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Inhibition of Toxigenic Fungi and Mycotoxin Formation by Nanoemulsions of Some Plant Essential Oils

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Abstract

Dried fruits and nuts may be contaminated with mycotoxins of fungi which are dangerous health problems. Forty samples of nuts and dried fruits were purchased from markets in Jeddah governorate, Saudi Arabia and were mycologically analyzed. Three nanoemulsions formulation of Clove, Basil and Sunflower were prepared. The mean droplets size for all formulations was found in the range 10.52-100 nm. The three nanoemulsions of Clove, Basil and Sunflower have droplet size of 89.99, 23.33 and 13.15, respectively. Antifungal activities of the three prepared nanoemulsions were studied against the two toxigenic fungi and two non-toxigenic fungi. MIC for NE of Clove oil examined against *A. flavus* and *A. parasiticus* was 0.02% and 0.03%, respectively. The effect of the nanoemulsions on reduction of mycotoxin production was also studied. Aflatoxin B1 and G1 produced by *A. parasiticus* and *A. flavus* were reduce by treatment with clove oil nanoemulsion in growth medium. In conclusion, nanoemulsions from plant essential oils specially clove inhibited both fungal growth and mycotoxin productions. In conclusion, nanoemulsion based on plant EOs can be used to control fungal growth and mycotoxins production in some food kinds.

Keywords: Dried Fruits; Mycotoxins; Fungi; Aflatoxins; Nanoemulsions; Essential Oil

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Introduction

Mould growth in agricultural products may cause an important hazard to human health by producing toxic metabolites called mycotoxins which are fungi secondary metabolites, occured naturally in food [1]. Mycotoxins represent a very large group of different substances including aflatoxins, ochratoxin A, patulin and *Alternaria* toxins which were produced by different mycotoxigenic species [2]. The major affected food commodities are cereals, nuts, dried fruit, coffee, cocoa, spices, oil seeds, dried peas, beans and fruit, particularly apples. Dried fruits are susceptible to mould growth and mycotoxin formation because of their high sugar content, method of harvest and drying conditions [3].

The most important dried fruits produced for human consumption are raisins, sultanas, figs, apricots, and dates ad their mycotoxin contamination may start with on the trees, increase during harvesting and sun drying, and continue to accumulate during storage. Because all these fruits are cultivated in warm climates, mycotoxins associated with these fruits are aflatoxins and ochratoxin A. Essential oils (EOs), which are aromatic and volatile oily extracts obtained from aromatic and medicinal plant materials, including flowers, buds, roots, bark, and leaves are one of the best such alternatives, given their strong antimicrobial activities [4,5]. They are also well-known for their antimicrobial, antiviral, antioxidant, antifungal, and insecticidal properties [6]. Viuda-Martos M, et al. (2008) demonstrated that citrus essential oils can be considered suitable alternatives in the food industry to control the growth of moulds commonly associated with food spoilage as *Aspergillus flavus* and *Penicillium verrucosum*, amongst others [7]. Moreover, these EO are generally considered safe to use in foods and beverages and are known as generally recognized as safe [8]. Volatile oils can exhibit antifungal activity at very low concentrations in a growth medium. For example, inclusion of 1 - 10 µL/mL of marjoram oil in a culture broth reduced the growth of five species of filamentous fungi by up to 89% compared to the control [9]. Many plant oils have been tested for antibacterial and antifungal properties [10]. Some of the natural products, such as cinnamon oil, clove oil, phenols, some spices, and many essential oils have been reported as effective inhibitors of fungal growth and aflatoxin production [11]. The volatile oil of lemon grass has been confirmed against spoilage and mycotoxigenic fungi [9]. The oil of basil (*Ocimum basilicum* L.) can inhibit the growth of a wide range of fungi (22 species) including mycotoxigenic strains of *A. flavus* and *A. parasiticus* Spear [12]. In recent years, the food industry has demonstrated a growing demand for natural compounds to develop novel food preservatives against spoilage and pathogenic microorganisms, as well as to sustain innovation in food packaging [13]. The use of EOs as a mild preservation technique in the food industry has gained considerable attention in recent years, mainly driven by the concern over the negative perception of consumers on chemical preservatives [14]. However, the high reactivity and hydrophobicity of EOs represent a formidable challenge to their direct incorporation in food and beverage products. Essential oils are hydrophobic compounds and usually have quite low solubility in water, which limits

