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Suppression effect of thyme and carvacrol nano-emulsions on *Aspergillus fumigatus* isolated from patients in the intensive care unit of Assiut University Hospital, Egypt

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Abstract

Background and Aim: *Aspergillus fumigatus* is a zoonotic fungus that causes several diseases in humans ranging from allergic reaction to fatal disseminated invasive infection, especially in immunocompromised patients. This study aimed to investigate the incidence of invasive *A. fumigatus* in patients admitted to the intensive care unit (ICU) of Assiut University Hospital, highlight the factors associated with their infection, and determine the antifungal effect of thyme nano-emulsion (TNE) and carvacrol nano-emulsion (CNE) on isolated *A. fumigatus* strains.

Materials and Methods: Mycological culture method and scanning electron microscopy (SEM) were used in the identification of *A. fumigatus* in 630 blood samples collected from 210 patients. TNE and CNE at five concentrations (1%, 2%, 4%, 6%, and 8%) and average sizes of 90.3 and 75.6 nm, respectively, were characterized by transmission electron microscopy. Their effect on *A. fumigatus* isolate growth was evaluated by the well-diffusion method and SEM, which was used for the detection of the degenerative effect of *A. fumigatus* ultrastructure.

Results: *A. fumigatus* was detected in 54 of 210 (25.7%) patients in the ICU. Advanced age and chronic diseases were considered important risk factors for invasive aspergillosis, especially in patients with more than 1 clinical disease. TNE and CNE showed an inhibitory effect on *A. fumigatus* isolates, which significantly increased with high concentrations. The respective values for TNE at concentrations of 6% and 8% were 6±0.41 mm and 15±0.67 mm. CNE completely inhibited *A. fumigatus* growth at concentrations of 4%, 6%, and 8%, while mean inhibition zones of 22±0.68 mm and 30±0.32 mm appeared at concentrations of 1% and 2%. SEM demonstrated degenerative changes in *A. fumigatus* structure.

Conclusion: TNE and CNE can be used in bioactive treatments against *A. fumigatus*, and additional studies are required to determine the safe and effective doses and best method for application in human and veterinary medicine.

Keywords: *Aspergillus fumigatus*, carvacrol nano-emulsion, intensive care unit patients, invasive aspergillosis, scanning electron microscopy, thyme nano-emulsion.

Introduction

Aspergillus species are widely spread in soil in which they inhabit as saprophytes. They have the ability to infect several living hosts, such as insects, animals, birds, plants, and human, causing fatal disseminated diseases in both animals and human [1]. A wide range of clinical conditions are caused by aspergillosis infection, ranging from allergic reactions to disseminated invasive aspergillosis (IA), especially in immunocompromised patients. Recently, *Aspergillus fumigatus* is considered the most common cause of IA. It is ubiquitous in the environment with small spores, which enhance its penetration and colonization to airways, especially in patients with compromised lung mucosal defense. This allows its spread into other organs, causing hematogenous fungal diseases [2-4].

IA is an emerging disease in patients in the intensive care unit (ICU), even without displaying apparent immunodeficiency, with a mortality rate of >80%. Factors that enhance the development of IA are chronic obstructive pulmonary disease (COPD), long-term corticosteroid treatment, HIV infection, lung transplant, liver cirrhosis, and malnutrition [5].

Antifungal drugs have a low effect in IA, as they are degraded in the bloodstream and do not directly reach the infected organ. Therefore, using nanoparticles as a drug delivery system may increase its viability [6]. Recently, antifungal-resistant isolates developed; thus, several studies are important to find accurate drugs and discover new effective materials for the treatment of fungal diseases [7].

Essential oils are naturally volatile and non-volatile compounds produced by aromatic plants. Thyme oil resulted from extraction of *Thymus vulgaris* in which thymol is the main component. Carvacrol compound was found in some aromatic plants, such as oregano, wild bergamot, and pepperwort. It is displayed in several pharmaceutical and biological fields as antioxidant, antifungal, anti-inflammatory, antibacterial, and vasorelaxant agents. Therefore, they are recently applied in different fields, such as pharmaceutical industries, cosmetics, and food [8,9]. Essential oil utilization is limited as they are low water-soluble. This problem can be resolved by encapsulation of essential oils in oil/water emulsions or water/oil nano-emulsions [10].

Nano-emulsions with nanosize are stable against coalescence, creaming, and flocculation, which enable them to be used in food industry and delivery systems for drugs, vitamins, and flavors [11]. It enhances the bioavailability of bioactive and hydrophobic drugs, in which they are characterized by improper absorption and slow oral bioavailability [12]. Thus, several studies in the application of nano-emulsions in agriculture and nanomedicine are important to determine the most effective and safe treatment methods for human.

