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## Efficacy of methanol extract of *Zingiber officinale* rhizome against acute pneumonia caused by *Pseudomonas aeruginosa*

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### Article Info

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#### Keywords

*Pseudomonas aeruginosa*

Lung infection

Liquid chromatography mass spectrometry

Toxicity

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Radiology

### ABSTRACT

*Pseudomonas aeruginosa* has become one of the major threats to public health as it causes serious diseases like pneumonia and the condition becomes sever if the disease is caused by drug-resistant strain. Thus, in the present study, the antibacterial activity of *Zingiber officinale* against *P. aeruginosa* causing a lung infection in Swiss albino mice was evaluated. Disease analysis includes bacteremia, x-rays based observation of patches formation in lungs, and destruction of lung tissue. Bacterial load showed a significant decline in the herbal treated group up to  $1.13 \pm 0.2 \text{ Log}_{10}$  CFU/ml at the 7<sup>th</sup> day as compared to the untreated group with the bacterial burden  $3.0 \pm 0.12 \text{ Log}_{10}$  CFU/ml. The histopathology reveals more distorted alveoli in an infected group with an accumulation of immune cells while in the treated group improved lung histology was seen. The findings stated that *Z. officinale* is effective may provide a suitable lead for future development and possible clinical utility as inhibitors of lung infection caused by *P. aeruginosa*.

### Introduction

Nosocomial pneumonia is a serious ailment in patients with more than 48 h of stay in hospital<sup>1</sup>. The nosocomial pneumonia is the second most common nosocomial infection associated with intensive care unit (ICU)<sup>2</sup>. The most common cause of nosocomial pneumonia is microaspiration of etiological agents that includes predominantly (50–80%) gram-negative bacteria such as *P. aeruginosa* (17.4%), *K. pneumonia*, *Enterobacter* spp. (18.1%), and *Haemophilus influenzae* (4.9%) and gram-positive bacteria such as *Staphylococcus aureus* (18%)<sup>2</sup>. *P. aeruginosa* is a frequently isolated pathogen from the clinical sample of hospitalized patients thus is responsible for a high percentage of infections<sup>3</sup>. This bacterium shows antibiotic resistance, by developing membrane impermeability, efflux pump systems and changes in drug targets thus posing a huge remedial problem<sup>4–6</sup>. WHO on 28 February 2017 have declared this bacterium as a critical antibiotic-resistant pathogen that requires the urgent need of a new antibiotic. All such devastating scenarios has confirmed that *P. aeruginosa* is definitely one of the critical pathogens with unmanageable virulence determinants. Additionally, this pathogen is also re-emerging with exhibiting trends of extensive drug resistance, which might lead to pan-drug resistance scenarios in the coming future, if not managed at present.

The exploration of the utility of ethnopharmacological

importance of medicinal plants is always a research quest to resolve this challenging issue of multi-drug resistance. Natural plant product(s) in the form of the pure compound or as the standardized extracts may provide an alternative to develop potent leads.

*Zingiber officinale* (ginger) is a perennial tropical herbaceous rhizome and a native to Asia<sup>7</sup>. China is known to be the first for the cultivation of *Z. officinale* then spread to India, South East Asia, West Africa and the Caribbean<sup>7</sup>. But in terms of its production, India is the biggest producer of *Z. officinale* in the world. In India, it is cultivated in almost all the states majorly in Orissa, West Bengal, North eastern states, and Kerala owing to favorable climatic condition<sup>7</sup>. Since ancient times the ginger is in use as a medicinal plant and used in Ayurvedic and Chinese medicine for curing heart problems, treat stomach disorders, diarrhoea, and nausea<sup>7</sup>. It is also reported to be enriched with anti-oxidant, immunomodulatory, anti-inflammatory, anti-rheumatic, anti-hepatotoxic, etc. medicinal traits<sup>7</sup>. The antimicrobial activity of ginger against gram positive, gram negative bacteria and fungi have been reported. The crude extract of *Z. officinale* was found to be lethal against *P. aeruginosa* as it causes membrane disruption and supresses the efflux activity and biofilm formation at in vitro level<sup>8</sup>. In order to build up novel therapeutic modalities for the lung infection caused by *P. aeruginosa*, the effect of crude extract of herbal on the infection was evaluated in this study.