their utilization in aqueous-based foods and beverages. This problem could be simply resolved by encapsulating EOs within emulsion-based delivery systems. After EOs are encapsulated into suitable emulsion delivery systems (Nanoemulsion), they can then be incorporated into aqueous-based foods (e.g., beverages) and other products by simple mixing [15].

Nanoemulsion is a heterogeneous system consisting of two immisible phases, one phase is oil phase and other is aqueous one. Now-a-day's nanoemulsions are frequently used for various the purposes like delivery of vaccine, DNA encoded drug, antibiotics, cosmetic and topical preparations and can be administrated via various routes like oral, pulmonary, ocular and transdermal, etc. Based on it is composition, there are three types of nanoemulsions: o/w (oil in water), w/o (water in oil), multiple emulsion {o/w/o (oil in water in oil), w/o/w (water in oil in water) [16]. To retain their biological activity and minimize at the same time the impact on the organoleptic properties of foods where incorporated, EOs need to be encapsulated in delivery systems, which are compatible with food applications [17]. The interest of incorporating essential oils in foods as preservatives is related to their recognition as safe natural compounds, being a potential alternative to produce foods free of synthetic additives. However, the incorporation of Eos with antimicrobial to foods still presents several drawbacks due to their poor water solubility as well as to toxicological and economic considerations [18]. Therefore, the aim of this study was to find out the effect of some essential oils and their nanoemulsions on some toxigenic and non-toxigenic fungi, isolated from different dried fruits.

Material and Methods

Preparation of Nanoemulsions

The essential oils of clove, basil and sunflower were purchased from local markets, Jeddah. Nanoemulsions from EOs Clove (*Syzygium aromaticum*), Basil (*Ocimum basilicum*) and sunflower (*Helianthus annuus*) were prepared. The NEs were prepared using the High Pressure Homogenization (HPH) technique [19]. The oil samples were diluted with a large amount of water (ratio 1:100). Pre-emulsions were obtained by high speed stirring using an Ultra Turrax T25 (IKA Labortechnik, Jahnke und Kunkel, Germany) at 24,000 rpm for 5 min. Then, the pre-emulsions were passed 5 times through an orifice high pressure homogenizer Nano DeBEE Electric Benchtop Laboratory (BEE International, USA) at 300 MPa. The resulting Nanoemulsions formulas were stored at room temperature, 25°C until used.

Size and morphological characters of nanoemulsion droplets using transmission electron microscope: The particle size analysis and morphology was determined using Transmission Electron Microscope at King Abdulaziz University, Faculty of Science. Three different diameters of each particle were determined and mean value was calculated and standard deviation (SD) was determined.

Preculture Preparation of Fungi

A suspension of fungi conidia for use as inoculum was prepared and the fungus was cultured on PDA slants for 7 d at 25, 28, and 30°C, by which time good levels of sporulation were observed. The conidia were harvested, suspended homogeneously in sterile distilled water containing 0.1% Tween 80, counted with a haemacytometer and diluted to achieve a concentration of 10^6conidia/ml for use as inoculum [20].

Antifungal Activity of Plant Oils and Nanoemulsions

Antifungal activity was determined by Agar well diffusion assay according to [21]. 15 ml of sterilized PDA medium was poured into each petri plate (90 mm diameter) and allowed to solidify. The plate were incubated with freshly prepared inoculums which were swabbed over the entire surface of the medium, rotating the plate 60 degree after each application by using sterile cotton swab, to ensure the spread of the tested fungus on the surface of the plate completely. One well of 6 mm diameter in the center of each plate was carried with the help of sterile cork-borer and 100 µl of different nanoemulsions of Clove oil, Basil oil and, Sunflower oil were used to fill each well with the help of micropipette. Sterile distilled water used as control. The inculated plates were left for 45 min. at room temperature to allow proper diffusion of the nanoemulsions to the medium. All plates were incubated at 25C° for 72 hr and the inhibition zones were measured (mm) at 3-equidistant points taken from the center of the inhibition zone and the average value was calculated. All experiments were carried out in triplicate and the reported data represents average values ± SD.

