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# *In vivo* Antimicrobial Activity of 1(4-(4-chlorobenzyloxy)phenyl)-3-(4-methyl Phenyl)-2-propen-1-ones (Chalcone 2) Against *klebsiella pneumoniae* Isolated from Pneumonia Patients.

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**Abstract:** We investigated the effects of 1(4-(4-chlorobenzyloxy)phenyl)-3-(4-methyl phenyl)-2-propen-1-ones (chalcon) 2 in the treatment of experimental *Klebsilla pneumoniae* in mice. Mice were infected with $5x10^6$ CFU/mL of *K. pneumoniae* nosely by bacterial suspension. After 5 days after infection the total WBC count raised from $4.5\times10^3$ to $11.3x10^3$ cell/μL, when treated with chalcon 2 at MIC concentration 600 μL/mL, the WBC count reduced to 10.5 cell/μL during 12 days from infection, while for untreated mice the total WBC raised from 4.5 to 9.7 cell/μL then 14.4 and 16.3 cell/μL during 12 days from infection. The type of Nutrophil elevated from $3.05\times10^3$ to$10.01\times10^3$ cell/μL then reduced to 9.30 after 8 or 12 days from infection, however for untreated mice the Nutrophil raised from 2.80 to 8.00 to 11.1 then 13.84 for day 0, 5, 8 and 12 from infection. The number of viable bacteria in infected mice only raised from 166 to 500 cell/mL then to 1000 cell/mL, while, when one or two doses of chalcon 2 at a dose 600 μg/mL at day 8 were treated to infected mice, the number of viable bacteria in the blood reduced from 333 to 166 cell/mL. After 15 days from infection the remaining mice for either untreated or treated mice with MIC of chalcon2 were died due to severe infection.

**Key words:** Antimicrobial agents, chalcone 2, *in vivo* and pathogenic bacteria, *Klebsiella pneumoniae*, pneomonia

## INTRODUCTION

Chalcones (1,3-diaryl-2-propen-1-ones) are α,β-unsaturated aromatic ketones, constitute a class of naturally occurring and synthetic compounds belonging to the flavonoid family (Buckingham, 1994) which synthesized by the base catalysed claisen-schmidt condensation reaction (Mirjalil and Zaghaghi, 2008), and considered as a very useful precursor for the preparation of different important heterocyclic compounds. Chalcone derivatives are found to possess a broad spectrum of biological activity, such as anti-malarial (Kolosov *et al*., 2007), anti-bacterial (Cherkupally *et al*., 2008), anti-fungal (Moustafa, 2003), anti-inflammatory (Spivey *et al*., 2000) anti-microbial (Arty *et al*., 2000).

The need for antimicrobial agents is urgent due to increasing resistance of pathogenic bacteria together with increased incidence of severe disseminated infections produced by *Klebsiella pneumoniae* in immunocompromised patients, have prompted to search for new antimicrobial agents, preferably those that can readily and simply be produced considering the increased incidence of severe opportunistic bacteria infections in immunologically deficient patients together with the development of resistance among pathogenic gram positive and gram negative bacteria (Adel and Sabiha, 2010), there is a great need in finding new classes of synthetic products that may be effective against antibiotic resiatant bacteria, there is a great interest search of new antimicrobial agents from the nature (Khider *et al*., 2010; Al-Fatimi *et al*., 2007). The aim of the current study was determine the antimicrobial activity of chalcone 2 *in vivo* against recent clinical *Klebsiella pneumoniae* isolated from pneumonia infected patients in Erbil city hospitals.

## MATERIALS AND METHODS

**Organisms collection:** Eight isolates of *Kliebsella pneumoniae* were isolated from sputum of patients that were infected with *K. pneumoniae* at Komary and Emergency Hospitals in Erbil city, Iraq during august 2010. All isolates identified by cultural, microscobical and biochemical tests. API20E (Biomerienex, Etoil, France) kit were include (Murray *et al*., 1995).