## Materials and Methods

### Isolation and identification of test organism

The isolation of *P. aeruginosa* from a medical sample was done in the Department of Microbiology, National Centre for Disease Control (NCDC), New Delhi, India. The battery of tests was performed for the recognition of *P. aeruginosa* from the sample as described in the previous studies<sup>9</sup>. The isolated bacterium was inoculated in sterile nutrient broth (at 37°C for 48 h). The antibiotic susceptibility test was performed by using Kirby Bauer disc diffusion technique in our previous studies, to determine the drug susceptibility pattern<sup>9</sup>. The result of the test was interpreted according to the Clinical Laboratory Standard Institute guidelines (CLSI) in 2008.

### Inoculum preparation

The fresh sterile nutrient broth was inoculated with the loop full of culture of *P. aeruginosa* grown on Mueller Hinton agar plate and incubated at 37°C for 17-18 h. 0.5 McFarland standard was used to maintain the turbidity by standard procedure. This suspension was used as inoculum.

### Preparation of herbal extract

250 g of shade-dried plant materials (rhizome) were

procured from a reputed supplier of medicinal herbs in New Delhi, India. Hot continuous percolation method at 65°C temperature was used to prepare aqua-alcoholic (water: methanol; 30:70) rhizome extract of *Z. officinale*<sup>9</sup>.

### Liquid Chromatography Mass Spectrometry Analysis

LC-MS analysis was performed using a LCQ MS mass spectrometer (Thermo-Finnigan, India). The solution of crude extract in methanol was analyzed on Acquity TQD QBB 1152 using Thermo Scientific™ Accucore™ RP-MS analytical column (100 x 2.1 mm, 2.6 µm) with auto-injector (SIL-10 AD VP) and diode array detector (SPD M-10 A). 0.1% formic acid in water as mobile phase was used at a flow rate of 400 µL/min for the elution. 30 and 650 L/h flow rate were set for the cone gas and desolvation, respectively. The desolvation temperature was 350°C. In the range of 150-1500 Dalton (Da), the positive and negative ions were scanned. The mass spectral data of reference compound(s) was used to identify known compounds in the crude extract by comparative analysis of the obtained peaks.

### Animals

The mammalian model system selected for investigation of the antibacterial efficacy of the herbal extract (*Z. officinale*) was Swiss albino Strain'A' mice. The experiments were conducted as per the guideline laid down by the Institutional Animal Ethics Committee (IAEC) of INMAS (Reg. No. 8/GO/a/99/CPCSEA)<sup>10</sup>. Swiss albino Strain 'A' male mice (06-07 weeks) weighing about 30 ± 2 g were obtained from INMAS animal facility and maintained under the controlled laboratory conditions. The animals were housed in fresh air conditioned room with temperature 25°C and the relative humidity 60 ± 5% RH. The animals were fed standard animal food pellets (Amrut Laboratory Animal Feed, India) and were offered drinking water. For the administration of herbal extract dissolved in drinking water, the oral route was chosen.

### Standardization of lung infection caused by *P. aeruginosa* in mice model

The empirical dose estimate for causing such infections using microbial count is ~10<sup>7</sup> CFU/mL<sup>11</sup>. Evidently, 10<sup>7</sup> CFU/mL was lethal dose while the expected outcome designed for the study was a non-lethal lung infection. Optimization and classical literature surge revealed that 10<sup>5</sup> CFU/mL could provide such outcome, accordingly chosen for the study<sup>12</sup>.

**Infection Protocol:** Mice were administered anesthesia (xylocaine) subcutaneously and infected intratracheally. Intratracheal instillation was performed with 0.1 mL inoculums (1 × 10<sup>5</sup> CFU/mL) suspension via 29 gauge needle on an insulin syringe. Pathogenesis was characterized by studying different parameters including (a) temperature,

(b) body weight, (c) nasal secretion, (d) piloerection, (e) locomotion, (f) grooming and (g) body condition were assessed. Blood was collected at the time interval (2<sup>nd</sup>, 4<sup>th</sup>, 8<sup>th</sup>, 24<sup>th</sup>, 28<sup>th</sup>, and 32<sup>nd</sup> h).