Determination of Minimum Inhibitory Concentration of the Test Fungi

The MIC for nanoemulsions clove oil was calculated using agar dilution method described by with some modifications [22]. About 500 µl of the nanoemulsions of clove oil are added to sterile tested tube containing 500 µl of distilled water and freshly prepared 20 µl of 10^6 spore/ml of the preculture. It was added and the number of living cells was counted on agar plate containing 10 ml of PDA medium and the plates were incubated at 28°C for 72hr to allow fungal growth. The developed colonies were counted and mean numbers were calculated. The MIC was determined as the lowest concentration of NE which developed no growth or small numbers colonies [22]. For every experiment, the control contained only sterile distilled water and the inoculums without nanoemulsions of clove oil. All experiments were carried out in triplicate.

Effect of the Nanoemulsions on Morphology of the Toxic Fungi

A. flavus and *A. parasiticus* were cultivated on potato dextrose broth at 28°C for 4 days. Then we were taken 2 ml of potato dextrose broth which containing preculture plus 400µl of nanoemulsion as treated samples and 400 µl of sterile distilled water as a control samples and we left all sample 2 hr. After centrifugation at 5000 rpm for 15 min. the cell was collected and stained using acridine orange (400 µmol L-1 acridine orange in 0.1 mol/l Na phosphate buffer, pH 7.0) according to the method described by [23]. After 2 min, the dye solution was removed by washing with distilled water and the samples were allowed to dry again at room temperature. The green and red fluorescence was visualized using a fluorescence microscope (Leica DM LB, Germany) at King Fahad Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia.

Statistical Analysis

All data were expressed as mean ± standard deviation (±SD). Statistical analysis was performed with two-way analysis of variance (ANOVA) and t-test. The statistical significance difference was considered when p-value≤0.05.

Results

Nanoemulsions from plant oils (Clove, Basil and Sunflower) were prepared using the High Pressure Homogenization (HPH) technique. The droplets morphologies for all of the nanoemulsions formulations were spherical and normally distributed (Figure 1).

Transmission Electron Microscope was used to determine size, shape and distribution. As shown in Figure 1 the droplets morphologies for all of the nanoemulsions formulations were spherical and normally distributed. In order to assess the droplet sizes trend and their distribution, approximate measurements of their sizes and coefficient of variation percentages (% VC), were calculated through dividing the standard deviation by the mean of six replicates of the droplet sizes as determined and demonstrated in Table 1.

According to Table 1, the variation coefficient percentages (% VC), was calculated through dividing the standard deviation by the mean diameter of three replicates of the droplet. % VC were 9.803%, 14.27% and 20% for nanoemulsions prepared from Clove, Basil and Sunflower. This meaning that the droplet was good distributed. The mean droplets size for all formulations was found in the range 10.52-100 nm. The three nanoemulsions of Clove, Basil and Sunflower have droplet size of 89.99, 23.33±3.33 and 13.15±2.630, respectively. According to the statistical analyses, which were based on measuring the p-value using the t-test that compare between two independent groups with unequal variances, it has been found that there were significant differences in droplets sizes of different formulations of the nanoemulsion.

Three nanoemulsions oils (sunflower, and basil) were tested against different fungi. The tested fungi were *A. flavus, A. Parasiticus, A. terreus* and *P. citrinum.* The effect of the three nanoemulsions on all tested fungi was studied. Very weak fungicidal activity was recorded for some the tested fungi (Table 2 and Figure 2). The color of some the fungi was changed by the presence of nanoemulsions in the medium.