**Antibiotic resistance test:** Antibiotics were used at the following concentrations (μg/mL). Ampicillin (Amp 50), Amikacin (Am 80), Erythromcin (Ery 10), Lincomycin (Lin 10), Chloramphenicol (Chl 30) and kanamycin (Kan 50). The antimicrobial activity of the antibiotics was screened by an agar disk diffusion test. Nutrient agar plates were seeded with *K. pneumoniae* to obtain semiconflucnt growth 10 mm-diameter paper disks of each drug were placed on the seeded plates, and the plates

were incubated overnight at 37ºC. Antimicrobial activity was recorded as the inhibition zone around the disks after incubation (Atlas *et al*., 1995).

**Preparation of chalcone 2:** Melting Point was determined by a Gallen Kamp electro thermal melting point apparatus, IR-Spectrum was recorded on a Bio-Rad Merlin, FT-IR spectroscopy Mod FTS 3000, in which taken as a disc KBr special for spectroscopy, the $^{1}$H-NMR, $^{13}$C-NMR and $^{13}$C-DEPT-135 were taken on a Bruker ultra shield 300 MHz with TMS as internal references, in Al-al-Bayt Central Labs, Amman, Jordan.

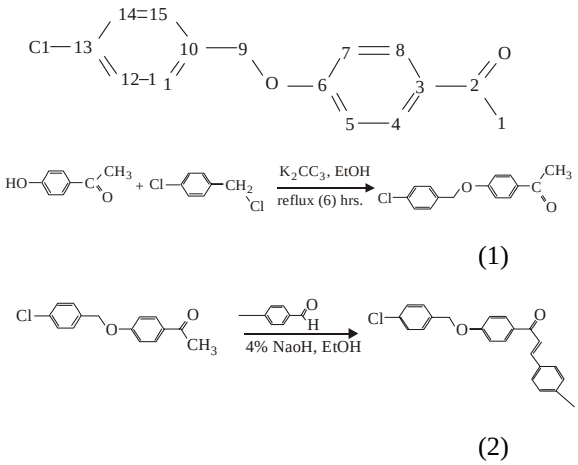
**1-Preparation of 4-(4-chlorobenzyloxy) acetophenone(1):** (Al-Hajjar, 2007). A mixture of 4-hydroxy-acetophenone (13.6 g, 0.1 mol), 4-chlorobenzylchloride (18.3 g, 0.12 mol) and anhydrous $K_2CO_3$ (27.6 g, 0.2 mol), in ethanol (100 mL - 96%) was refluxed with stirring for 6 h. When the reaction is completed, the cooled solution poured into water, solid materials immediately was obtained. The product filtered off, washed several times with water and cold ethanol, dried and recrystallized from ethanol to obtain white crystals of 4-(4-chlorobenzyloxy)acetophenone (1) ($C_{15}H_{13}ClO_2$), m.p. (91-93ºC), and in the yield of (21.8 g, 83.9%).

IR($cm^{-1}$) ; 1666 (C=O) , 1597 (C=C), 1260 and 1175 (C-O-C) .

$^{1}$H-NMR: 2.55(s,3H,$COCH_3$); 5.1(s, 2H, $H_5$); 6.98(d, 2H, $H_{5,7}$);7.37(s, 4H, $H_{11,12,14,15}$); 7.95(d, 2H, $H_{4,8}$) $^{13}$C-NMR: 26.6:$C_1$; 69.32: $C_9$; 114.5.12:$C_{5,7}$; 128.78: $C_{11,15}$; 128.88:$C_{12,14}$;130.6:$C_{4,8}$; 130.7:$C_3$; 134:$C_{13}$; 134.6:$C_{10}$; 162.3:$C_6$; 196.7:$C_2$.

$^{13}$C-DEPT: 26.6:$C_1$; -69.32: $C_9$; 114.5.12:$C_5$, $_7$; 128.78: $C_{11,15}$ ; 128.88:$C_{12,14}$; 130.6:$C_{4,8}$.