### **Acute toxicity by Maximum Tolerable Dose (MTD) Assay**

Limit test: The experimentation was done in order to evaluate the acute toxicity of the extract in terms of percent survival, and gross changes in behavior alterations. A study was performed over a period of 24 h following the oral administration of single dose of 2000 mg/kg body weight with six animals (male mice). The mice were constantly observed for the first four hours in order to visualize signs of toxicity and behavioral changes. The maximum dose up to which no lethality was observed referred as Maximum Tolerable Dose (MTD)<sup>13</sup>.

### **In vivo antipseudomonal activity**

Animals were divided randomly into 03 groups (control or no infection, negative control or untreated and D1 i.e., herbal treated) each group contains 06 mice. 2% Xylocaine was used to anaesthetise the mice by giving local anesthesia subcutaneously, and the intratracheal route was used to administer infection via a 29-gauge needle. 100 µl of a suspension of bacterial cells ( $10^5$  CFU/mL) in early-logarithmic-phase<sup>12</sup>. The herbal treatment was given twice a day at the interval of 2 and 4 h of post-inoculation on the same day. The treatment included an oral dose of D1= 98 mg/kg body weight and continued for 7 days. Bacteremia was evaluated in terms of CFU/mL for this, blood sample was collected from untreated and herbal treated group once daily and streaked on agar plate. Radiological imaging of lungs was done prior to killing at day 7. The lungs were aseptically collected and were analyzed for the histopathological study. The biomedical waste bags were used to pack the dead animals after experiment further these bags were picked up by Delhi Pollution Control Committee authorized Biomedical waste disposal vehicle for the incineration of the dead body of animals at their own facility.

### **Radiological study**

The radiology imaging of lungs of mice was performed. Subcutaneously ketamine was given and the anaesthetized mice were placed on a radio-graphic sensor (Krystal X easy digital, Owandy software) at a distance of 80 cm from the X-ray source. Radiographic assessment was done by using X-ray machine (Intra Scan DC Skan Ray, with IV- 1 phase 230 V, IF- 50 Hz, Momentary & Stand by current -0.25A, Made in India) with a 48 kVp exposure for 0.5 mAs.

### **Hematology**

Three animals from each group were used to conduct

hematological and biochemical testing. Samples of blood (200 µL) for hematological and biochemical analysis was withdrawn from saphenous vein in a 3 mL ethylene-diamino-tetracetic-acid (K3-EDTA) tube (Becton Dickinson, BD Vacutainer). Blood sample was then analyzed for complete blood profile including red blood cell (RBC) count, hemoglobin, platelet count, total and differential white blood cell (WBC) count. The measurements were executed by Hematology Analyzer (Medonic CA530).

### **Histopathology**

The cervical dislocation was used to sacrifice the mice on 7<sup>th</sup> day of experiment. The whole lungs were expunged and fixed in 4% paraformaldehyde followed by decalcification in 5% formic acid. The lungs were embedded in paraffin for tissue sectioning. Haematoxylin-eosin stains were used for staining the tissue sections (5 µm thick) and these sections were observed under Olympus microscope (Olympus U-TV 0.63×C; SN 9L01588 T2, Tokyo, Japan (Made in the Philippines).

### **Statistical analysis**

Results are expressed as Mean ± SEM. Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Turkey's test for all parameters. The p < 0.05 was considered significant.