From Table 3, the result showed that using oil nanoemulsions with high concentration had good effect on some tested fungi. Overall Clove oil nanoemulsions have the greatest effect by 14.1, 15.1 and 17.8 mm

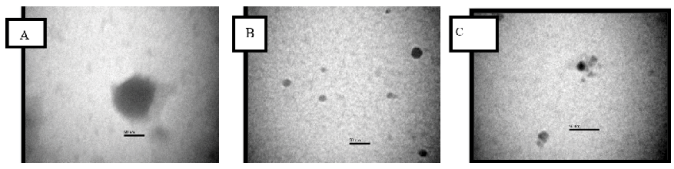

Figure 1: Transmission Electron Microscope (TEM) of the thee NEs showing diameter of the droplet, A: clove, B: basil and C: sunflower.

Table 1: Droplet diameter sizes and variation coefficient of NE formulations.

NE	Droplet diameter (nm)	Mean of Droplet diameter (nm)	Variation coefficient (% VC)
NE clove	83.66 -100.33	89.99±8.82a	9.80%
NE basil	20.66 - 23.33	23.33±3.33bc	14.27%
NE sunflower	10.78-13.15	13.15±2.63c	20%

Where: * Significant difference at P-value ≤ 0.05, ND: no inhibition zone

Table 2: Antifungal activity of clove, basil oil and sunflower oils on some toxigenic and non-toxigenic fungi using diffusion assay method.

Fungi	Mean diameter of inhibition zone (mm) ± (SD)			
Toxigenic fungi	**Clove oil**	**Basil oil**	**Sunflower oil**	**DMSO**
Aspergillus flavus	7±0.000	7±0.000	ND	ND
Aspergillus parasiticus	7.5±0.055	7.5±0.055	ND	ND
Non-toxigenic fungi				
Aspergillus terreus	ND	ND	ND	ND
Penicillium citrinum	ND	ND	ND	ND
Statistical analysis	Sum of Squares	Mean Square	F	Sig.
Fungi type	0.015	0.015	10	0.005*
Oil type	0	0	0	1
Fungi x Oil	0	0	0	1

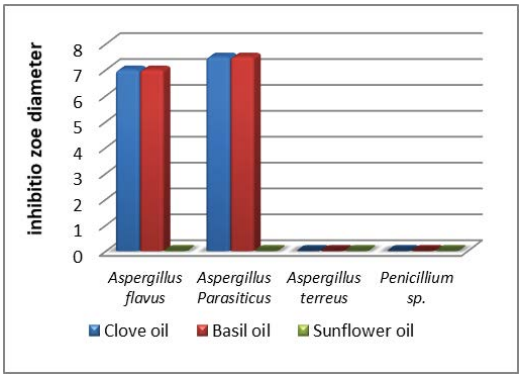

Figure 2: The antimicrobial activity of clove, basil oil and sunflower oils against the tested fungi.

Table 3: Antifungal activity of clove, basil oil and sunflower oils against some toxigenic and non-toxigenic fungi.

The tested fungi	Mean diameter of inhibition zone ± SD (mm)			
Toxigenic fungi	**Clove oil**	**Basil oil**	**Sunflower oil**	**DMSO**
Aspergillus flavus	14.1±0.041	10.5±0.055	11.1±0.117	ND
Aspergillus parasiticus	15.1±0.041	11.8±0.075	ND	ND
Non-toxigenic fungi				
Aspergillus terreus	17.8±0.240	ND	7.8±0.232	ND
Penicillium citrinum	ND	ND	ND	ND
Statistical analysis	**Sum of Squares**	**Mean Square**	**F**	**Sig.**
Fungi type	0.013	0.007	0.341	0.714
Oil type	2.987	1.493	76.302	0.000*
Fungi x Oil	0.804	0.402	20.552	0.000*

