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Synthesis and Antimicrobial Ctivity of 3-(4-(4-chlorobenzyloxy)phenyl)-1-phenyl-5-(2-chlorophenyl) Pyrazoline (3) on *E. coli* in Mice

[1]Adel K. Khider, [2]Farouq E. Hawaiz, [3]Sarwen O. Taha and [2,4]Mohammed S. Ahmed
[1]Biology Department,
[2]Chemistry Department, College of Education/Scientific Departments,
University of Salahaddin, Erbil, Iraq
[3]College of Education, University of Soran, Erbil, Iraq
[4]Jolamerg Basic School, Directorate of Hawler Education, Genera Directorate of Hawler Education, Erbil, Iraq

Abstract: The *in vivo* antimicrobial activity of synthesized compound Pyrazoline 3-(4-(4-chlorobenzyloxy) phenyl)-1-phenyl-5-(2-chlorophenyl) pyrazoline (3) against *E. coli* ATCC25922 strain was investigated. *E. coli* ATCC25922 strain was inhibited at concentration of 400 μg/mL. Acute Toxicity of pyrasoline, cleared that all treated mice with 400 μg/mL increase the survival time, and no significant differences of mortality and behavior of animals within a week could be seen between pyrasoline treated group and control group. Twelve days after infection, all immunological indicators in the blood were increased for infected mice, while for infected and treated was the same as day seven. Sixteen days from infection all immunological parameters for infected mice were increased, while for infected and treated group was decreased. The remaining mice infected with bacteria alone died. Survival rate was observed in the group of mice infected and treated with tested chemical compound.

Key words: Antibiotic resistant, antimicrobial, *E. coli, in vivo,* pathogenic bacteria, pyrazoline

INTRODUCTION

Antimicrobial compounds have become indispensable to the current health care system assisting and are complementing the natural immune system against microbial infections. However, because conventional antibiotics are offend abused to treat microbial infections, because microorganisms have developed resistance to these antimicrobials, due to easily transfer of genetic materials among pathogenic bacteria by transformation, conjugation or transduction and appearance of new antimicrobial resistance strains, the continuous development of novel antimicrobial agents is more crucial than ever (Lee and Lee, 2008; Nassar, 2010; Khider, 2010). Therefore, the medical community faces problems against infections caused by pathogenic bacteria, and the need for an effective therapy and search for effective antimicrobial agents (Neela *et al*., 2009). There is a real perceived need for the discovery of new compounds endowed with higher antimicrobial properties and lower risk.

Pyrazolines are an important class of heterocyclic compounds that attracted considerable attention due to their significicant biological activity which includes potential application as, anti-inflammatory (Hoffman *et al*., 2007), antimicrobial activity (Habib *et al*., 2010; Khider 2011), antifungal (Nassar, 2010), anti-tumor (Abunada *et al*., 2008), anti-histamic (Sridevi *et al*., 2009), anti-depressant (Li *et al*., 2007) and anti-viral activities (Hajos, 2002 ; Alam and Mostahar, 2005).

In the present study, the synthesis and antimicrobial activity of pyrazoline (3) against *in vivo Escherichia coli* was investigated in mice.

MATERIALS AND METHODS

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 *E. coli* 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).

Determination of MIC: The anti Escherichia coli activity of pyrazoline 3 was carried out by broth microdilution method according to (Atlas *et al*., 1995). Serial delusions

Corresponding Author: Adel Kamal Khider, Biology Department, College of Education/Scientific Departments, University of Salahaddin, Erbil, Iraq

of the tested material was 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 1×10^5 CFU mL of *Escherichia coli* and incubated at 37ºC for 24 h. Minimum Inhibitory Concentration (MIC) of the tested material was determined.

Preparation of pyrazoline(3): Melting Points were determined by a Gallen Kamp electro thermal melting point apparatus, IR-Spectra were recorded on a Bio-Rad Merlin, FT-IR spectroscopy Mod FTS 3000, in which solid materials were 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 (Jordan).

1-Preparationof 4-(4-chlorobenzyloxy)acetophenone(1) (Hoffman *et al*., 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 hours. 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 gm, 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:$C_{4,8}$.