## **Results**

### **Liquid Chromatography Mass Spectrometry Analysis**

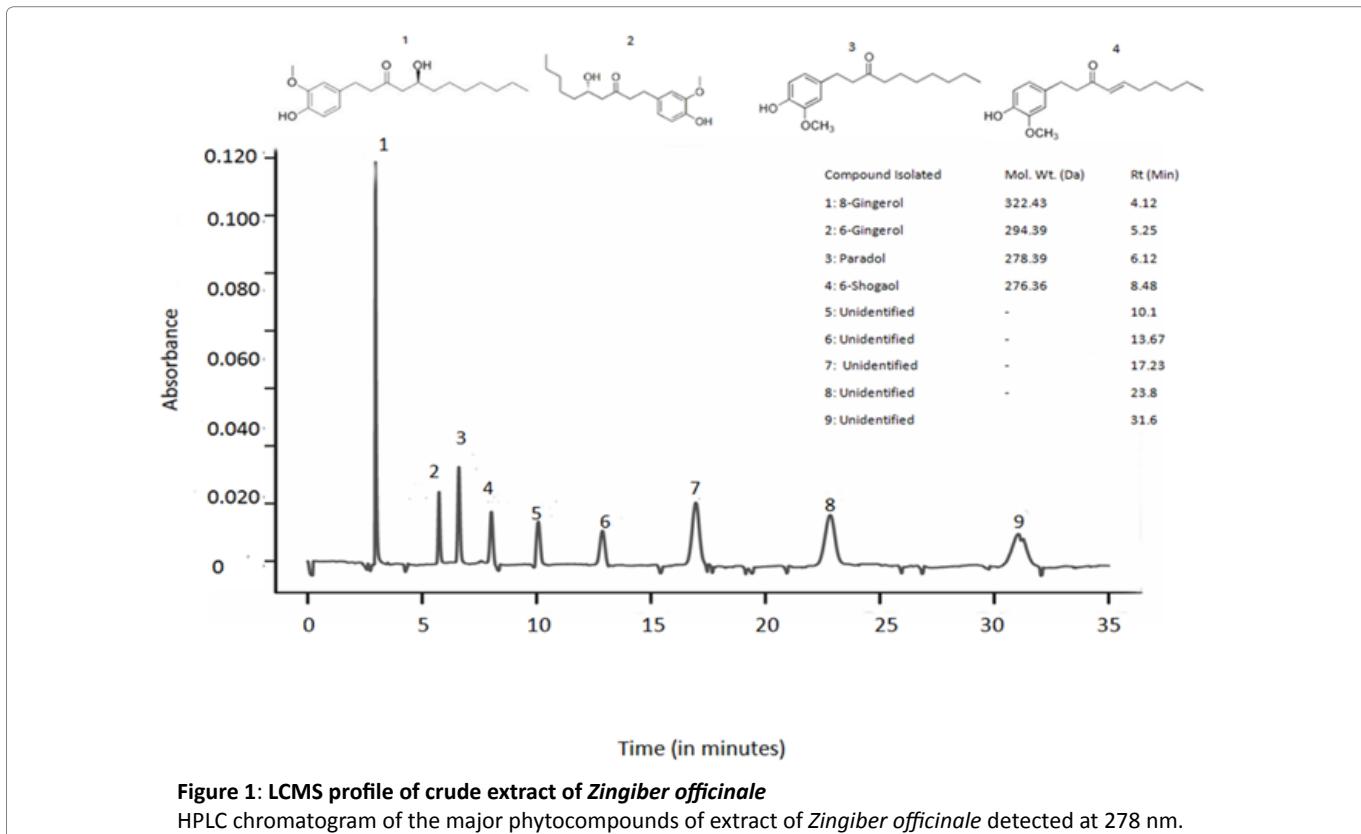
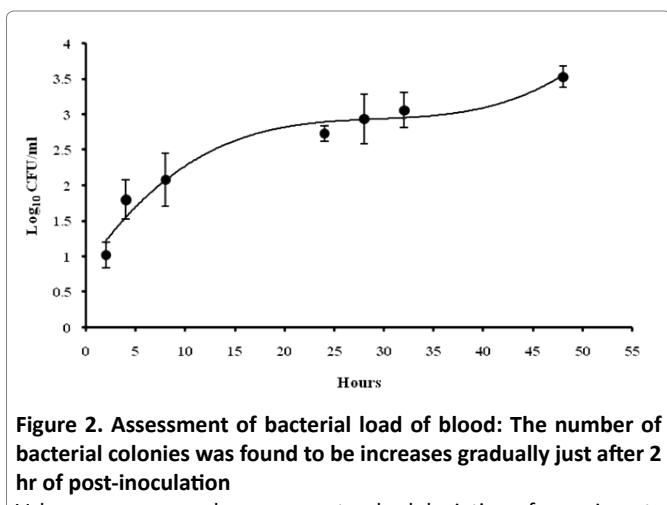
Mass spectral analysis of the eluted samples was performed in order to record fragmentation pattern. LCMS analysis revealed 09 peaks out of which 04 peaks were recorded and the identified compounds were 8-gingerol (RT: 4.12), 6-gingerol (RT: 5.25), paradol (RT: 6.12) and 6-shogaol (RT: 8.48) (Figure 1). In addition to this 05 unidentified peaks were also obtained.

