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# THE SYNERGESTIC EFFECTS OF EDTA / ANTIBACTERIAL COMBINATIONS IN *KLEBSIELLA PNEUMONAE* INDUCED PNEUMONIA RAT MODEL

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**Keywords:**

Ethylene diamine tetra acetate, *Klebsiella pneumoniae*, Oxidative stress, Free Radicals

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**ABSTRACT:** The study was conducted to evaluate the synergistic effect of EDTA with antibacterial (Ceftriaxone + Sulbactum) as a combinational therapy against antioxidant enzyme activity, lipid peroxidation level, some hematological and biochemical parameters in *Klebsiella pneumoniae* induced nosocomial pneumonia followed by histopathology of lungs tissue in experimental exposed Sprague Dayle (S.D) rats. Total 24 rats were selected and divided into 4 groups of 06 rats each for *in-vivo* and *in-vitro* EDTA / antibacterial therapy. Group I Control normal saline treated group; group II was infected *via K. pneumoniae* bacterial strain. Group III and IV were infected plus treated group with antibacterial and EDTA / Antibacterial drugs. The results indicated that a significant ($p<0.005$) decrease in enzymes activities (Superoxide dismutase, catalase and ascorbic acid) along with increased lipid peroxidation level as well as cytokine parameters (tumour necrosis factor-α, interlukin-6) and biochemical parameters in plasma of infected group as compared to control group. These activities were increased along with decreased in lipid peroxidation level and biochemical parameters in both treated group as compared to infected group after seven days treatment with antibacterial and EDTA/Antibacterial drugs. When antibacterial treated group was compared with EDTA/Antibacterial treated group, all above parameters were improved in the plasma of EDTA / Antibacterial treated group. These findings concluded that EDTA / Antibacterial has better synergistic efficacy effect than antibacterial alone in *Klebsiella pneumoniae* induced nosocomial pneumonia infection.

**INTRODUCTION:** Respiratory tract infections are severe and most common types of infection treated by medical practitioners all over the world. Nosocomial pneumonia is the second most common infection, causing high morbidity and mortality and about 80% of nosocomial infections caused by *Klebsiella pneumoniae* are due to multidrug-resistant strains.

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The emergence of antibiotic-resistant bacterial strains necessitates the exploration of alternative antibacterial therapies [1]. Important causes of Gram-negative resistance includes extended-spectrum *β*-lactamases (ESBLs) in *Klebsiella pneumoniae*, high level third-generation cephalosporin (Amp C) *β*-lactamase resistance among *Enterobacter* species observed in pneumonia.

Recent data suggest that because of ESBLs and high-level amp C *β*-lactamase resistances, use of third-generation cephalosporins may be ineffective in many patients with nosocomial infections [2]. EDTA is a polyamino carboxylic acid, a colorless water-soluble metallo-chelator and is known to have intrinsic antimicrobial activity.

Such activities include direct antimicrobial effects, potentiating the activity of other classes of antibiotics, detoxication, and neutralization of bacterial toxins / enzymes. One of the recognized modes of action of EDTA is to potentiate other antibiotics by having synergistic effect in combinational use with antibiotics [3].

Simple experiment was done for testing the effect of antibacterial / EDTA combination on isolates of *Pseudomonas* sp. significantly improves the efficiency of using such combination. This made the medical community to re-evaluate older agents with combinational therapy to other agents having synergistic effects. Combinational therapy most often refers to the simultaneous administration of two or more medications to treat the single disease [4]. In the present study experimental nosocomial pneumonia induced by *Klebsiella pneumoniae* was established in S.D. rats and the effectiveness of EDTA / antibacterial (ceftriaxone with sulbactum) as a combinational therapy was studies.

**MATERIALS AND METHODS:** Study was conducted in pre-clinical lab division of Venus Medicine Research Centre, Baddi, Himachal Pradesh, India. Chemicals used in the study were obtained from Sigma, St. Louis, MO, USA and analytical grade chemicals were purchased locally. Biochemical kits used in the study were purchased from Bayer Diagnostics India Ltd., Baroda, Gujarat, India. The study was approved by the institutional animal ethical committee (IAEC/CS/ 10/2010) of Venus Remedies Pvt. Ltd.

**Laboratory Animals:** Healthy adult male Sprague Dayle rats weighing in 65 - 105 g were procured from animal house facility of Venus Medicine Research Centre, Baddi, Himachal Pradesh, India. They were housed in polypropylene cages, maintained under standard control conditions (12 h light and 12 h dark condition), temperature (23 ± 2 °C) and humidity (65 ± 5%).