**2-Synthesis of chalcone: 1(4-(4-chlorobenzyloxy) phenyl)-3-(4-methyl phenyl)-2-propen-1-ones:** (chalcone 2): (Khan *et al*., 2006) Chalcone (2) was synthesized by dissolving 4-(4-chlorobenzyloxy)-acetophenone (1) (2.6 g, 0.01 mol) in ethanol (25 mL - 96%), and added to the solution of an (4-Methylbenzaldehyde) (0.01 mol) in 96% ethanol (25 mL) and (20 mL) of 4% ethanolic sodium hydroxide. The mixture was stirred at room temperature for (5 min.) until the formation of white- pale yellow crystals of chalcone, and then kept the solution at room temperature for (2 h). Chalcone crystals were separated by suction filtration, washed with ethanol and water to neutralize, dried and purified by recrystalization from ethanol and xylene as a suitable solvents, chalcon (2) ($C_{23}H_{19}ClO_2$), m. p. (192-194ºC), and in the yield of (89.5%). IR ($cm^{-1}$); 1655 (C=O), 1607 (C=C), 1260 and 1175 (C-O-C).




Scheme (1)

**Chemical material chalcone 2 testing:** chalcone 2 was synthesized in the laboratories of Chemistry department, College of Education/Scientific Departmrnts, Salahaddin University, Erbil, Iraq. All organisms were tested by broth microdilution method according to (Atlas *et al*., 1995).

**Determination of MIC:** The anti *Klebsiella pneumoniae* activity of chalcone 2 was carried out by broth microdilution method according to (Atlas *et al*., 1995). Serial delusions of the test material were prepared in saline solution to obtain 1 mg/mL. antimicrobial growth inhibition was determined at 100-1000 µg/mL concentrations. The tubes were inoculated with $1x10^5$ CFU/mL of *K. pneumoniae* and incubated at 37ºC for 24 h. Minimum Inhibitory Concentration (MIC) of the tested material was determined.

**Animals:** Thirty laboratory mice *Mus musculus* (whit albino) (kindly provided by Medical College animal house, Erbil Medical University, Erbil Iraq) that was 7 week old and had a body weight of approximately 150 g were used.

**Acute toxicity:** The acute toxicity of the chemical material was evaluated in 30 normal albino mice. The animals were fasted for 24 h, after which they were treated once orally with MIC dose of the chemical material, the mice were then observed for at least 72 h and up to 7 days, for death, lethargy, jerkiness, sensitivity to noise and touch (Rene *et al*., 2007).

**Treatment protocol:** Mice were divided into 3 groups, each comprising 7 animals.

Group1: was control group
Group 2: was infected with *K. pneumoniae* of $5\times10^6$ CFU/mL suspention

Table 1: Antibiogram groups of *Klebsiella pneumoniae* isolated from pneumonia infected patients

| Antibiogram | Amp | Am | Chl | Ery | Lin | Kan | Isolate no. | No. of resistant antibiotics |
|---|---|---|---|---|---|---|---|---|
| A1 | + | + | - | - | - | - | 1,3,4 | 2 |
| A2 | + | + | - | - | - | - | 5,2 | 3 |
| A3 | + | + | + | - | - | - | 7,8 | 3 |
| A4 | + | + | + | + | + | + | 6 | 6 |

Table 2: The absorbance reading of synthetic chemical material chalcone 2 against *Klebsiella pneumoniae* K6 isolate

| | Concetration of chalcone 2 (μg/mL) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Chemical material | 100 | 200 | 300 | 400 | 500 | 600 | 700 | 800 | 900 | 1000 |
| chalcone 2 | 1.93 | 1.84 | 1.44 | 1.23 | 0.98 | 0.53 | 0.34 | 0.21 | 0.09 | 0.02 |

Group 3: infected with *K. pneumoniae* and treated with MIC of chalcon 2 at a concentration of 600 μg/mL.

**Respiratory tract infection:** Mice were infected by method (Rene *et al*., 2007), in brief mice were anaesthetized with diethyl ether in a special apparatus. Then, mice were intranasal injected with the bacterial suspension of $5x10^6$ CUF/mL for 3 times within 3 days, and each time the injection volume was 500 μL/kg by spatial soft tube was intubated into the mouse through its nose to its pharynx. Through this tube the suspension was injected with a trace injector to each the animal pharynx to simulate inhalation. The concentration of bacteria in the suspension was determined by measuring the amount of absorbance at 600 nm. A standard of absorbencies based on known CFU was used to calculate the inoculums concentration.

