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Zingiber officinale: Its antibacterial activity on *Pseudomonas aeruginosa* and mode of action evaluated by flow cytometry

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ABSTRACT

Biofilm formation, low membrane permeability and efflux activity developed by *Pseudomonas aeruginosa*, play an important role in the mechanism of infection and antimicrobial resistance. In the present study we evaluate the antibacterial effect of *Zingiber officinale* against multi-drug resistant strain of *P. aeruginosa*. The study explores antibacterial efficacy and time-kill study concomitantly the effect of herbal extract on bacterial cell physiology with the use of flow cytometry and inhibition of biofilm formation. *Z. officinale* was found to inhibit the growth of *P. aeruginosa,* significantly. A major decline in the Colony Forming Units was observed with 3 $\log_{10}$ at 12 h of treatment. Also it is found to affect the membrane integrity of the pathogen, as 70.06 ± 2.009% cells were found to stain with Propidium iodide. In case of efflux activity 86.9 ± 2.08% cells were found in Ethidium bromide positive region. Biofilm formation inhibition ability was found in the range of 68.13 ± 4.11% to 84.86 ± 2.02%. *Z.officinale* is effective for killing Multi-Drug Resistant *P. aeruginosa* clinical isolate by affecting the cellular physiology and inhibiting the biofilm formation.

1. Introduction

Pseudomonas aeruginosa, is a foremost pathogen of nosocomial and community acquired infections and is demonstrating a jeopardize epitome for immunocompromised individuals [1,2]. The pathogen by different means including low permeability of outer membrane, efflux activity and by biofilm production presents a serious therapeutic challenge [3–5]. Under harsh environment the bacteria construct phenotypically altered biofilm which is engineered to resist these conditions more efficiently. As initial empiric treatment of pseudomonal infections, beta-lactam antibiotic with an aminoglycoside is recommended to the patient [6]. But, due to their innate and progressively developed capability of antimicrobial resistance, the condition has become difficult and the eradication of this notorious pathogen has turned into a tedious task [7]. The condition necessitates the development of effective, safe and cost worthy drugs.

Bioactive phytoconstituents including, phenols, phenolics, alkaloids, tannins, terpenoids, essential oils and polypeptides are mainly responsible for the medicinal activity. Extraction of medicinally active ingredient from different part of plants is based on solvent selection hence natural plant product preparation is a peculiar step in the herbal based medicine production [8]. Further, screening of herbal extract against the pathogen may provide a potent lead against the stubborn microbial threat.

Earlier, we have selected *Zingiber officinale* as a probable agent against Multi-drug resistant *P. aeruginosa,* using *in silico* bioprospection study of 38 plants [9]. A broad range of biodynamic activities of *Z. officinale* with anti-oxidant, immunomodulatory, anti–inflammatory, antirheumatic, antihepatotoxic etc. are known [10–12]. The aim of the present study was to investigate the antibacterial efficacy of methanolic extract of *Z. officinale*.

2. Methods

2.1. Plant material

The rhizome extract of *Z. officinale* (ginger) was prepared by hot continuous percolation method using aquo-alcoholic solvent

Abbreviations: ICU, intensive care unit; MIC, minimum inhibitory concentration; HCl, hydrochloric acid; $AlCl_3$, aluminium chloride; TBA, thiobarbituric acid; SDS, sodium dodecyl sulfate; FTIR, fourier transform infrared; TA, tannic acid; NCDC, national centre for disease control; PBS, phosphate buffer saline; PI, propidium iodide; EtBr, ethidium bromide; LB, luria broth.

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system (70: 30; Methanol: Water) and stored in cryovials at 4 °C.

2.2. FTIR spectroscopic analysis of extract

Spectrum was obtained with the help of OMNI- sampler attenuated total reflectance accessory on a Nicolet FTIR spectrophotometer (Thermoscientific Nicolet is10, USA). A small amount of extract (2 mg) was placed directly on the germanium piece of the infrared spectrometer and the data was collected over the wave number ranged from 4000 to 500 cm^{-1} and analyzed by using Omnic software (version 5.2) [13].

2.3. Qualitative analysis of phytoconstituents

Different tests were performed to determine the presence of different phytoconstituents. Dragendroff test was used for alkaloids, Alkaline reagent test was followed for flavanoids, Lead acetate test for Tannins, and Biuret test for Proteins [14–16].