**Collection and Preservation of *Klebsiella pneumoniae* Culture Medium:** The Culture medium of *Klebsiella pneumoniae* organisms were obtained from IMTECH (Institute of Microbial Technology), Chandigarh, India. A single isolated colony of bacterial strain were maintained on nutrient agar slant, nutrient agar plate was transferred to 50 ml nutrient broth and incubated at 37 °C for 18 h. Organism were harvested by Centrifugation at 2500x for 20 min and washed to 3 - 4 times with sterile PBS (0.2 M, pH 7.2).

**Animal Grouping and Investigation:** Thirty male Sprague Dayle rats were divided into four groups (six rats in each group): Group 1: Control normal saline treated group (0.5 ml NaCl), Group 2: *K. pneumoniae* infected (50 µl log $10^6$ colony-forming units (CFU)/ml) group, Group 3: Antibacterial treated group, {Infected + ceftriaxone plus sulbactum treated group (155 mg/Kg bodyweight / bid)}and Group 4: EDTA / Antibacterial {Infected + ceftriaxone plus sulbactum + EDTA treated group (186 mg/Kg bodyweight/bid)}.

After pre ($7^{th}$ day) and post ($14^{th}$ day) treatment blood samples were collected for the estimation of blood parameters. Rats were sacrificed at the end of experiment; lung tissues were removed aseptically and lung tissue's photograph were taken immediately for gross observation changes in each group. Lung tissue was sectioned into two halves, one half of lung was homogenized in 1ml normal saline and histological examination was done in other half part of lung tissue.

**Experimental:**

**Induction of Nosocomial Pneumonia:** For induction of nosocomial pneumonia in the rat model, bacterial dose 50 µl of log 106 CFU/ml and the method was employed for intranasal instillation of the bacterial inoculums adopted by Kumar *et al*., 2016 [5]. For induction of nosocomial pneumonia, bacterial dose was instilled into the nasal opening while holding the rat upright for 7 days twice daily (at 10.00 a.m. and 5.00 p.m.). Total 18 rats (six rats in each group) were infected and confirmed by increased body temperature, sneezing, coughing, labored movement (Walking with difficulties), cell count (WBC) and presence of bacterial count in blood sample. While six were treated with 0.9% NaCl only (Group I).

**Treatment of Infection:** Antibiotics ceftriaxone (1000 mg) and sulbactum (1500 mg) with EDTA were obtained from Venus Remedies, India. After confirmation of disease, ceftriaxone + sulbactum and ceftriaxone + sulbactum + EDTA drugs were given *via* intravenous route for 7 days. The ratio of

fixed dose combination of ceftriaxone + sulbactum with EDTA was 1: 2 respectively.

**Preparation of Plasma and Lung Homogenate:** Blood samples were collected by retro orbital vein In the sodium citrate (3.8%) containing vial and centrifuged at 4 °C at 6000 rpm for 15 minutes and plasma was collected for determine enzyme activity along with malonaldialdehyde level and biochemical parameters of a all groups. Lung tissue homogenates (10%) were prepared in chilled phosphate buffer - NaCl solution containing 0.15 mol/L NaCl in 0.05 mol/L, $Na_2HPO_4$-$NaH_2PO_4$ buffer (pH 7.2) and left for at least 1 h at 2 - 8 °C before measurement of MDA.

**Determination of Biochemical Parameter:** The automated cell counter (Sysmex XT 2000i) was used for the determination of WBC and platelets levels. The biochemical parameters (Urea, Uric acid, Creatnine, Total Billurubin, SGOT, SGPT, ALP, total protein) were measured by fully automatic biochemical analyser (Erba Manheim; Model EM200) in all groups.

**Determination of Antioxidant Enzymes Assay in Plasma:** Superoxide dismutase (SOD) activity was determined by the Method of Misra and Fridovich[6]. Catalase activity was measured by the method of Luck [7]. The ascorbic acid was determined by the method of Roe *et al*.,1954 [8].

**Determination of Antioxidant Enzymes Assay in Lung Homogenates:** Lipid peroxidation measurement to determine the extent of lung tissue damage was assessed by the measurement of malonaldialdehyde (MDA) according to Verma *et al*., 2013 [9].