Therefore, this study aimed to investigate the incidence of *A. fumigatus* in patients in the ICU and use two natural compounds, namely, thyme nano-emulsion (TNE) and carvacrol nano-emulsion (CNE), as therapeutic agents against isolated strains of *A. fumigatus*.

Materials and Methods

Ethical approval and Informed consent

Ethical approval was obtained from our local ethical committee of Assiut University Hospital. Patients' participation was optional and collection of samples and data was done after their consent or their guardian consent.

Study period and location

This study was conducted from December 2019 to January 2021. All samples and data were collected from patients admitted to Assiut University Hospital. The samples were transferred in an insulated icebox to the Faculty of Veterinary Medicine, Sohag University, for the laboratory work.

Data and sample collection

As *A. fumigatus* existence in the blood is intermittent, 630 blood samples were collected from 210 patients in the ICU (three samples from each patient represented one sample) with chronic diseases, such as respiratory disease, renal failure, liver cirrhosis, infected vascular diseases, diabetes mellitus (DM), hepatitis C, and cardiac disease. Data were collected from patients by standard form, including age, sex, and medical history.

Mycological examination

Potato dextrose agar (PDA) with antibiotics was cultured by patient's blood samples for 5 days at 25°C. *A. fumigatus* was identified macroscopically and microscopically and then by scanning electron microscopy (SEM) (JEOL, JSM-5400LV, Japan) in Electron Microscope Unit of Assiut University [13].

TNE and CNE preparation

At room temperature (25ºC), Tween 80 2v/v% was dissolved in double-distilled water. Then, a magnetic stirrer (Daihan, Korea) was used in shaking the mixture for 10 min to obtain a homogenous solution. The essential oils were slowly added and mixed for 15 min using direct-driven stirrer. Subsequently, the resulting emulsions were sonicated with ultrasonic homogenizer (25 kHz, 650 W) [9]. The nano-emulsions were prepared in Nanotechnology Research Unit, Animal Health Research Institute, Assiut, Egypt. The size of both TNE and CNE was measured in the Electron Microscopy Unit, Assiut University, using TEM (Jeol, USA).

TNE and CNE effect

A. fumigatus isolates (n=54) were cultured for 6 days at 25°C in liquid potato dextrose medium; then, 1×10^6 CFU/mL was inoculated on PDA containing 50 µL of the prepared nano-emulsions with different concentrations (1%, 2%, 4%, 6%, and 8%) on each well and incubated at 25°C for 6 days. Nano-emulsion free plates of PDA were applied as control (Figure-1). The antifungal effect of nano-emulsions was detected by inhibition zone growth [14]. Then, SEM was used for the identification of nano-emulsions effect on the fungal ultrastructure.

Statistical analysis

SPSS 14 (IBM Corp., NY, USA) was used for describing the antimicrobial effect of TNE and CNE on *A. fumigatus* isolates by mean and standard error. A significant difference was indicated by $p<0.05$.

Results

A. fumigatus detection and risk factors

Of 210 patients in the ICU, 54 (25.7%) were positive for *A. fumigatus*. Patients aged >60 years

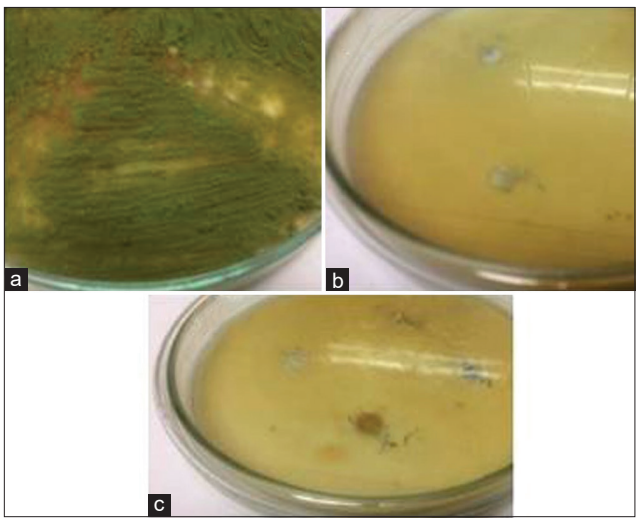

Figure-1: *Aspergillus fumigatus* in potato dextrose agar plates: (a) Positive control, (b) negative control for thyme nano-emulsion, (c) negative control for carvacrol nano-emulsion.