### **Standardization of lung infection caused by P.aeruginosa in mice model**

A significant bacterial load of  $3.53 \pm 0.17 \text{ Log}_{10}$  CFU/ml was recorded at 48<sup>th</sup> h of post-infection (Figure 2). A weight loss up to 32% was observed at 48<sup>th</sup> h with significant Hypothermia with the temperature drop of  $27 \pm 0.5^\circ\text{C}$  (48<sup>th</sup> h) from  $38.47 \pm 0.69^\circ\text{C}$  (0 h) (Figure 3). Absence of grooming was observed, eyes were partially opened, very less locomotion and weakened after 24 h. From 8<sup>th</sup> h nasal discharge along with piloerection stays, Hyperventilation after 4<sup>th</sup> h of inducing infection was noticed upto one day of infection cycle and later it goes down.

### **Maximum Tolerable Dose assay**

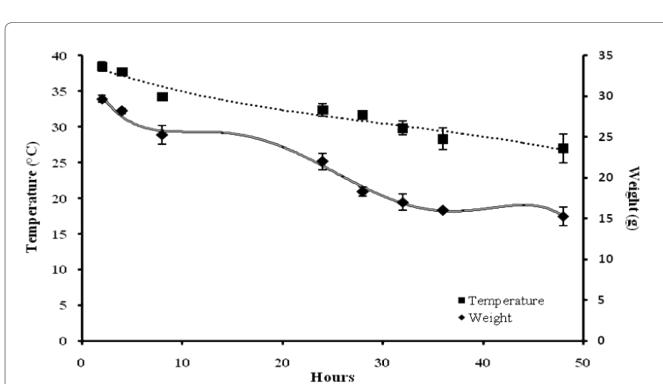
In order to perform the preclinical animal studies the lethal dose or maximum tolerated dose of the extract

**Figure 1: LCMS profile of crude extract of *Zingiber officinale***HPLC chromatogram of the major phytocompounds of extract of *Zingiber officinale* detected at 278 nm.**Figure 2. Assessment of bacterial load of blood: The number of bacterial colonies was found to be increases gradually just after 2 hr of post-inoculation**Values are expressed as mean  $\pm$  standard deviation of experiments each performed in triplicates and analysed using univariate ANOVA.

was determined. As shown in the Table 1, no mortality was observed in the mice at 2000 mg/kg body weight, administered orally. Also, no significant changes were observed in the physical parameter and no any adverse effects were observed on mice.

### In vivo antimicrobial activity

After 2h of the commencement of herbal treatment the bacterial burden was recorded  $1.02 \pm 0.27$  and  $1.04 \pm 0.17$   $\text{Log}_{10}$  CFU/ml for negative control (infected), and D1 group, respectively. A gradual increase of bacterial load

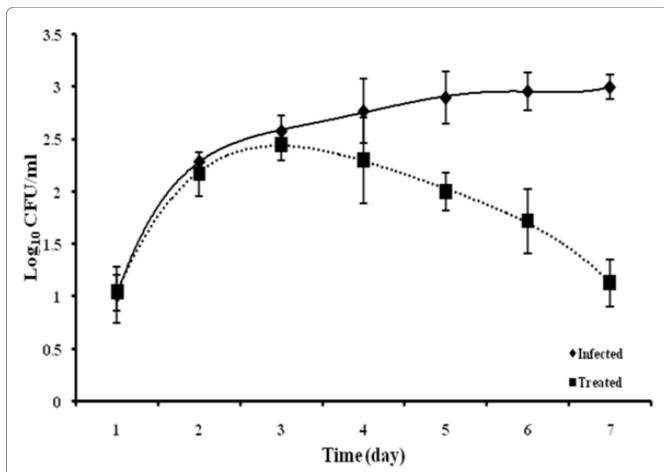
**Figure 3. Evaluation of physical parameters: Decrease in body temperature commenced at 4th h of post-inoculation; Persistent decrease in weight was also observed**Values are expressed as mean  $\pm$  standard deviation of experiments each performed in triplicates and analysed using univariate ANOVA.

in the infected group was observed that reaches up to  $3.0 \pm 0.12$   $\text{Log}_{10}$  CFU/ml. A significant decline of the bacterial loads in blood and reduces by  $1.13 \pm 0.2$   $\text{Log}_{10}$  CFU/ml at 7<sup>th</sup> day (Figure 4). It was observed that all the animals have recovered from the infection with herbal dose D1. In negative control group all the animals died on day 7 of the study.