Synthesis of chalcone: 1(4-(4-chlorobenzyloxy) phenyl)-3-(2-chlorophenyl)-2-propen-1-ones: (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 2-chloro benzaldehyde (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 suitable Solvents,

($C_{22}H_{16}Cl_2O_2$), m.p. (138-140ºC), and in the yield of (92%)
IR (cm^{-1}); 1660 (C=O), 1608 (C=C), 1260 and 1175 (C-O-C)

Synthesisofpyrazoline3-(4-(4-chlorobenzyloxy)phenyl)-1-phenyl-5--(2-chlorophenyl) pyrazoline (3)(Yar *et al*., 2009): A mixture of phenyl hydrazine (0.16 g, 0.0015 mol), chalcone (2), (0.001 mol) and sodium hydroxide (0.001 mol) in (25 mL - 96%) ethanol was refluxed with stirring about (6 h) until complete the reaction which was monitored by the formation of ppt. of the pyrazoline product (3). The ppt. was isolated by suction filtration, washed with ethanol and water to neutralize, dried and purified by recrystalization from (xylene-ethanol) as suitable double solvent. 3 ($C_{28}H_{22}Cl_2N_2O$), m.p. (169-171ºC), and in the yield of (58%). IR(cm^{-1}) ; 1608 (C=C), 1597 (C=N) 1260 and 1175 (C-O-C) (Fig. 1).

Bacterial strain: The standard strain Escherichia coli ATCC25922 Kindly provided by Media Diagnostic Center, Erbil, Iraq was used as infected bacteria at concentration of 6×10^6Cell/mL according to (Song *et al*., 1997).

Animals: The 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 as experimental units. Complete Randomized Design was used, and each comprising six mice for each treatment as follows: Group A: The control treatment did not receive any bacteria or therapy. Group B: Resaved the bacterial suspension of standard strain E. coli of 6×10^6 CUF/Ml suspension. Group C: Mice were infected with *E. coli* and treated with MIC of pyrazoline at a concentration of 400 μg/mL. The investigation performed in medical College animal house, and Komary Hospital Laboratories, Erbil city during June-Augest, 2011.

Acute toxicity: The acute toxicity of pyrazoline 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 pyrazoline, the mice were then observed for at least 72 h and up to 7 days, for death, lethargy, jerkiness, sensitivity to noise and touch.

Infection process: The mice were infected according to the method (Rene, 2007), in brief, the mice were infected with the bacterial suspension of 5×10^6CUF/mL orally for 3 times within 3 days, and each time the volume was 500 μL/kg by spatial soft tube was intubated directly into the

stomach. 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 pyrazoline 400 μg/mL at a dose of 7 ml/kg of body weight and were tracheotomized. After 7, 12, 16 and 20 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. Anchrome 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 White Blood Cells (WBC) count and differential WBC count were detected according to the method that explained in (Alfred, 2005).

RESULTS

The new synthesized compound pyrazoline 3-(4-(4-chlorobenzyloxy) phenyl)-1-phenyl-5-(2-chlorophenyl) pyrazoline(3) (Fig. 1) show *in vitro* antimicrobial activity against *Escherichia coli* ATCC25922 strain, Table 1 show the Minimal Inhibitory Concentration (MIC) of pyrazoline compound, the following concentrations of tested compound were used (100-1000) μg/mL, tested *E. coli*