represented the highest infection rate (34.6%), followed by those aged 51-60 (23.8%), 41-50 (17.8%), and 30-40 (14.3%) years. The infection rates were 26.8% in men and 24.1% in women (Table-1). Based on the medical history of patients, the highest percentage of *A. fumigatus* was noted in patients with COPD, liver cirrhosis, and hepatitis C (42.1%), followed by patients with COPD, cardiac diseases, and DM (38.9%); respiratory and rheumatic heart diseases (37.9%); cardiac and vascular surgeries (35%); cardiac diseases and renal failure (29.4%); renal failure (19.2%); infected vascular surgeries (17.6%); and liver cirrhosis (11.8%). The lowest result (10%) was reported in patients with cardiac diseases (Table-2).

TNE and CNE effect

TNE has an inhibitory effect on *A. fumigatus* at concentrations of 6% and 8%, with mean inhibition zones of 6±0.41 mm and 15±0.67 mm, respectively. Complete resistance was detected at concentrations of 1%, 2%, and 4%. Moreover, CNE represented inhibitory effect on fungal growth at concentrations of 1% and 2% with mean inhibition zone of 22±0.68 mm and 30±0.32 mm, respectively. Complete suppression of *A. fumigatus* growth was reported in concentrations of 4, 6, and 8% (Table-3). SEM described the damage of *A. fumigatus* ultrastructure due to the effect of TNE and CNE (Figure-2).

Table-1: Incidence of *Aspergillus fumigatus* among intensive care unit patients.

Patients characteristics	Blood samples n=210		Patients with *Aspergillus fumigatus* n=54	
	n	%	n	%
Age				
30-40	21	10	3	14.3
41-50	45	21.4	8	17.8
51-60	63	30	15	23.8
>60	81	38.6	28	34.6
Gender				
Male	127	60.5	34	26.8
Female	83	39.5	20	24.1

Table-2: Medical history of *Aspergillus fumigatus* patients.

Medical history	Blood samples n=210		Patients with *Aspergillus fumigatus* n=54	
	n	%	n	%
Infected vascular surgeries	34	16.2	6	17.6
Cardiac diseases	30	14.3	3	10
Renal failure	26	12.4	5	19.2
Liver cirrhosis	17	8.1	2	11.8
Respiratory diseases and rheumatic heart disease*	29	13.8	11	37.9
COPD, liver cirrhosis, and hepatitis C*	19	9.05	8	42.1
Cardiac and vascular surgeries*	20	9.5	7	35
Cardiac diseases and renal failure*	17	8.1	5	29.4
COPD, cardiac diseases, and DM*	18	8.6	7	38.9

*Patients have more than 1 disease, COPD=Chronic obstructive pulmonary disease, DM=Diabetes Mellitus

Discussion

Table-1 reveals that *A. fumigatus* was detected in the blood of patients in the ICU with percentage of 25.7%. Higher results were reported by Peláez *et al*. [15] and Steinmann *et al*. [16]. The lower results of blood culture may be attributed to that the blood culture only detected the existence fungal hyphae in blood circulation and, in case of IA, only the detached fungal hyphae that enter the bloodstream can be detected, so its presence in the circulation is intermittent [17].

A. fumigatus infection was increased in patients with old age and decreased in patients with younger age. It was detected with highest percentage in patients aged >60 years. Therefore, advanced age is considered an important factor in IA [18]. Patients with more than 1 disease represented the highest infection rate, especially those with respiratory diseases (Table-2). From our results, patients with COPD, liver cirrhosis, and hepatitis C represented the highest percentage of *A. fumigatus* (42.1%). According to Chen *et al*. [19], the incidence of IA was increased in decompensated liver cirrhosis, neutropenia, pulmonary diseases, and diabetes. The majority of patients received antibiotics and steroids in hospitals to prevent inflammation and infections, but they decrease human immune function and do not cover all pathogens; thus, patients are prone to several pathogens, such as *Aspergillus* species. Evidence of aspergillosis was increased with some risk factors, such as chronic lung diseases, liver failure, surgery, immaturity, and sepsis. Inhalation of *A. fumigatus* resulted in several diseases, depending on human immunological status, which varies from slight hypersensitivity to serious invasive infection in internal organs [1].

Normal air contains a high concentration of *Aspergillus* due to their high capacity of sporulation, resulting in conidia of small size (2-3 μm), which enables them to reach the lung parenchyma through

Table-3: Suppression effect of thyme and carvacrol nano-emulsions on *Aspergillus fumigatus* isolates.