### Radiological study

A severe soft tissue damage of lungs was observed in radiological analysis of the negative control (untreated) group (Figure 5A). The herbal was found to be capable

of seizing and healing the tissue damage (Figure 5C) as moderate tissue damage was observed in the form of lesions in treated group. In case of uninfected group normal image of lungs was observed (Figure 5B).



**Figure 4.** *In vivo* antibacterial activity of *Zingiber officinale* extract Bacteremia in two groups (Infected and Treated) of mice, observed at pre-decided time interval for the study

## Hematology

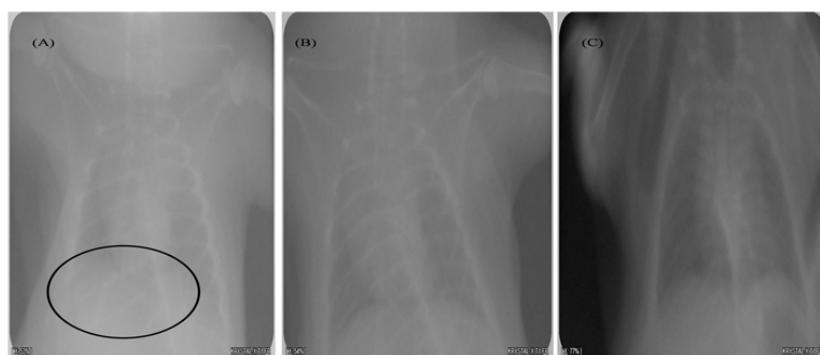
There were changes observed in the hematological parameters during the dosing period. An increased level of white blood cells ( $12.65 \pm 0.12 \times 10^3/\mu\text{L}$ ) was observed in case of infected group during the study while in treated groups no such significant changes were observed. WBC count peaked in the infected group with significant raise in lymphocytes as compared to not infected group as well as treated group. Neutrophils percentage was also peaked in infected group with the value  $78.89 \pm 1.8\%$  as compared to other groups in the study. There are no aberrations were observed in count of RBC, eosinophils, and monocytes and other hematological parameters like hemoglobin in all the groups (Table 2).

## Histopathology

Histopathology of lungs showed more diffuse and patchy accumulation of inflammatory cells within the alveolar space along with the infiltrates was noted in all the lung section of infected mice (Figure 6A). No exudate was observed in uninfected mice also the alveoli were found to be intact with undamaged lung parenchyma (Figure 6B). In herbal treated animal group improved lung histology was

**Table 1.** Acute toxicity study.

Herbal	No. of animal (male mice)	Dose (mg/kg body weight oral)	Observations					
			Mortality	Body weight	Temperature	Behavioral pattern	Lethargy	Diarrhea
<i>Z. officinale</i>	06	2000	Not observed	No significant changes	No significant changes	Active	Not observed	Not observed

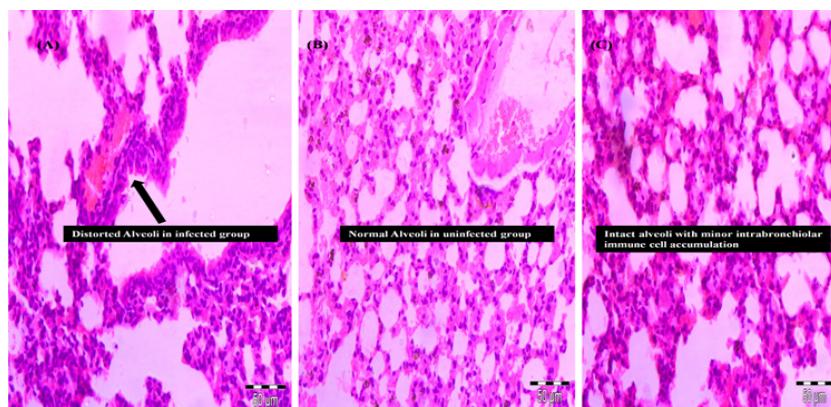


**Figure 5.** Radiological analysis of thoracic cage

(A) Lesions were observed in infected group (under circle), (B) Normal X-ray image is obtained with uninfected group, (C) Mild lesions were obtained