Where: * Significant difference at P-value ≤ 0.05, ND: no inhibition zone

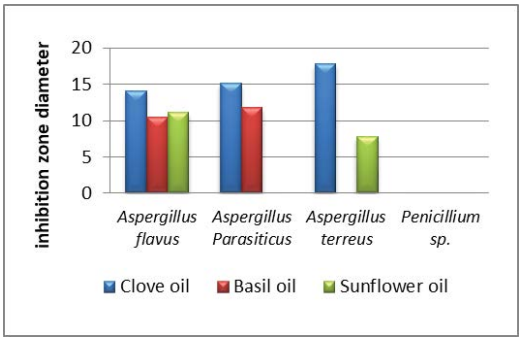

Figure 3: The antimicrobial activity of clove, basil oil and sunflower oil nanoemulsions against the tested fungi.

on *A. flavus*, *A. parasiticus* and *A. terreus,* respectively while *P. citrinum* showed no inhibitory effect (Figure 3 and Figure 4).

In the other hand, the basil oil nanoemulsions, the diameter of the inhibition zones were 10.5 and 11.8 mm for *A. flavus* and *A. parasiticus* respectively, but *A. terreus* and *P. citrinum* showed no inhibitory effect (Figure 3). Finally, it was found that the Sun flower oil nanoemulsions, the diameter of the inhibition zones were 11.1 and 7.8 mm for *A. flavus* and *A. terreus,* respectively but the tested solutions showed no inhibitory effect on *A. parasiticus* and *P. citrinum* (Figure 3). The obtained inhibition zone of the colve oil nanoemulsion on *A. parasiticus* was shown in Figure 4.

MIC for nanoemulsions clove oil was determined for the tested fungi, *A. flavus* and *A. parasiticus* using PDA agar dilution method.

Different concentration of nanoemulsions clove oil was added to the agar media. After incubated, numbers of colonies was calculated for each plate. MIC was determined as the lowest concentration which gave no growth or very small numbers of colonies. The calculated MICs of clove oil nanoemulsion for *A. flavus* was 0.02 (Figure 5) and the calculated MIC for *A. parasiticus* was 0.03% (Figure 6).

Both *A. flavus* and *A. parasiticus* were grown in PDA broth medium with or without the nanoemulsions of clove oil. The Aflatoxins were extracted and detected on TLC. Then, TLC was examined for aflatoxins which were detected using UV at 254 nm as shown in Figures 7 and 8. In the case of control, aflatoxins were detected at high concentration, which appeared as dark color. In case of fungal treatment with the nanoemulsion of clove oil, the result showed that the color was pale which mean low quantity of aflatoxins. The effect of clove oil nanoemulsions on the production of Aflatoxin B1 and G1 by *A. parasiticus* was determined. Figure 8 showed the reduction in Aflatoxin B1 and G1 quantities that are produced by *A. parasiticus* using clove nanoemulsions compared to control.

Treated and untreated mycelia of *A. flavus* and *A. Parasiticus* were stained and examined under fluorescence microscope. Figure 9 and Figure 10 showed normal live bright red-stained control mycelia of the both fungi compared to treated mycelia with clove oil nanoemulsion which appeared dark no cell content inside the mycelium.

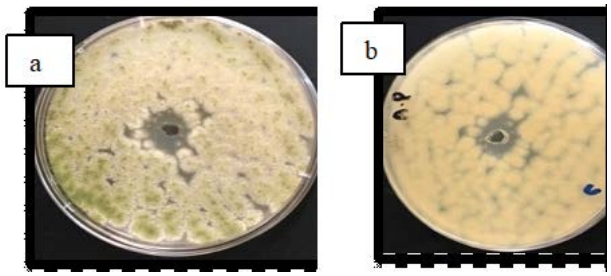

Figure 4: The antimicrobial activity of Clove oil nanoemulsions against *Aspergillus parasiticus* using well diffusion method, a: surface view and B: bottom view.