**Challenge procedures and blood samples:** At the time of the challenge, all mice were treated with MIC concentration of (chalcon 2) 600 μg/mL at a dose of 7 mL/kg of body weight and were tracheotomized. After 5, 8, 12 and 16 days after challenge, all mice were sacrificed by 20% pentobarbital at 3 mL/kg of body weight, and blood samples were obtained by cardiac puncture.

**Bacterial count:** A standard loop method (Baron and Finegold, 1990) for bacterial colony counts of blood was used. An anichrome loop with an internal diameter of 3 mm, delivery approximately 0.006 mL, was used for inoculation of the samples in the standard loop technique. Approximately fixed and spread it over agar plate. The plates incubated at 37ºC for 24 h, the number of colonies is counted, and this number is used to calculate the number of viable bacteria per milliliter blood.

**White BC count:** The total whit blood cells (WBC) count and differential WBC count were detected according to the method that explained in (Alfred, 2005).

## RESULTS

The in vitro antimicrobial activity of newly synthesized compound chalcone 2 was tested against more selected resistant *K. pneumoniae* K6 isolated from pneumonia infected patients in Erbil Hospitals Iraq (Table 1). The table shows that all *K. pneumoniae* isolates tested were resistant to ampicillin and amikacin at final concentrations. In contrast, one isolate K6 was resist to all tested antibiotics.

In order to determine the minimal inhibitory concentration (MIC), the following concentrations of tested compound were used (100-1000) μg/mL, and all *K. pneumoniae* isolates tested were inhibited by chalcone 2 at concentration of 600 μg/ mL (the MIC was 600 μg/mL) (Table 2).

Acute toxicity of chalcone 2 at concentration of MIC after 48 hr on the survival time and mortality within a week. Almost all treated mice with 600 μg/mL chalcone 2 increased the survival time. Further more, no significant differences of mortality and behavior of the animals within a week could be seen between chalcone 2 treated groups and the control group, also all organs normal when dissected the mice.

All immunological parameters for treated or untreated mice were similar for all groups at zero time after infection, the total WBC was 4.3-4.6, Ne was 2.8-3.05. As shown in the Table 3, 8 days after infection the total WBC, Ne, Ly, Mo, and bacterial number increased in group infected with bacteria, and stile decreasing observed for infected and treated group. Twelve days after infection the all immunological indicators in the blood were increased, while for infected and treated group was the same, moreover 16 days from infection the remaining treated mice with bacteria alone were died. Higher survival rate was observed in the group of mice infected and treated with tested chemical material than in the group of mice infected only with *K. pneumoniae* alone.

Chalcone 2 administration 5 days after to the infection did not prevent deposition of bacteria in the blood. The blood count was similar to those in the infected with bacteria only, mice show raped increasing to 500 viable CFU/mL, while in infected and treated remain 333 CFU/mL, 12 days after infection the blood of mice treated with chalcon 2 and infected with *K. pneumoniae*, the number was retracted and reached to 166 CFU/mL, in infected treatments only were 1000 CFU/mL.

**Gross pathological finding in the lungs:** On days 8 and 12 all of the lungs from infected animals showed strong

Table 3: Some immunological characteristics and number of viable bacteria in blood of infected mice with *Klebsiella pneumoniae* K6 isolate

| Days after infection | Treatments | $TWBC_s X 10^3$ | Ne X $10^3$ | LY X $10^3$ | Mo X $10^3$ | Eo X $10^3$ | Ba X $10^3$ | No. of bacteria mL blood |
|---|---|---|---|---|---|---|---|---|
| 0 | A | 4.60 | 3.05 | 1.18 | 0.38 | 0.0 | 0.0 | 0.0 |
| | B | 4.30 | 2.80 | 1.08 | 0.34 | 0.09 | 0.0 | 0.0 |
| | C | 4.50 | 3.05 | 1.17 | 0.38 | 0.0 | 0.0 | 0.0 |
| 5 | A | 4.60 | 3.05 | 1.18 | 0.38 | 0.0 | 0.0 | 0.0 |
| | B | 9.70 | 8.00 | 1.02 | 1.00 | 0.0 | 0.0 | 166 |
| | C | 11.3 | 10.01 | 1.18 | 0.10 | 0.0 | 0.0 | 333 |
| 8 | A | 4.6 | 3.10 | 1.18 | 0.38 | 0.0 | 0.0 | 0.0 |
| | B | 14.4 | 7.13 | 0.62 | 0.69 | 0.06 | 0.0 | 500 |
| | C | 10.5 | 9.30 | 1.17 | 0.12 | 0.0 | 0.0 | 333 |
| 12 | A | 4.60 | 3.05 | 1.18 | 0.38 | 0.0 | 0.0 | 0.0 |
| | B | 16.3 | 13.84 | 1.32 | o.95 | 0.10 | 0.0 | 1000 |
| | C | 10.5 | 9.31 | 1.13 | 0.12 | 0.0 | 0.0 | 166 |
| 15 | A | 4.60 | 3.05 | 1.18 | 0.38 | 0.0 | 0.0 | 0.0 |
| | B | -- | -- | -- | -- | -- | -- | -- |
| | C | -- | -- | -- | -- | -- | -- | -- |