**Table 2.** Hematological profile.

Parameters	No infection	Only infection	Infection + <i>Z. officinale</i> dose
Haemoglobin (g percentage)	$14.13 \pm 1.68$	$14.98 \pm 1.67$	$14.23 \pm 1.78$
RBC count ( $\times 10^6/\mu\text{L}$ )	$5.75 \pm 1.82$	$5.55 \pm 1.79$	$5.35 \pm 1.52$
Platelet count ( $\times 10^6/\mu\text{L}$ )	$8.47 \pm 2.88$	$8.31 \pm 1.08$	$8.7 \pm 1.80$
Total WBC ( $\times 10^3/\mu\text{L}$ )	$5.37 \pm 0.84$	$12.65 \pm 0.12$	$6.37 \pm 0.84$
Differential percentage	Neutrophil	$20.67 \pm 0.80$	$78.89 \pm 1.8$
	Lymphocytes	$88.2 \pm 1.84$	$121 \pm 2.4$
	Eosinophil	$2.17 \pm 0.75$	$2.07 \pm 0.15$
	Monocyte	$0.67 \pm 0.28$	$0.65 \pm 0.38$

**Fig 6. Lung pathohistology photomicrographs**

(A) Severe histological damage occurs in lungs in particular massive inflammatory infiltrates within the alveolae and alveolar damage (arrow), (B) Normal lung histology in uninjected mice with intact alveoli (arrow), (C) Intact alveoli with normal lung histology in treated (D1) group with minor intrabronchiolar immune cell accumulation

observed with the disappearing of the exudates from the lungs. Also, the inflammatory cell infiltrates of bronchi and perivascular edema were less seen in the examined section of herbal treated group (Figure 6C).

## Discussion

Antibiotics have been established as the mainstay in the treatment of bacterial infections, there was a conviction in the medical field that this would gradually eradicate the onset of infectious diseases. However, the world wide emergence of such bacterial strain has become a customary problem. WHO in February 2017 released a list of antibiotic resistant bacteria and categorized them into critical, high and moderate severity agents posing a threat to human health. *P. aeruginosa* has been placed in the critical category as it has been reported to account for higher morbidity and mortality rate in humans. This bacterium is an opportunistic pathogen that accounts for a high percentage of nosocomial pneumonia. No absolute treatment modality is available for MDR *P. aeruginosa*. Additionally, all the available 3<sup>rd</sup> line drugs exhibit serious hepato-renal toxicities. Numerous natural plant products have also been tested for their antibacterial efficacy against *P. aeruginosa* across the globe.

We have reported previously 05 herbs against multi-drug resistant *P. aeruginosa* by utilizing bioprospection approach followed by validation by using molecular docking analysis<sup>8</sup>. The aquo-alcoholic extract of rhizome of *Z. officinale* was also assessed at in vitro level to determine its Minimum Inhibitory Concentration (10 $\mu$ g/mL) and their mechanism of activity were additionally assessed to repress the biofilm formation and efflux movement of the pathogen<sup>9</sup>. The present study concentrates on the assessment of various parameters of disease and affirmation of efficacy of the herbal extract by utilizing a pre-clinical pneumonic murine model.

MTD was determined to estimate the highest dose of the herbal that does not cause unacceptable side effects. Limit test was performed in order to determine the MTD by using OECD guideline 423, wherein, the results revealed that the aquo-methanolic extract of *Z. officinale* have not been found to be toxic with an oral dose of 2000 mg/kg body weight with no significant morbidities. No aberrations were observed in the body weight and other physical parameters (Table 1). Lethargy and diarrhea did not occur in animals. Thus, it is concluded that the extracts does not show any toxic effects.