**Cytokines Assay:** Serum cytokines TNF-α and IL-6 were assayed using invitrogen ELISA kits (Camarillo, CA, USA) following manufacturer's instructions.

**Histopathological Analysis of Lung Tissue:** Lungs were removed aseptically and collected in 10% buffered formalin saline for histological studies. The formalin fixed tissues were washed overnight in running tap water and dehydrated in ascending series of alcohol (70 - 100%) and cleared in benzene. For routine histopathology, the 4 - 5 micron thick tissue sections were cut from the paraffin embedded tissues and was stained with haematoxylin and eosin stain (H & E) (Luna 1968). Morphological changes were assessed by using routine light microscopy.

**Statistical Analysis:** All values were presented as Mean ± SD. One-way analysis of variance (ANOVA) followed by paired t-test was used to determine statistical difference between Control (saline treated) group *vs.* disease induced group and disease induced group vs. both treated groups. The p - values <0.05 were considered statistically significant.

**RESULT:**

**EDTA / Antibacterial Combinations on Biochemical and Hematological Parameters:** The levels of Urea, uric acid, creatnine, Total Bilurubin, ALP, SGOT, SGPT, WBC, and PLT were found to be statistically ($p<0.005$) increased in infected group as compared to control group. These biochemical parameters were statistically lowered in antibacterial treated group as well as in EDTA / antibacterial treated group after seven days of treatment as compared to infected group. When EDTA / antibacterial treated group was compared with antibacterial treated group, Urea, uric acid, creatnine, ALP, SGOT, SGPT and WBC ($p <0.005$) were statistically lowered in EDTA / antibacterial treated group after treatment of seven days. But the level of Total Bilurubin and PLT were slightly lowered in EDTA/antibacterial treated group **Table 1**. Total protein level was statistically lowered in infected group in comparison with control group and statistically improved in EDTA / antibacterial treated group but significantly improved in antibacterial treated group after seven days of treatment.

**EDTA / Antibacterial Combinations on Enzymatic and Non-enzymatic Antioxidant Enzymes Activities:** Superoxide dismutase, Catalase and Ascorbic acid were statistically decreased in *K. pneumoniae* infected diseased control group as compared with control group. These enzyme activities were significantly improved in antibacterial treated group as well as in EDTA/antibacterial treated group, when compared with infected group after treatment of seven days. Comparison of antibacterial treated group *vs.* EDTA / antibacterial treated group, EDTA / antibacterial treated group showed statistically

increased level of Superoxide dismutase, Catalase and Ascorbic acid. The Lipid peroxidation (MDA) level was significantly elevated in infected group and after seven days treatment; it statistically lowered in both treated group. MDA level was statistically improved in EDTA/antibacterial treated group than the antibacterial treated group after seven days of treatment **Table 2**.

**TABLE 1: EFFECT OF EDTA / ANTIBACTERIAL THERAPY ON HEMATOLOGICAL AND BIOCHEMICAL PARAMETERS IN NASOCOMIAL PNEUMONIA INDUCED RAT MODEL**

