samples, whereas it decreased in the filmed sample (peanut and almond). Except in case of hazelnut the oleic acid not changed in storage samples compared with the control. Table 6 shows that the total MUFA increased in all non-coated samples, while the total PUFA decreased. The total UFA was increased only in non-coated samples (peanut and almond). The high PUFA/SFA ratio also demonstrated the oil's high degree of unsaturation and propensity for oxidative processes. Concerning the PUFA/SFA, it only slightly decreased in non-filmed hazelnut and remained constant in the case of almonds and peanuts compared to the control samples. In contrast, it decreased in coated almond and peanut samples compared to uncoated samples. A slight variation in the Cox value for treated samples was noticeable.

4.7. Anti-aflatoxigenic properties of oil-emulsion using simulating experiment

The results recorded for the simulated experiment of lime-oil-emulsion used in nut-seeds filming to prevent *A. flavus*-producing strain infection reflect emulsion efficacy. They expressed that oil emulsion has the potency to reduce the fungal *A. flavus*-producing strain infection for the nut seeds. The inhibition efficiency of the lime oil emulsion on the fungal-spore growth in the three nuts is ordered as follows; almond filmed seeds > hazelnut filmed seeds > peanut filmed seeds.

Mycelia growth weight inhibition ratios regarding the lime oil filming of seeds were between 78.02% for peanut-filmed seeds and 84.5% for almond-filmed seeds (Fig. 2a).

Concerning the aflatoxin production by the applied strain of *A. flavus* on the nut seeds, non-filmed seeds of peanuts, hazelnut, and almond were recorded with high concentrations of aflatoxinB_1 (AFB_1). The concentration of the AFB_1 was determined at 934.27 ± 7.21 ng/kg for peanut seeds, 597.34 ± 11.05 ng/kg for hazelnut seeds, and 621.55 ± 10.77 ng/almond seeds. The AFB_1 contaminated peanut seeds at 18.71 ± 2.14 ng/kg, which is still less than the regulation limit for the seeds coated with a film of lime oil emulsion. While nut seeds filmed of almonds and hazelnut were recorded as not detected for AFB_1 contamination (Fig. 2 b).

Generally, the lime oil emulsion was found to have a noticeable effect and high potency to protect the nuts seeds from the fungal effect during storage and reduce the oxidation of their oils.

5. Discussion

Bioactive components of lime essential oil were studied. The high antimicrobial activity was expected for lime oil due to the presence of a high amount of limonene (the principal components), pinene, and γ–terpinene, which was previously reported to have antimicrobial activity [26–29].

These terpene components are considered volatile materials and need to be loaded on inert material that prevents their damage and supports their efficiency. The encapsulation technique is the most critical application in achieving stability and bioavailability of bioactive compounds to ensure they reach the target systems [41].

The formed emulsion, consisting of novel composite components, showed high stability. This feature was cleared by the parameter values recorded in Table 2. The emulsion stability represents 99.61%, and the zeta potential value is (−21.16 mV), where the poly dispersing index recorded less than (0.5). The acidity and the pH values reflect the semi-acid properties of the formed emulsion that can

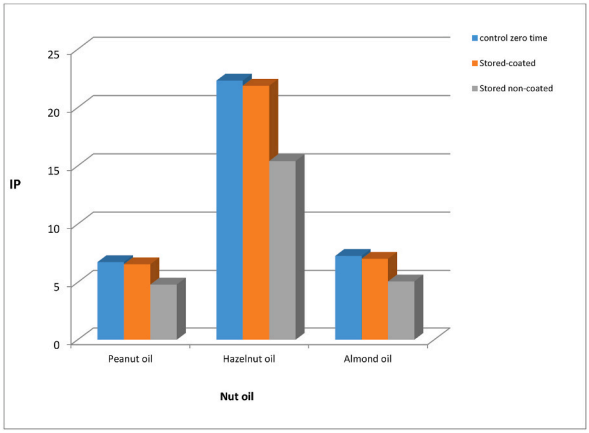

Fig. 1. The change in induction period (IP) of the coated and non-coated three nuts after storage for one year in comparison to the control at zero time.