A: Control; B: Infected mice with *K. pneumoniae* K6 isolate; C: Infected mice with *K. pneumoniae* K6 isolate and treated with MIC concentration of synthetic chemical material chalcone 2; $TWBC_s$: Total White Blood Cells; Ne: Neutrophile; Ly: Lymphosite; Mo: Monosite; Eo: Eosinophil; Ba: Basidohpile

inflammatory changes, which were often accompanied by formation of small multiple abscesses. Abscess formation was less common and inflammatory changes were moderated in the lungs of mice infected and treated with chalcone 2. In control mice (not infected group) no change notes during the experimental time.

## DISCUSSION

Infections, especially acute bacterial pneumonia are common and are major of morbidity and mortality in immunocompromised patients. In this study, we investigated the effects of a synthesized chemical material chalcon 2 in mice. Chalcone 2 is a chemical material prepared by Department of Chemistry, college of Education/Scientific departments, Salahaddin University Erbil, Iraq, and has been reported during production to be effective against some bacterial infections. The assayed doses represented $5\times10^6$ CFU/mL since the chalcone 2 at MIC did not provoke any change in the behavior of animals, the reagent may be considered safe for medical uses.

The bacteriological findings in the blood of the mice infected with *K. pneumoniae*, and treated with chalcone 2 has clear effects in prophylaxis and in treatments of acute pulmonary Klebsiella infections. Prophylactic use of chalcone 2 treatment in particular brought about the results. After 5 days of infection, the symptom of disease appeared on infected mice, such as swallowing, lung raised to out of the body, and become weak. The mice were infected nosily with $5\times10^6$ CFU/mL, and the total WBC count raised from $4.5\times10^3$ to $11.3x10^3$ cell/µL as shown in Table 3, the type of leukocytes elevated, Neutrophil raised from $3.05\times10^3$ to $10.1\times10^3$ cell/µL. The type of WBC used as immunological parameters to determine the case of infection (Provan *et al*., 2004). While normal range was $4.2\times10^3$ cell/µL (Hoffman *et al*., 2000 and Provan *et al*., 2004), because the main type of phagocytic cells which is required to participate in the phagocytosis in the ingestion of foreign bodies are Neutrophil and Macrophage (Kevn, 2002; Henderson and Oyston, 2003; Evnest and Stendahl, 2006), so during infection with bacteria the range of Nutrophils increased comparing with the control. While Basophil and Eosinophil are role in immunity, Eosinophil increasing in cancer and parasitic infections (Bain and Flower, 1996), high level of Basophils generally corresponds to an active allergic response (Wikipedia, 2010).

Further studies should be under taken to clarify how chalcone 2 produce protective effects against microbial infections. However, it is already known that chalcone 2 decreased the number of *K. pneumoniae* cells in the blood gradually Table 3, in infected mice and treated with chalcone 2, while the number of viable cells in the blood of infected mice with *K. pneumoniae* only were increased from 166 CFU/mL at day 5 to 1000 CFU/mL at day 12 from infection. Effective host defense against bacterial blood infection is primarily dependent on the raped clearance of microorganisms from the blood treated (Sibille *et al*., 1990). Further more, studies now under way to evaluate the efficiency of chalcone 2 in combination with other antibiotics and study its toxicity and its mutagencity.

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