| Biochemical Parameters | Duration | Healthy Control (N=6) | Disease control (N=6) | Antibacterial (N=6) | EDTA/Antibacterial (N=6) |
|---|---|---|---|---|---|
| Urea | Pre | 27.07 ± 1 .37 | 40.03 ± 2.15** | 39.37 ± 2.02* | 40.11 ± 1.71* |
| (mg/dL) | Post | 27.1 ± 1.32 | 43.61 ± 0.94** | 36.78 ± 1.86** | 29.93 ± 1.96** |
| Uric Acid | Pre | 1.66 ± 0.04 | 2.95 ± 0.12** | 3.02 ± 0.22* | 3.25 ± 0.42* |
| (md/dL) | Post | 1.66 ± 0.06 | 4.04 ± 0.14** | 2.74 ± 0.30** | 1.90 ± 0.44** |
| Creatnine | Pre | 0.24 ± 0.01 | 0.96 ± 0.11** | 0.97 ± 0.12* | 0.98 ± 0.11* |
| (mg/dL) | Post | 0.23 ± 0.01 | 1.21 ± 0.10** | 0.88 ± 0.11** | 0.29 ± 0.06** |
| Total Bilurubin | Pre | 0.36 ± 0.06 | 0.63 ± 0.05** | 0.60 ± 0.09* | 0.61 ± 0.07* |
| (mg/dL) | Post | 0.38 ± 0.05 | 0.89 ± 0.18** | 0.46 ± 0.05** | 0.39 ± 0.04** |
| Total Protien | Pre | 8.40 ± 0.56 | 5.10 ± 0.68** | 4.89 ± 0.50* | 4.72 ± 0.29* |
| (g/dL) | Post | 8.42 ± 0.55 | 3.55 ± 0.43** | 4.05 ± 0.09** | 7.79 ± 0.46** |
| ALP | Pre | 122.13 ± 4.06 | 216.53 ± 2.31** | 216.03 ± 0.2.69* | 217.4 ± 2.16* |
| (IU/L) | Post | 122.26 ± 4.08 | 219.55 ± 1.47** | 211.41 ± 2.18** | 134.23 ± 2.83** |
| SGOT | Pre | 47.22 ± 3.22 | 84.99 ± 3.45** | 85.52 ± 2.37* | 86.36 ± 2.52* |
| (IU/L) | Post | 47.14 ± 3.43 | 93.85 ± 6.95** | 83.09 ± 1.50** | 46.52 ± 3.47** |
| SGPT | Pre | 38.65 ± 3.60 | 63.39 ± 0.98** | 64.03 ± 1.42* | 65.08 ± 1.98* |
| (IU/L) | Post | 38.50 ± 3.66 | 67.16 ± 1.08** | 60.63 ± 1.75** | 42.52 ± 1.67** |
| WBC ($10 \times 10^3$/µL) | Pre | 7.3 ± 0.92 | 11.06 ± 1.15** | 11.93 ± 1.87* | 12.6 ± 1.68* |
| | Post | 7.46 ± 0.80 | 13.66 ± 0.99** | 9.25 ± 1.24** | 6.65 ± 0.37** |
| PLT ($10 \times 3$/µL) | Pre | 258.66 ± 80.32 | 388.66 ± 52.38** | 395.33 ± 54.04* | 413.66 ± 49.34* |
| | Post | 254.5 ± 77.88 | 486.33 ± 47.75** | 265 ± 63.79** | 184.33 ± 14.81** |

N = Number, Data presented as mean ± SD. Analysis was done between control *vs.* disease control as well as disease control *vs.* Cef + Sul and disease control *vs.* Cef + Sul + EDTA and Cef + Sul *vs.* Cef + Sul + EDTA. * = p>0.05; not significant and ** = p<0.05 significant 5 significant. N = Number, Data presented as mean ± SD.

**EDTA / antibacterial combinations on cytokines levels:** In diseased condition, level of cytokines IL-6 and TNF-α both statistically increases (p<0.005) as compared to healthy rats. The levels of both cytokines were significantly lowered after seven days of treatment with antibacterial and EDTA / antibacterial. When we compared both treated groups with each other, EDTA / antibacterial treated rats showed slightly lower level of TNF-α and IL-6 **Table 2**.

**TABLE 2: EFFECT OF EDTA / ANTIBACTERIAL THERAPY ON BIOCHEMICAL PARAMETERS IN NASOCOMIAL PNEUMONIA INDUCED RAT MODEL**

| Enzymatic Parameters | Duration | Healthy Control (N=6) | Disease control (N=6) | Antibacterial (N=6) | EDTA/Antibacterial (N=6) |
|---|---|---|---|---|---|
| SOD | Pre | 0.202 ± 0.007 | 0.090 ± 0.002** | 0.085 ± 0.008* | 0.080 ± 0.007* |
| (n mole min/mL) | Post | 0.208 ± 0.006 | 0.067 ± 0.008** | 0.123 ± 0.003** | 0.175 ± 0.008** |
| Catalase | Pre | 0 .352 ± 0.187 | 0.134 ± 0.007** | 0.127 ± 0.005* | 0.127 ± 0.006* |
| (nmolemin/mL) | Post | 0.359 ± 0.17 | 0.117 ± 0.004** | 0.156 ± 0.009** | 0.263 ± 0.34** |
| Ascorbic Acid | Pre | 8.085 ± 0.60 | 5.35 ± 0.67** | 4.86 ± 0.56* | 4.36 ± 0.39* |
| (mg/mL) | Post | 8.11 ± 0.56 | 3.72 ± 0.48** | 5.89 ± 0.51** | 6.93 ± 0.82** |
| MDA | Pre | 3.39 ± 0.39 | 5.21 ± 0.60** | 5.62 ± 0.90* | 5.19 ± 0.48* |
| (n mole min/mL) | Post | 3.44 ± 0.41 | 6.63 ± 0.45** | 4.95 ± 0.97** | 3.62 ± 0.62** |
| TNf-alpha | Pre | 333.30 ± 7.35 | 428.30 ± 63.89* | 469.56 ± 72.35* | 528.23 ± 58.09** |
| (pg/Ml) | Post | 334.55 ± 5.27 | 557.95 ± 89.58** | 415.11 ± 45.26** | 352.11 ± 20.23** |
| IL-6 | Pre | 425.67 ± 8.22 | 522.03 ± 35.97** | 534.69 ± 40.58* | 603.13 ± 73.06** |
| (pg/Ml) | Post | 424.08 ± 10.31 | 655.39 ± 40.56** | 453.71 ± 32.06** | 436.92 ± 14.02** |

