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Inhibitory effects of rosemary (*Rosemarinus officinalis* L.) essential oil on pathogenicity of irradiated and non-irradiated *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa is considered one of the most important opportunistic pathogens, which is responsible for nosocomial infections. The complex pathophysiology of infections associated with *P. aeruginosa* is due to its ability to grow in a biofilm mass and to produce a large number of virulence factors. This study aimed to investigate the antipseudomonal efficacy of rosemary essential oil (REO) against 25 clinical isolates control and gamma irradiated (at 24.4 Gy). Susceptibility testing towards 15 standard antibiotics and REO was carried out using disc diffusion method. The potency of REO on motility, biofilm and pyocyanin production was evaluated. Total protein profile was estimated by SDS polyacrylamide gel electroporesis. REO has showed a broad spectrum of antimicrobial activity compared to the standard antibiotics. According to their high sensitivity to REO, six isolates were chosen for MIC_{90} with minimum inhibitory concentration (MIC_{90}) values ranged from 5 to 25 μl/ml. REO exerted marked effects on irradiated and non-irradiated tested isolates. The results displayed high significant reduction in biofilm and pyocyanin production. Significant reduction in cell-surface hydrophobicity was observed for some isolates. As for motility, REO altered twitching, swarming and swimming ability of the tested isolates. The ultra structure of bacterial cell results in ruptures of the bacterial cell, leakage of the cytoplasmic components and consequently cell death. Total protein analysis of control, irradiated and irradiated with treatment of REO revealed some changes in protein profile as a result of treatment. **Conclusion**: This study may hasten the application of REO in the treatment and prevention of pseudomonas resistant isolates in nosocomial infections.

1. Introduction

The increase of microbial resistance to antibiotics threatens the public health on a global scale as it reduces the effectiveness of treatments and increases morbidity, mortality and health care costs, especially in developing countries. Bacteria have the genetic ability to transmit and acquire resistance to drugs [1]. Consequently, common strategies adopted by pharmaceutical companies to supply the market with new antimicrobial drugs include changing the molecular structure of the existing medicines in order to make them more efficient [2]. Radiotherapy for malignant human neoplasms is relatively safe and effective form of treatment, but it may become limited due to its undesired side effects. These effects, as reduction in host defense mechanisms and changes in the flora, play a major role in radiation death [3]. Furthermore, the low doses of gamma irradiation had an effect on the resistance of some bacterial isolates to different antibiotics resulting in increasing difficulty in treating infections caused by these organisms [4].

The interest in medicinal plants has increased in the recent years, this interest has led to the discovery of new biologically active molecules by the pharmaceutical industry and the adoption of crude extracts of plants for self-medication by the general public. Essential oils are complex volatile compounds, naturally synthesized by various parts of the plant during its secondary metabolism. The presence of a large number of alkaloids, phenols, terpenes derivatives compounds and other antimicrobial compounds makes the essential oils more précised in their mode of action against the pathogenic microorganisms [5]. *Rosmarinus officinalis* L. (Lamiaceae), commonly known as rosemary, is widely used in folk medicine, cosmetics and phytopharmacy [6]. More than 30 compounds have been identified in the essential oil from *R. officinalis* (REO) [7].

Pseudomonas aeruginosa is a ubiquitous Gram-negative rod-shaped bacterium responsible for many infections among immunocompromised hosts, burned patients and individuals suffering from cystic fibrosis. Beside the well known swimming and twitching motilities, this bacterium is capable of another type of migration called swarming. This complex type of motility is usually defined as a rapid and coordinated translocation of a bacterial population across a semi-solid surface. Swarmed cells display enhanced resistance to a variety of antibiotics. Pyocyanin production and biofilm formation are also from the most important virulence factors of *P. aeruginosa*. There is a complicated relationship between swarming motility and biofilm development [8].

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The objective of this study is to evaluate the antibiotic profile of *P. aeruginosa* isolates from different clinical specimens and the potentiality of REO against some virulence factors for irradiated at a dose of 24.4 Gy and non-irradiated isolates as biofilm formation, motility and pyocyanin production. Also, this study determines which changes in protein synthesis could be detected when the cells were treated with essential oil.

2. Materials and methods

2.1. Bacterial isolates and antibiotics susceptibility test

Twenty five *P. aeruginosa* isolates were obtained over 3-month period from Specialized Ain-Shams Hospital, Cairo, Egypt, from various clinical specimens (ear, urine, skin, eye swab and pus samples). All isolates that were identified as *P. aeruginosa* on the basis of their typical colonial appearance, characteristic pigments, and citrate, oxidase, catalase and urease tests were positive. Finally the identification was carried out to species level data base (API 20 E from Biomerieux France). All *P. aeruginosa* isolates were tested for their susceptibilities to antibiotics by the disc diffusion agar method [9] in accordance with Clinical and Laboratory Standard Institute's recommendations [10]. The following antimicrobial disks (μg) (Oxoid) were used: fusidic acid (FD10), sulphamethoxazole-trimethoprim (SXT25), tobramycin (TOB10), polymyxin (PMX), ceftazidime (CFZ 30), tetracycline (TE30), nalidixic acid (NA), clarithromycin (CLAR), gentamicin (CN10), azithromycin (AZM15), teicoplanin (TEC30), ticarcillin/clavulanic acid (TIM85), cefepime (FEP 30), piperacillin tazobactam (TZP110) and imipenem (IPM10). Petri plates were prepared with 10 ml of sterile Mueller Hinton Agar. The tested cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. Then antibiotic discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. The plates were incubated for 24 h at 37 °C. Zones of inhibition were recorded in millimeters and the experiment was repeated in triplicate.