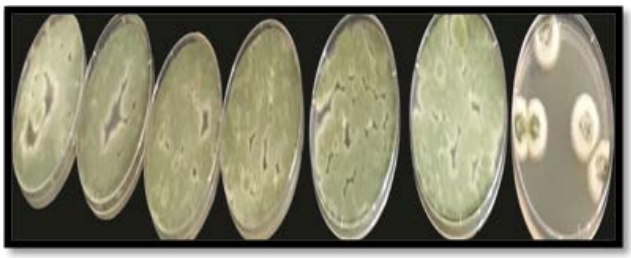

Figure 5: Minimum inhibitory concentration of nanoemulsions clove oil for *Aspergillus flavus*.

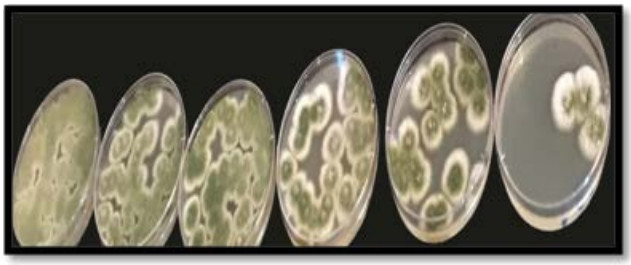

Figure 6: Minimum inhibitory concentration of nanoemulsions clove oil for *Aspergillus parasiticus*.

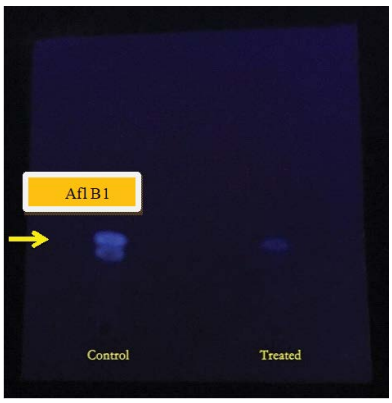

Figure 7: The effect of nanoemulsions clove oil on Aflatoxin B1 from *Aspergillus flavus* compared with control (without nanoemulsions clove oil).

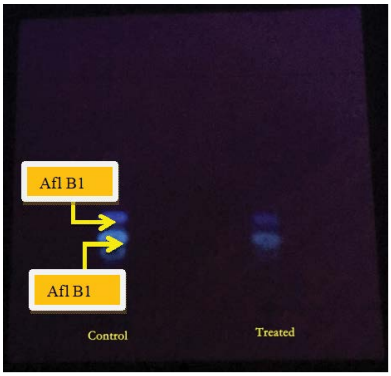

Figure 8: The effect of nanoemulsions clove oil on Aflatoxin B1 and G1 from *Aspergillus parasiticus* compared with control (without nanoemulsions clove oil).

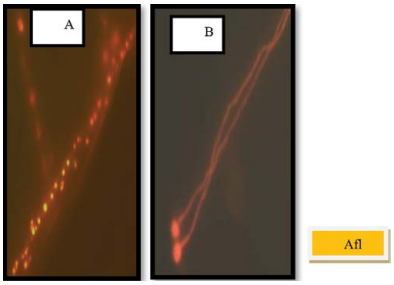

Figure 9: Parasiticus stained by Acridine orange at 60 X magnification, A: untreated mycelia of A. parasiticus, B: treated mycelia with clove oil NE.

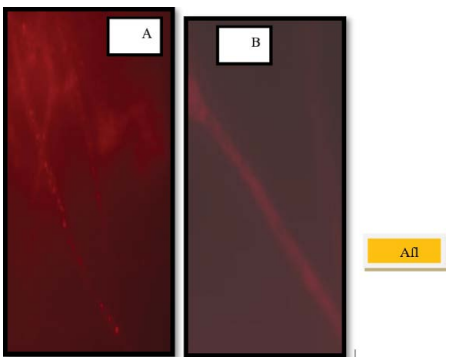

Figure 10: A: *A. flavus* (control) stained by Acridine orange at 60X magnification B: *A. flavus* treated with NE of clove and stained by Acridine orange at 60X magnification.