N=Number, Data presented as mean ± SD. Analysis was done between control *vs.* disease control as well as disease control *vs.* Cef + Sul and disease control *vs.* Cef + Sul + EDTA and Cef + Sul *vs.* Cef + Sul + EDTA. Where * = p>0.05; not significant=** p<0.05 significant. N=Number, Data presented as mean ± SD.

**Histopathology Study:** In histopathological study, the microscopic examination of lungs showed normal histological picture in control group **Fig. 1A**. In infected group, Lung sections showed increased intravascular neutrophils (N), sepal thickening, and inflammation (I) along with hemorrhages and congestion **Fig. 1B**. In antibacterial treated group, mild focal emphysematous changes were seen microscopically **Fig. 1C**. The EDTA / antibacterial treated group showed mild congestions were seen in the lung tissue **Fig. 1D**.

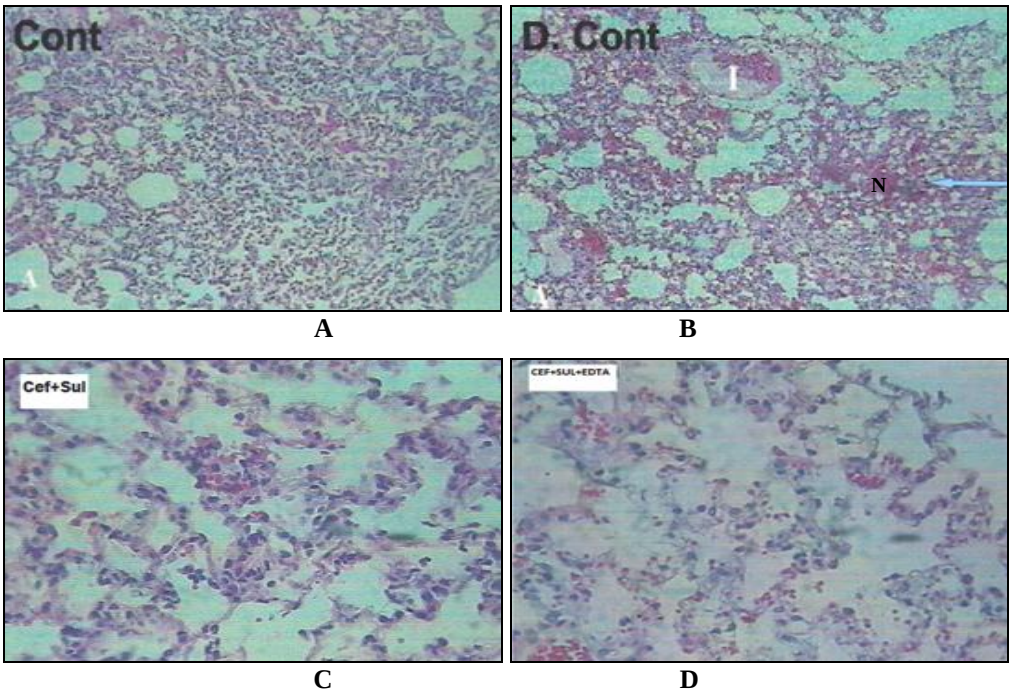


**FIG. 1: HISTOPATHOLOGY STUDY**

**DISCUSSION:** *Klebsiella pneumoniae* is a common pathogen that causes community-acquired pneumonia and nosocomial infections due to multidrug - resistant strains [10]. It is observed that combination drug therapy is more effective due to their synergistic effect than individual drug therapy [11, 12]. Ceftriaxone is antibiotic drug with potent bactericidal activity against a wide range of gram positive and gram negative bacteria and sulbactum is a *β*-lacatamase inhibitor with better broad spectrum activity against gram negative organisms [13, 14]. Ethylene diamine tetra acetate (EDTA) a third vector with these two drugs as a combinational therapy has antioxidant and free radical scavenger properties that act against the microorganism for any of the biological role of metal dependent proteins which are associated to the maintenance of their transcription factors [15].