To study the effect of the herbal extract on a pulmonary infection model, first of all the pathogenesis of *P. aeruginosa* was determined. The animal was inoculated through intratracheal instillation in order to bypass the upper airways and to cause acute pneumonia thereby delivering a high load of bacteria in the distal bronchi. Similar route was used by Munder and coworkers, to study the acute *P. aeruginosa* lung infection<sup>14</sup>. The virulence of the pathogen is measured by pathogenesis in mice and hypothermia along with bacteremia which was studied as an accurate predictor of illness. Soothil et al., (1992) also studied the hypothermia for the measurement of bacterial virulence<sup>11</sup>. The pathological manifestation was observed from 2<sup>nd</sup> h of post-inoculation by observing the bacteremia, hypothermia and weight loss (Figures 2 and 3). As it is mentioned in the literature that the bacteremia often arises in pneumonia and is associated with the high rate of morbidity and mortality<sup>15</sup>. The occurrence of bacteremia was employed as the primary end point. A sudden drop in body temperature and significant decline in weight were found in all the infected animals. These findings are in line with the symptoms obtained by Soothil et al., in their study on mice<sup>11</sup>. Exposure to *P. aeruginosa* did not cause fever, but it leads to hypothermia, as reported by Munder and coworkers<sup>14</sup>.

At day 7 of post-administration of herbal extract there is a significant drop of bacterial count of *P. aeruginosa* was observed. X-rays analysis was used for the assessment of the progression of pneumonia in mice (Figure 5). The patchy parenchymal tissue in the x-rays report of the lungs suggesting the presence of pneumonia (Figure 5A). At the termination point of the study the lungs were removed from euthanized mice shows and there have been numerous distinct areas of hemorrhage and alveolar inflammation was observed.

There were significant changes observed in the hematological parameters during the dosing period. An increased level of white blood cells ( $12.65 \pm 0.12 \times 10^3/\mu\text{L}$ ) was observed in case of infected group during the study while in treated groups no such significant changes were observed. WBC count peaked in the infected group with the significant rise in lymphocytes as compared to the control group as well as the treated group. It has been reported that high WBC count is associated with pneumonia as well as Chronic Obstructive Pulmonary Disease (COPD). Gan et al., pointed out that there is a direct relationship between the high WBC count and induction of pulmonary disease that demonstrated the severity of exacerbation<sup>16</sup>. Sin et al., reported the association between high blood WBC count with an increased severity of symptoms in case of pulmonary diseases<sup>17</sup>. Thus, it is reasonable to check WBC count as it presents a physiological marker for lower respiratory disease<sup>18</sup>. The neutrophil percentage was also found to be increasing in the infected group with the value  $78.89 \pm 1.8\%$  as compared to other groups in the study. Neutrophil accumulation during lower respiratory tract bacterial infection was also demonstrated by Mizgerd (2002) in mice as one of the innate immune response in the lung against bacterial infection<sup>19</sup>. There is no aberrations were observed in count of RBC, eosinophils, monocytes and hemoglobin in all the groups (Table 2).

Severe damage with infiltration of exudates in the alveoli was observed during histopathological examination (Figure 4A). In herbal treated group there were better condition of lung tissues was obtained with normal to mild inflammation as compared to untreated group (Figure 4C). The anti-inflammatory action during pneumonia along with the bactericidal activity of *Z. officinale* was observed by the reduced tissue inflammation in the treated group. Thus, the findings indicating the protective effect and symptomatic relief providing capabilities of the *Z. officinale* extract.

In any case, no preclinical efficacy investigations of *Z. officinale* focusing on *P. aeruginosa* or its drug resistant strains causing pneumonia have yet been conducted.

LCMS analysis of the rhizome extract had confirmed the presence of alkaloids mainly (Figure 1). Gingerol have been

known to possess antibacterial activity against periodontal bacteria and vancomycin-resistant enterococci<sup>20-21</sup>. Also phenolic compounds of ginger have been reported to show the therapeutic potency against cancer and show activity in cell signaling pathway<sup>22-23</sup>.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Ethical approval

The experiments were conducted as per the guideline laid down by the Institutional Animal Ethics Committee (IAEC) of INMAS (Reg. No. 8/GO/a/99/CPCSEA)

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