2.4. Quantitative estimation of tannin

About 0.01 ml of extract was added to 0.75 ml of distilled water followed by 0.05 ml Folin- Ciocalteau reagent, 0.1 ml of 35% sodium carbonate and 0.1 ml of distilled water and incubated at room temperature for 30 min. A set of reference standard solutions of tannic acid were prepared in the same manner as described above. Absorbance was recorded at 725 nm using UV visible spectrophotometer (Electronics Corporation of India Ltd, Hyderabad, India). The tannin content was expressed in terms of mg of TA/g of extract [17].

2.5. Quantitative estimation of alkaloid by using harborne method

0.5 g extract was dissolved in 20 ml of 10% acetic acid and concentrated upto one quarter of the initial volume on water bath at 50–55 °C. Concentrated ammonium hydroxide was added drop wise to settle the precipitate. The precipitate was washed with dilute ammonium hydroxide. The residue is the alkaloid present in the extract which was dried and weighed [18].

2.6. Anti-lipid peroxidation assay using TBARS (thiobarbituric acid-reactive substances)

Egg yolk (100 μl) and 50 μl of extract or standard Ascorbic acid (10, 25, 50, 75, 100 μg/ml) were added. 300 μl of 20% acetic acid and 300 μl of 0.8% TBA in 1.1% SDS were added. The resulting mixture was vortexed and heated for 1 h at 90 °C. After cooling at room temperature, 750 μl of butanol was added to each tube, the mixture then centrifuged at 3000 rpm for 10 min [19]. The absorbance of upper organic layer was measured at 532 nm using UV visible spectrophotometer. The anti-lipid peroxidation is calculated in terms of %inhibition and is calculated by using the formula:

$$\% \text{ Inhibition} = 1 - \frac{T}{C} \times 100$$

where, C: absorbance value of the fully oxidized control; T: absorbance of the test sample.

2.7. Hydrogen peroxide scavenging activity

The hydrogen peroxide radical scavenging activity was estimated by using by the modified method described by Nabavi et al. (2008) [20]. A solution of hydrogen peroxide (4 mM) in 0.1M PBS (pH 7.4) was prepared. 4 ml of extract at different concentration (5, 10, 25, 50, 75, 100, 125 μg/ml) was added to 0.6 ml of hydrogen peroxide solution. The mixture was incubated at room temperature for 10 min and the absorbance was taken at 230 nm. The percentage of hydrogen peroxide scavenging by the extract and gallic acid (standard) was calculated as follows:

$$\% \text{ Scavenged} = \frac{Ao - A1}{Ao} \times 100$$

where, A_0: absorbance of the control; A1: absorbance of test sample(extract or standard.

2.8. Nitric oxide radical scavenging activity

The nitric oxide radical scavenging activity was determined using the method of Ebrahimzadeh et al. (2008). One ml of Sodium nitroprusside (5 mM in PBS with pH 7.4) was added to 3 ml of the different concentration of extract (5, 10, 25, 50, 75, 100, 125 μg/ml) and incubated for 190 min. After incubation 0.5 ml of Griess reagent was added and the absorbance was observed at 546 nm [21]. Gallic acid was used as standard. The percentage inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{Ao - A1}{Ao} \times 100$$

where, A_0: absorbance of the control; A1: absorbance of test sample (extract or standard.

2.9. Bacterial strain

P. aeruginosa isolated from clinical sample in Department of Microbiology, National Centre for Disease Control (NCDC), New Delhi, India. The strain was resistant to colistin, clindamycin, ceftazidime and piperacillin. The culture was maintained on Mueller Hinton agar plate incubated at 37 °C.

2.10. Determination of antimicrobial activity

The antimicrobial effect of extract of *Z. officinale* was studied by using agar well diffusion method. To prepare inoculums the bacterial density was adjusted to 10^6 CFU/ml in peptone water and diluted by 100 times to prepare working inoculum. The working inoculum was evenly distributed on Mueller Hinton agar plate and wells were made. The test broth solution containing variable concentration of extract *i.e.,* 50, 100, 150 and 200 μg/ml was added to the well. The control used was amikacin with similar concentration range of the extract. The results were measured in mm after 24 h incubation at 37 °C [13].

2.11. Minimum inhibitory concentration

The test broth solutions containing variable concentrations of testing samples (extract and amikacin) separately (1, 2.5, 5, 10, 25, 50, 100, and 200 μg/ml), were inoculated with inoculum (10^6 CFU/ml) in microtitre plate followed by incubation at 37 °C for 24 h. The MIC was evaluated by recording Optical density (OD) at 600 nm, after 24 h. The colony count was also evaluated on MacConkey agar plate (by seeding 100 μl of test culture with different concentration of extract) after 24 h of incubation to determine minimum inhibitory concentration [13].

2.12. Time-kill study

Time-kill study was conducted with twice of MIC, obtained in