Antimicrobial Activity of Different Essential Oils against *Aspergillus flavus* and *Klebsiella pneumoniae* Isolated from Respiratory Tract

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ESSENTIAL oils from *Cinnamomum verum* (Cinnamon), *Lavandula angustifoli* (Lavender), *Lepidium virginicum* (Peppergrass oil), *Zingiber officinale* (Ginger) and *Cinnamomum camphora* (Camphor) have been investigated for their antifungal and antibacterial activities against 6 fungal isolates of *Aspergillus flavus* and 25 bacterial isolates of *Klebsiella pneumoniae*. The sensitivity was determined using agar well diffusion method and the inhibition zones were compared with the standard drug itraconazole for fungi and Amikacine for tested bacteria. The tested essential oil showed a wide spectrum of inhibition against the tested isolates. Treating the *Klebsiella pneumoniae,* with cinnamon essential oil led to an external changes, irregular cell shape and disintegration of bacterial cell wall under transmission electron microscope. GC-MS technique was used for cinnamon essential oil; the composition of cinnamon oil was dominated by cinnamaldehyde. The *in vivo* efficacy of cinnamon essential oil in treatment of *Klebsiella pneumoniae* in mice lungs was proven.

Keywords: Essential oils, Antifungal, Antibacterial, GC-MS, TEM.

Introduction

The drug resistant pathogens are one of the most serious to successful treatment of microbial diseases. The use of medicinal plants became the first medicines is global phenomenon. In Nature, essential oils play an important role in the attraction of insects to promote the dispersion of pollens and seeds or to repel other ones. In addition, essential oils may also act as antifungal, antibacterial, antiviral, insecticides and herbicides (Bakkali et al., 2008). This search intends to respond to the increasing resistance of pathogenic microbes to antibiotics and there is increasing acquaintance acceptability of the use of herbal drugs in today's medical practice. There is no effective machinery to regulate manufacturing practices and quality standards (Kunle et al., 2012). It is important to find out the particular microorganisms for which the herbal extracts are active (Pesavento et al., 2015). Essential oils and other extracts of plants have evoked interest as sources of natural products concerning their potential uses as alternative remedies for the treatment of many infectious diseases (Nor et al., 2016). Essential oils possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties against microorganisms (Lakehal et al., 2016). Essential oil exhibited maximum inhibitory activity against respiratory disease causing microbes like *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, H*aemophilus influenzae*, and *P. aeruginosa* (Skocibu et al., 2004).

There is a belief that herbal medicines might be effective benefit in the treatment of certain diseases, that are free from side effects (Bouzidi et al., 2016). The detection of some of these biological properties needed for the survival of plants has also been the base for searching similar properties for the combat of several microorganisms responsible for some infectious diseases in humans and animals (Cornelia et al., 2017).

The objective of this study was to determine the antifungal and antibacterial effects of five plant essential oils against fungal and bacterial respiratory tract infections.

Materials and Methods

Test organisms

Six fungal isolates were obtained from patient had cough and breathing problems which had bronchial asthma. Aspergillus was isolated and maintained on Sabouraud Dextrose Agar (SDA) medium for 7 days at 28 ± 2°C with

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adding 0.05 g/L chloramphincol as antibacterial agent (Mahmoud et al., 2011). The isolate was identified microscopically under light microscope at Mag 40x according to Moubasher (1993). Pure cultures of 25 tested bacterial isolates used in the study were obtained from cultivation of medical specimen of sputum from patients admitted to Tanta University hospital, Egypt. These specimens were taken from the Central Lab. of the hospital. The specimen were cultivated on nutrient broth overnight at 37°C for 24 h, then subcultured on nutrient agar, macConkey agar and blood agar for another 24 h. The investigated isolates were identified morphologically and biochemically according to Bergey's Manual (Garrity, 2001). The bacterial isolate was maintained on nutrient agar slants and stored at 4ºC prior to use.