Discussion

Presence of fungi and their toxic metabolites (mycotoxin) in food for example dried fruits and nuts is virtually inevitable particularly in developing areas. Mycotoxins are a group of toxic fungal secondary metabolites which can contaminate agricultural products under pre- and postharvest conditions. They can cause acute or chronic toxic effects such as carcinogenic, mutagenic, teratogenic, atherogenic and oestrogenic effects in human and animals [24]. Mycotoxins affect food quality, resulting in huge economic losses in addition to being hazardous to consumer health for producing countries [25].

The numbers of microorganisms on most dried fruits vary from a few hundred per gram of fruits to thousands and they are mostly on the outer surfaces. Spores of bacteria and moulds are likely to be the most numerous. When part of the fruit has supported growth and sporulation of mould before or after drying, mould spores may be present in large numbers. If drying trays are not clean and improperly loaded, a marked increase in the numbers of bacteria and fungi may take place during the drying process. Spoilage of most dry fruits usually occurs during storage, handling and transport [26]. Different fungi like *Aspergillus*, *Penicillium*, *Rhizopus*, *Mucor*, *Alternaria* and *Paecilomyces* usually contaminate dried fruit and nut samples [27-29].

Currently, there is a growing interest in the utilization of new preservative methods that are from natural origin. Essential Oils (EOs) are natural compounds that have been shown promising treatment for food application because of its strong antifungal, antiviral, and antibacterial activities [6,30]. Essential oils are volatile, natural, aromatic oily liquids that can be obtained from several parts of the plants especially the aerial ones as leaves and flowers [31]. Although essential oils have been shown to be a promising alternative to chemical preservatives against foodborne pathogens. They present special limitations that preclude its use in food products. Low water solubility, high volatility, and strong odor of EOs are the main properties that make it difficult for food application [32].It is also a big challenge to incorporate oil-based compounds in aqueous food products because it shows physical and chemical instability when it is applied in food systems [33]. However, several studies have shown that the use of nanoemulsions can be a great choice for application of EOs in food matrix [34].

Nanoemulsions are dispersions of nano-scale droplets formed by shear-induced rupturing. Nanoemulsions are defined as O/W (oil in water) or W/O (water in oil) emulsion producing a transparent product that has a droplet size from 20-200 nm and does not have the propensity to coalesce. Nanoemulsions have many interesting physical properties that are different from or are more extreme than those of micro-scale emulsions. Nanoemulsions appear visibly different from micro-scale emulsions since the droplets can be much smaller than optical wave lengths of the visible spectrum. So nanoemulsions can appear nearly transparent in the visible spectrum and exhibit very little scattering [35]. In the present study, the oil in water (O/W) nanoemulsions from plant oils (clove, basil and sunflower) were prepared by using the High Pressure Homogenization technique [19]. Similarly, nanoemulsion containing tween 20 (0.5%) as an emulsifier, *Achillea* oils and water prepared by [36,19]. Also, the nanoemulsion containing Neem oil, Tween 20 and distillated water was successfully optimized by the high-energy method prepared, with mean droplet size of 67.85 nm [37]. The droplet size increases as the amount of water increases and decreases with the amount of surfactant due to the increase in interfacial area and the decrease in the interfacial tension [38]. It can be concluded that the amounts of components used in the preparation of NEs affects the droplet size of the nanoparticles.

The three NEs of clove, basil and sunflower were examined under transmission electron microscope. Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts [39,40].

In this study, the three formulated NEs of clove, basil and sunflower oils were tested on two toxigenic fungi, *A. flavus* and *A. parasiticus* by using different methods for evaluating antimicrobial activity. Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants or microbial extracts [41]. Another method to determine the antimicrobial activity of NEs was minimum inhibitory concentrations which is a quantitative method and considered as the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing [42].

Agar well diffusion method was assessed as mentioned in our result, it was observed that NEs showed weak activity on the tested fungi in compare with the control that showed excellent activity, this indicated that tested fungi maybe resistant or intermediately resistant to NEs compared to control, or because of other factors that affected the test such as agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria [42] while MIC method was assessed as mentioned in our result our result revealed that the NEs of colve oil had inhibitory effects against all the tested fungi, *A. flavus* and *A. parasiticus*.