Due to the role of metal dependent protein, it enters into the cell membrane and opens the $Ca^{+2}$ channel and increased the concentration of combination drug in the bacterial cell leading to inhibition of microbial cell transcription and death. EDTA with divalent metal ions and make them unavailable for the bacteria which are essential for cellular replication and growth [16].

*K. pneumoniae* causes acute inflammation and increases neutrophil activities with increased oxidative stress and various inflammatory biomarkers in Nosocomial pneumonia infection [17]. In this study EDTA / Antibacterial combination showed better antibacterial synergistic efficacy against gram negative bacteria than antibacterial alone. Nosocomial pneumonia infection causes some alterations in hepatic and renal enzymes. This study shows, SGOT and SGPT levels were statistically increased in disease control group as compared with control group. Author in his study showed that, elevated levels of hepatic enzymes were frequently noted in nosocomial pneumonia infection in children [18]. The elevated level of SGOT and SGPT may be associated with possible hepatocellular dysfunction due to severe inflammation and sepsis. Nikolic *et al.*, 2006 has

been reported the higher plasma activities of SGOT and SGPT and increased malonaldialdehyde level production in damage liver [19]. The level of renalenzymes (total bilirubin, alkaline phosphatase, urea, uric acid and creatinine) were significantly increased in infected group as compared to control group. Many studied showed that the renal enzymes induced by vasoactive mediators triggered by bacterial components. Another author reported that in pneumonia infected immunocompetent rats have elevated of bilirubin and creatinine levels [20].

Due to respiratory infections by the distal airways, the phagocytic cells are activated and these activated cells release excess free radicals as a host defence against infection. Peroxidative lipid damage occurred whenever reactive oxygen free radicals are produced, so this damage may be reduced by antioxidant enzymes. Superoxide dismutase (SOD) and catalase are free radical scavenging enzymes that inhibits the generation of free radicals [21]. EDTA controls free radical damage due to lipid peroxidation by serving as a powerful antioxidant [22].

In this study, antioxidant enzymes activities (SOD, Catalase, MDA), SOD and Catalase activities were statistically increased ($p<0.005$) in antibacterial treated group as well as in EDTA / antibacterial treated group when compared with infected group after treatment of seven days. SOD and Catalase activity significantly ($p<0.005$) reduced in EDTA / antibacterial treated group than antibacterial treated group after seven days treatment. MDA level was significantly decreased in both treated group and the level was statistically lowered in EDTA / antibacterial treated group when compared to antibacterial treated group alone **Table 2**.

In pneumonia the initiation, maintenance, and resolution of inflammation is dependent upon the complex network of pro-inflammatory and anti-inflammatory cytokines. This study showed that cytokines were significantly increased due to bacterial infections in diseased control group as compared with control group. Many studies have been showed that the cytokines is important factor for host defense against the invasive pathogen 23. The levels of TNF-α and IL-6 were decreased comparatively more in the combination drug of EDTA / Antibacterial treated group than anti-bacterial alone after seven days of treatment in diseased condition MDA level increases significantly Table 2. *Klebsiella pneumoniae* causes oxidative stress in respiratory infection [24]. During *Klebsiella pneumoniea* infection, the levels of antioxidant can be decreased due to generation of oxidative stress in the lung tissue.

Many studies have shown that Ceftriaxone + Sulbactum have neutrophils inhibition function and free radical scavenger property [16]. Pneumonia infection is treated by the empirical therapy of antibiotics. A combination of antibiotics provides a better and broad spectrum of coverage than any single antibiotic alone. Combination therapy should be synergistic and provides an antibacterial spectrum greater than the sum of individual activities. Several studies reported that combination therapy have a better efficacy than monotherapy in nosocomial pneumonia infection [25].

In this study we also observed that EDTA / Antibacterial showed better free radical scavenger property than antibacterial combination which reduced lung tissue injury.

**CONCLUSION:** To conclude, study showed that a novel of EDTA / antibacterial combination showed better efficacy in terms of antimicrobial and free radical scavenger property by inhibition of the free radical, which causes tissue injury and inflammatory response in nosocomial pneumonia infections in SD rats.

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