Molecular identification of the fungal isolate and accession number

DNA extraction

The mycelium of *Aspergillus* was scratched off the surface of 2% Potato Dextrose Agar PDA Petri plate. The mycelia (50 mg) were ground using a mortar and pestle. DNA was extracted from the powdered tissue using i-genomic DNA extraction Mini Kit (INTRON Biotechnology, Inc, Cat. No. 17371) according to manufacturer's instructions. The eluted DNA was stored at -20ºC (Denning et al., 1990).

PCR condition

Amplification of internal transcribed spacer (ITS) region was conducted in thermal cycler (C1000TM Thermal Cycler, Bio-RAD) using ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`GGAAGTAAAAGTCGTAACAAGG-3`) primers (White et al., 1990). The following parameters were used: 35 cycles of 94ºC for 30 sec, 51ºC for 1 min, 72ºC for 1.5 min, and a final extension at 72ºC for 3 min. Each PCR mixture (25 µl) as follow, (1 µl) of nucleic acid, 1 µl of each primer (10 pmol), (12.5 µl) of GoTag® Master Mix (Promega Corporation, USA) and 9.5 µl of nuclease free water (Promega). 15 µl of all PCR products were analyzed by electrophoresis through a 1% agarose gel, stained with ethidium bromide.

Sequence analysis of the ITS region

The amplified PCR amplicon was submitted by City of Scientific Research and Technology Applications, New Borg El Arab City, Alexandria, Egypt to Macrogen Company (Seoul, Korea) to be sequenced. The DNA nucleotide sequence was analyzed using DNA BLASTn (NCBI). Pair wise and multiple DNA sequence alignment were carried out using Clustal W (1.82) (Thompson et al., 1994).

Identification of the most sensitive bacterial isolate by using the Biomerieux VITEK® 2 system

The tested bacterium mostly affected by the cinnamon essential oil was cultured on the appropriate liquid nutritional medium and was incubated overnight at 37°C. Then the cultures were centrifuged at 3000 rpm for 20 min, washed with sterile saline solution and the turbidity of the bacterial suspensions was adjusted with a densitometer to match that of a McFarland 0.5 standard in 0.45% sterile sodium chloride solution, then the VITEK 2 cards were filled with bacterial suspension and manually loaded into the VITEK 2 system.

Essential oils

Five natural essential oils; Cinnamon, Pepper grass oil, Lavender, Camphor and Ginger were purchased from Captain Company in the local market and different dilutions of the oils were prepared (1:1, 1:3, 1:5 and 1:10 v/v) using Dimethyl sulphoxide (DMSO).

Antifungal and antibacterial susceptibility assay of isolated fungi and bacteria

Amphotericin B, itraconazole, Fluconazole, ketoconazole, Metronidazole and Nystatin were used as positive antifungal controls for fungal isolates. The plates were incubated at 25°C for 2-3 days. The results were expressed in terms of the diameters of inhibition zones and the mean were then calculated. All experiments were carried out in triplicates, and the mean of 3 readings was calculated. Antibiotics susceptibility was assessed using the disc diffusion method for all bacterial isolates as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2014) using fourty selected antibiotic discs (Bioanalyse, Turkey) from different classes of antibiotics. These antibiotics (µg/disc) were Imipenem (10), Clavunic acid (10), Amikacin (30), Streptomycin (10), Gentamicine (10), Chloramphenicol (30), Azithromycin (15), Cephradine (30), Cefaclor (30), Cefotaxime (30), Cefadroxil (30) Ceftazidime (30), Levofloxacine (5) and Amoxicillin (30). The test was carried out by placing 6 mm diameter of paper disc containing antibiotic onto a microbial inoculated plate. Bacteria were incubated at 30-37°C for 16-24 h and the inhibition zones were measured after incubation period. Each inhibition zone around the antibiotic disc was measured and values were interpreted as sensitive or resistant