Many researches showed that microemulsions and nanoemulsions may have antimicrobial properties. A soybean oil based nanoemulsion which extensive data have been reported, has been shown to have bactericidal properties against Gram-positive but not against enteric Gram-negative species [43]. It is sporicidal, in dilutions up to 1:1000 and has antiviral properties on enveloped viruses and it is fungistatic, but not fungicidal [44-46].

Despite the significant interest in the use of EO for food preservation against spore-forming microorganism, only sparse data are available. For example, eugenol nanoemulsions demonstrated to be effective in the inhibition of *Fusarium oxysporum* [47]. Eugenol oil nanoemulsion inhibited not only radial growth, but also sporulation and pigmentation of some pathogens. The mycelial tips were also swelled, branched and distorted.

Clove and eugenol oil showed good potential to inhibit growth of *A. niger*, *Penicillium sp*., and *Rhizopus sp*. on media. Eugenol, a main component of clove oil, was proposed as the agent responsible for clove oil's antifungal activity against a wide range of plant pathogens [48,49].

Antimicrobial nanoemulsions are highly stable oil-in water emulsions composed of nanometer-sized, positively charged droplets that have broad-spectrum activity against enveloped viruses, fungi, and bacteria [45,50, and 51]. Nano biocide a product prepared by mixing several bio-based chemicals was reported to eliminate the causal agent of rice blast disease [51].

Peppermint oil nanoemulsions stabilized with modified starch exhibited more effect on *Listeria monocytogenes* and *Staphylococcus aureus* [52]. Significant reduction of *Bacillus cereus* population was observed after treatment by cinnamon oil nanoemulsion formulated with Tween 80 prepared with ultrasonic emulsification [53].

Carvacrol and eugenol nanoemulsions were found less effective

than macroemulsions against *Escherichia coli* and *Listeria innocua* [54]. Thyme oil nano-emulsified by ionic antimicrobial surfactant lauricarginate and sodium dodecyl sulfate demonstrated reduced antimicrobial efficacy against four strains of acidi-resistant spoilage yeasts [55]. Specific mechanisms of antimicrobial activity of plant oils and their contents of monoterpenes are explicated in different ways. All associated with their lipophilic character that lead to accumulation and disruption of biomembranes; leakage of cellular components; alteration of fatty acids and phospholipids that lead to energy depletion; changes in the synthesis of DNA and RNA; and destruction of protein translocation [56]. Dilution methods are the most appropriate ones for the determination of MIC values. MIC for NE of Clove oil examined against *A. flavus* and *A. parasiticus* was 0.026 % and 0.03 % ml/ml, respectively. The X8W60PC nanoemulsion has great potential as a topical anti-fungal agent. Using MIC assay, 0.08% of the nanoemulsion was inhibitory to *C. albicans* and *C. parapsilosis* in addition to filamentous fungi [50].

Selective staining of cell walls with fluorescent dyes has been useful in morphological and developmental studies of fungi. Many studies have been reported in which acridine orange, a basic fluorescent dye was used in conjunction with fluorescent microscopy for the demonstration of *Candida* species [57]. In this study we utilized staining method described by [23]. The tested fungi are *A. flavus* and *A. parasiticus*. The treated and untreated mycelium was observed under using a fluorescence microscope. In the study of [58], stained *A. nidulans* with acridine orange was observed. The nucleus appeared as a green sphere containing a smaller red sphere; this distinct nuclear unit was readily distinguishable from all other cell constituents in the same fraction.

Other fluorescent, dyes like Calcofluor white and Fungiqual have also been used to demonstrate *Candida* though they have been shown to have nonspecific fluorescence and other artifacts [59]. AO fluorescence of fungi is due to nuclear binding of the organism [60]. The intensity of color depends upon the concentrations of nucleic acids.

Conclusion

Many fungi produce different types of myvotoxins that cause many health problems. Using essential oil or their nanoemulsions solutions inhibited both fungal growth and mycotoxis production in the growth medium. Thus, these materials can be used to preserve food from fungi infection.

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