2.2. Antibacterial activity of REO

All selected isolates were used to estimate the antibacterial activity of REO using the disc diffusion technique [11]. Filter paper discs (Whatman No.1, 6 mm diameter) containing 20 μl of REO were applied to the surface of agar plates that were previously seeded by spreading of 0.1 ml (containing 4×10^5 cfu/ml) from overnight culture. Plates were incubated at 37 °C for 24 h. and the resulting inhibition zone was measured. All data collected for each assay are the averages of three determinations.

2.3. Minimal inhibitory concentration (MIC_{90}) determination

The highly sensitive isolates (six) towards REO were selected to estimate minimal inhibitory concentration of REO. MIC_{90} was defined as the lowest concentration of antimicrobial agent that inhibited 90% of bacterial growth, the 96-well micro-titre assay using resazurin as an indicator of cell growth was employed [12]. Under aseptic conditions; the tested oil was dissolved using dimethylsulfoxide (DMSO) and diluted from 2.5 to 1250 μl ml^{-1} with sterile distilled water, a volume of 100 μl of REO dilutions were pipetted into the first row of the plate. To all other wells, 50 μl of nutrient broth or normal saline was added. Serial dilutions were performed using a multichannel pipette. Tips were discarded after use that each well had 50 μl of the REO in serially descending concentrations. To each well, 10 μl of the resazurin indicator solution was added. Using a pipette nutrient broth was added to each well. Finally, 10 μl of bacterial suspension (2×10^5 cfu/ml) was added to each well. The plate was wrapped loosely with cling film to ensure that bacteria did not become dehydrated. The plate had a set of controls: a column with all solutions with the exception of the test compound, and a column with all solutions with the exception of the bacterial solution adding 10 μl of nutrient broth instead. The plates were prepared in triplicate, and placed in an incubator set at 37 °C for 18–24 h. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive indications. MIC_{90} was determined as the lowest oil concentration at which color change occurred. The average of the three values was calculated and was the MIC_{90} for the test material and bacterial strain.

2.4. Irradiation Process

This was done to study the antimicrobial activity of the tested oil against the tested pathogen causing infections in radiotherapy treated cancer patients. Clinical isolates, under study, were subjected to gamma irradiation at a dose level of 24.4 Gray (Gy) using ^{137}Cesium gamma cell, at the National Centre for Radiation Research and Technology (NCCRT), the Atomic Energy Authority, Egypt. The dose rate at the time of the experimentation was 0.751 rad/s. This dose is biologically equivalent to the fractionated multiple therapeutic dose given on daily fraction schedule used in treatment of cancer patients [13]. Each isolate was inoculated in 5 ml of Tryptone Soya broth medium and incubated at 32 °C for 24 h. The cell density was adjusted at 10^8 cells/ml. The cultures obtained were diluted in 20 ml fresh medium and incubated for 2–3 h, then divided under aseptic conditions into three groups. Group I exposed *in vitro* to 24.4 Gy gamma radiations, group II was subjected to treatment with REO and exposed to the same dose of radiation and was used immediately for different investigations and the third group remained as a control.

The following experiments were performed on the selected isolates (control, irradiated and treated with irradiated & REO).

2.5. Biofilm formation assay by (tissue culture plate method)

Biofilm formation was determined by using a spectrophotometric method described by Christensen *et al.* [14]. Briefly, stationery 18 h culture of the tested bacterial isolates in 5 ml of TSB were washed, diluted using fresh medium and standardized to contain about 10^5 (cfu/ml). Aliquots of 0.2 ml of the diluted cultures were added to the wells of sterile flat-bottom polystyrene tissue culture plates. Following 48 h of incubation at 37 °C, the contents of tissue culture plates were gently aspirated with a micropipette. The plates were then washed with sterile buffer. Adherent organisms were fixed by incubating them for 1 h. at 60 °C [15] and then staining them with crystal violet (1%) for 5 min. After washing using water to remove the excess strain, the plates were dried for 30 min at 37 °C. Then, after drying the optical densities (OD_{595}) of isolates, adherent biofilms were read with Microplate Reader-SunoStick SPR-960B at 595 nm. Adherence measurements were performed in quadruplicate and repeated at least three times, the values were then averaged.

2.6. The anti-biofilm activity of the tested oil

The anti-biofilm activity of REO was evaluated through testing its ability to prevent the bacterial adherence by spectrophotometric method as follows: In each well of tissue culture plate 150 μl of 18 h broth culture of the applied isolate and 50 μl of REO were mixed at the same time. In case of control wells, 50 μl of sterile buffer was used and proceed as described above.

2.7. Scanning electron microscope (SEM)

The biofilm formation of the selected *P. aeruginosa* (control and treated) was observed using SEM. The cultures were grown on glass cover slips. After 24 h of incubation, the cover slips were rinsed with distilled water to remove planktonic cells and processed for (SEM) examination as described by Nakamiya et al. [16].