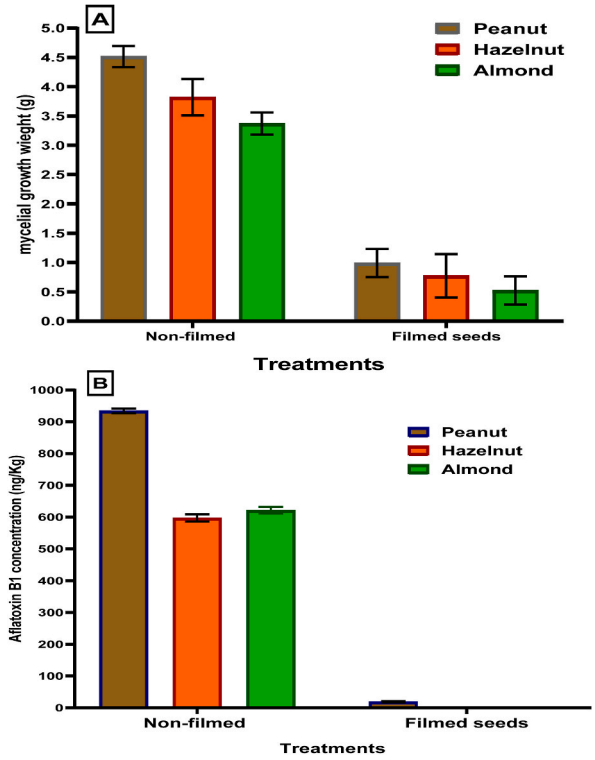

Fig. 2. Fungal growth and reduction of aflatoxin B_1 production of a simulated experiment using *A. flavus* strain on filmed and non-filmed nut seeds.

assess the antimicrobial properties of the applied film material using this emulsion. This feature is also emphasized where the oil's antibacterial and antifungal potency was enhanced by its loading on the emulsion composite (**Table 3**). The changes in chemical composition values (PV, P-Av, FFA%, IP) of peanut, hazelnut, and almond nut seeds after one year of storage were recorded (Table 5). The best results were given by the filmed nut seeds indicating more preservation effect of the coating film on these seeds. In contrast, the non-filmed models of nut seeds significantly deteriorated their chemical composition. It was shown to have a considerable decrease in carbohydrate, protein, and fat values and an increase in moisture, ash, and fiber content levels compared to the control after storage.

The oxidative stability of nut oils affects how long it may be stored [42,43]. In this regard, Table 5 reflects how the fatty materials are stable for those oils gained from filmed nut seeds (coated seeds). FFA% increase in non-filmed samples but slightly changed in filmed ones, this may be due to the protection of the film from the growth of some fungi, such as *Aspergillus*, that were able to hydrolyze the nut oils leading to the liberation of FFAs [44]. A higher increase in PV in non-filmed samples than in filmed samples may be due to the effective protection of lime oil film against the growth of fungi which helps in oil stability. They also increase seed heat,

accelerating the process of producing primary oxidation products such as hydroperoxides and conjugated dienes [13]. The following secondary oxidation process has secondary products such as ketones and aldehydes, increasing the *P*-AV and TOTOX value in non-filmed seeds, as shown in the results. The presence of lime oil film on the nuts seeds protects them from these adverse effects of the fungi due to its high antimicrobial potency, shown clearly in Table 3. IP of all coated samples were stable compared to non-coated samples.

The fatty acid double bonds react with the single oxygen, decreasing the double bond numbers and decreasing its unsaturation. This decrease in double bonds is evident in the reduction of PUFA % and increase in MUFA %. The cox value, which calculated from the equation (eq. (3)).

It also decreased due to the decrease of the oil unsaturation by the oxidation process in non-filmed samples. These changes were not found in the filmed samples due to the protective effect of the seeds by lime oil film from the oxidation process. In this regard, the stability or quit-little changes in the fatty acid composition of filmed (coated) nut seeds (Table 6) can indicate the extension in the nut seeds' shelf life.

Utilizing a simulation experiment on nut seeds, the antifungal effect that reflects toxigenic fungal inhibition was reflected as a reduction in the mycelia growth weight of spiked samples with fungal spores. Again, aflatoxin B_1 produced by the infected fungi was recorded as undetected for filmed hazelnut and almond seeds (Fig. 1). These results agreed with the previous work of potato slices coated by essential oil emulsion [45,46]. The results showed an inhibition rate of more than 70%, which can primarily be ascribed to the limonene content of the oil.

6. Conclusion

Lime oil was loaded on a novel composite of whey protein, Arabic gum, CMC, and gelatin and applied to coat some nuts. The characterization results of the prepared emulsion showed promising results for zeta potential, particle size, and PDI. Antifungal properties of oil emulsion showed effective potency against toxigenic fungal strains. Using a simulated experiment for infecting nut seeds by toxin-producing fungi of *A. flavus*, results reflected the efficiency of oil-emulsion loaded as film coating on nuts to inhibition of mycelia growth weight between 78.02% for peanut-filmed seeds and 84.5% for almond-filmed seeds.

Regarding the production of the AFB_1, film coating material possessed an influence on fungi to reduce aflatoxin production in peanut seeds, and it was not detected for hazelnut and almond. However, stored nuts coated by oil emulsion showed limited changes in proximate analysis and oxidation parameters of fatty content. The oxidation stability of oils from the filmed nuts almost did not change during storage, indicating the positive effect of lime film on the oxidation stability and shelf life of the studied oils. These results recommended using fruit lime byproducts as safe natural preservers applied in coating film as a novel way of nut seed prevention against mycotoxigenic fungal contamination and mycotoxin production.

Author contribution statement

Minar M. Hassanein, Eman F. Al-Amrousi: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Adel G. Abdel-Razek, Ahmed N. Badr: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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