Concentration	Inhibition zone (mm)		p-value
	Thyme nano-emulsion	Carvacrol nano-emulsion	
	Mean±SdE	Mean±SdE	
1%	NZ	22±0.68	0.05
2%	NZ	30±0.32	
4%	NZ	No growth	
6%	6±0.41	No growth	
8%	15±0.67	No growth	

Mean±SdE = Mean±standard error, *NZ = No zone formed

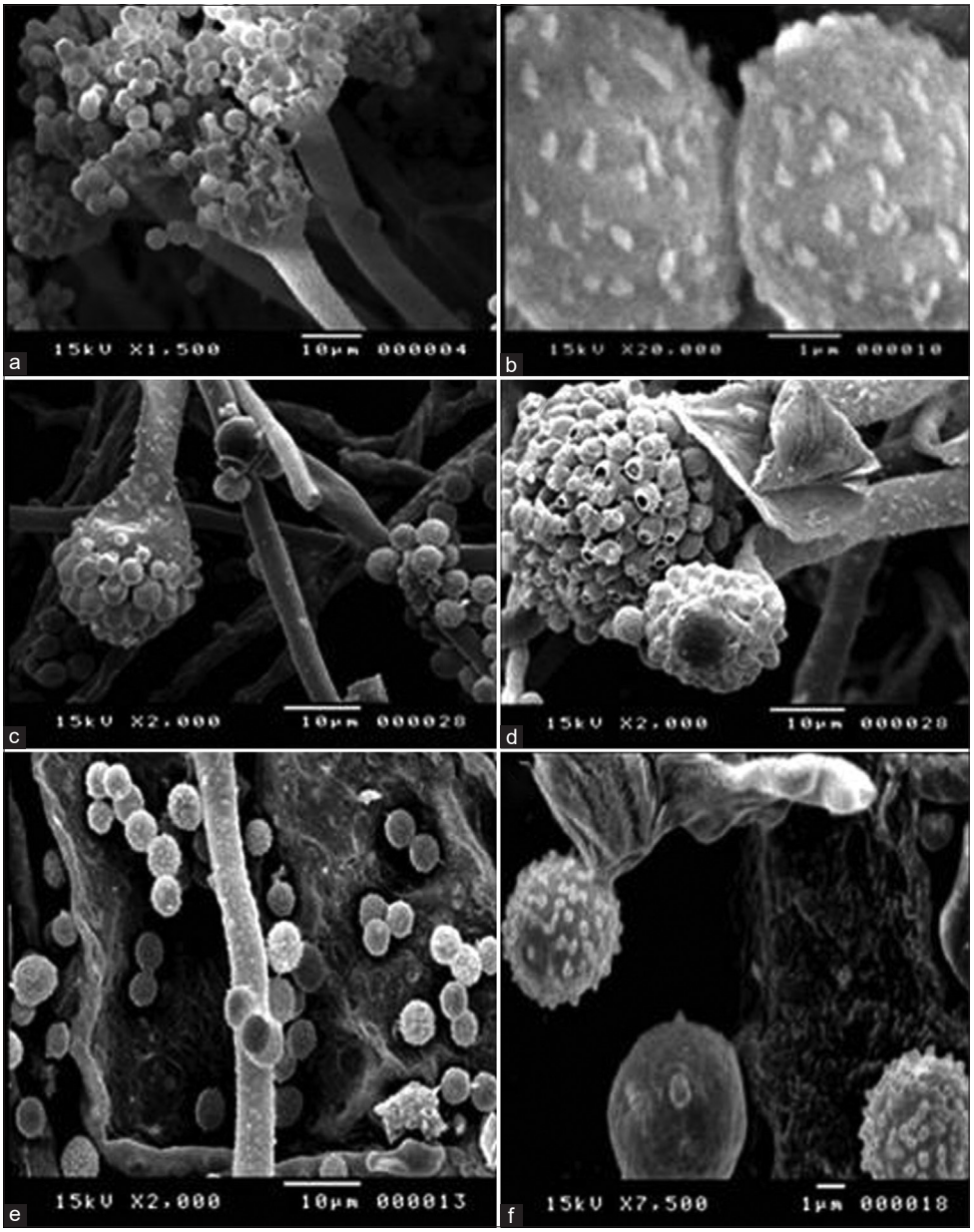

Figure-2: Scanning electron microscopy for (A and B) untreated *Aspergillus fumigatus*, (C and D) effect of thyme nano-emulsion at 6% on *A. fumigatus,* and (E and F) effect of CNE at 1% on *A. fumigatus*.

the airways [20]. Host defense against inhaled fungal spores acts through two lines: The first starts in the mucous layer and ciliary action of the respiratory tract, and the second comprises the phagocytic system of alveolar macrophage. Normally, macrophages, on identifying the beta-D-glucan of the fungal cell wall, produce inflammatory mediators, which stimulate neutrophils to start cellular immunity. In case of chronic diseases, immunosuppression resulted in neutrophil dysfunction and decreased its level. Moreover, *Aspergillus* secretes toxic metabolites, which interfere with macrophage action and neutrophil phagocytosis, such as mycotoxins, ochratoxins, and aflatoxins [21]. Therefore, disruption of host defense mechanism facilitates the attachment of the fungus into the lung parenchyma and dissemination into other organs with prolonged immunity impairment.

Nano-emulsions effect

Nano-emulsions have gained attention as antimicrobial agents, so they are applied in the delivery of drugs through the parenteral, oral, transdermal, and transmucosal routes and effectively enhance their bioavailability [22]. They are also used for delivering bioactive food ingredients, which facilitate their solubilization and physiochemical stability in gastrointestinal tract [12].

TNE with an average size of 90.3 nm and CNE with an average size of 75.6 nm (Figure-3) were used in five concentrations, and their impact is described in Table-3. CNE at 1% and 2% inhibited *A. fumigatus* growth, while complete suppression of growth was represented in concentrations of 4%, 6%, and 8%. However, TNE's inhibitory effect was observed only at concentrations of 6% and 8%. The effect of both nano-emulsions significantly increased with concentration ($p<0.05$).

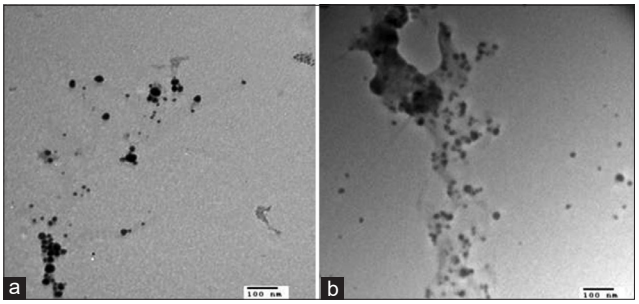

Figure-3: Transmission electron microscopy showing (A) thyme nano-emulsion with average size 90.3 nm, (B) carvacrol nano-emulsion with average size 75.6 nm.

The improvement of CNE formulation in drug delivery makes it more potent for the treatment of several diseases, because nanoformulation in liposomes can solve many problems of confrontation, such as solubility, bioavailability, and stability [23]. Moreover, CNE increases the capacity level of total antioxidant and prevents lipid peroxidation inside the cell [24].

The antimicrobial effect of volatile compounds may be attributed to their lipophilicity, as they can perforate the cell membrane, disrupt cell metabolism, and increase membrane fluidity and permeability. Moreover, they prohibit respiration, disrupt membrane protein function, induce ion leakage, and alter the ion transportation process in the fungal cell [25].

SEM was used to assess the effect of TNE and CNE on *A. fumigatus* ultrastructure by describing their morphology surface (Figure-2). The untreated fungi showed abundant growth and strong hyphal attachment with conidia. CNE at lower concentration (1%) resulted in changes in conidia structure with irregular, loose, and rough structure and distortion in its surface, damage of the fungal hyphae, and fallout of conidia. Treatment with TNE represented limited development and distortion of fungal hyphae. These results are in agreement with Nguefack *et al*. [26], who found complete inhibition of conidial germination and mycelial growth of *A. fumigatus* using different essential oils. Ergosterol is the main sterol present in the cytoplasmic membrane of *A. fumigatus*. The modifications in its morphology, which resulted from the effect of TNE and CNE, might be linked to the interference in cell wall synthesis, which affects fungal growth and morphology [27]. Therefore, nano-emulsions can be effective antifungal agents. They resulted in morphological deformation, collapse, and deterioration of fungal conidia and hyphae [28].

Conclusion

The presence of *A. fumigatus* in the blood of patients in the ICU may comprise invasive infection, especially in patients with advanced age and those with more than 1 disease. TNE and CNE as natural compounds represent the antifungal effect on *A. fumigatus* growth, but CNE has a suppression effect in low concentrations. Therefore, they can be used in various fields associated with medicine, such as antifungal and bioactive supplements.

Authors' Contributions

AAH: Conceived the idea and designed the study. RH: Collected data and samples. AAH: Analyzed the data and performed the laboratory work. WME: Prepared and characterized the nano-emulsions. AAH and RH: Drafted the manuscript. AAAH Supervised and revised the manuscript. All authors have approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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