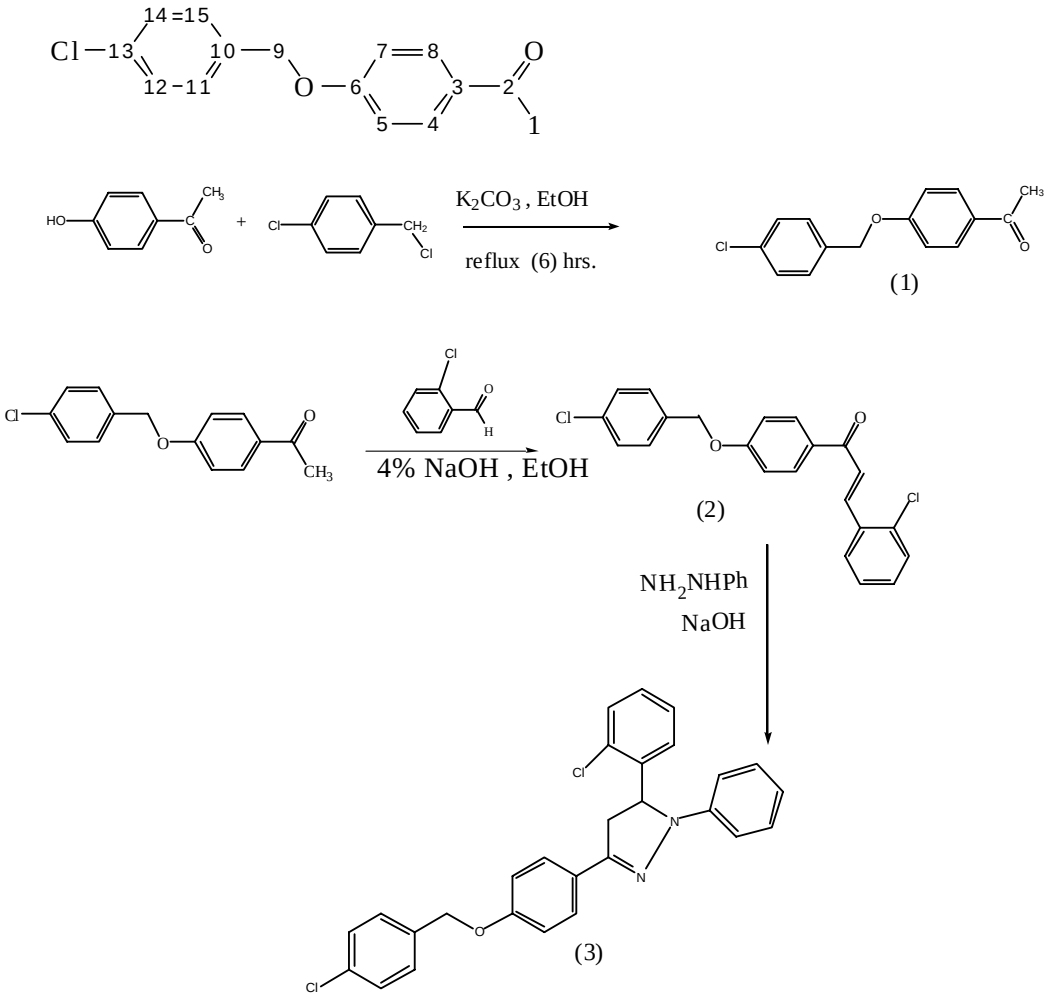

Fig. 1: Preparation of pyrazoline(3)

Table 1: The absorbance reading of synthetic chemical material pyrazoline against *E. coli* isolate

	Concetration of pyrazoline (μg/mL)									
Chemical material	100	200	300	400	500	600	700	800	900	1000
Pyrazoline	1.12	1.08	0.75	0.28	0.15	0.12	0.10	0.04	0.007	0.004

Table 2: Some immunological characteristics and number of viable bacterial cells in blood of infected mice with *E. coli*.

Days after infection	Treatments	TWBCs $\times 10^3$	Ne $\times 10^3$	LY $\times 10^3$	Mo $\times 10^3$	Eo $\times 10^3$	Ba $\times 10^3$	No. of bacteria per mL blood
0	A	4.60	3.05	1.18	0.38	0.0	0.0	0.0
	B	4.3	2.80	1.08	0.34	0.09	0.00	0.00
	C	4.5	3.05	1.17	0.38	0.00	0.00	0.00
7	A	4.6	3.05	1.18	0.38	0.00	0.00	0.00
	B	12.4	10.97	1.18	0.32	0.00	0.00	166
	C	10.8	9.32	1.18	0.30	0.00	0.00	0.00
12	A	4.6	3.05	1.18	0.38	0.00	0.00	0.00
	B	13.00	11.88	1.19	0.03	0.00	0.00	500
	C	10.4	9.31	1.17	0.01	0.00	0.00	0.00
16	A	4.6	3.05	1.18	0.38	0.00	0.00	0.00
	B	13.5	11.17	1.13	0.74	0.00	0.00	500
	C	10.0	9.02	1.07	0.01	0.00	0.00	0.00
20	A	4.6	3.05	1.18	0.38	0.00	0.00	0.00
	B							
	C							

A: Control; B: Infected mice with E. coli strain; C: Infected mice with *E. coli strain* and treated with MIC concentration of synthetic chemical material pyrazoline; $TWBC_s$: Total White Blood Cells; Ne: Neutrophile; Ly: Lymphosite; Mo: Monosite; Eo: Eosinophil; Ba: Basidohpile

were inhibited by pyrazoline at concentration of 400 μg/mL.

Acute toxicity of pyrazoline at concentration of MIC on the survival time and mortality within a week was tested. Almost all treated mice with 400 μg/mL pyrazoline increased the survival time. Furthermore, no significant differences of mortality and behavior of the animals within a week could be seen between pyrazoline treated group and the control groups all organs normal when dissected the mice. After seven days of infection, the symptom of disease appeared on infected mice, such as swallowing anus, and all immunological parameters for treated or untreated mice were similar for all groups at zero time after infection, the total WBC and neutrophil were 4.3×10^3 and 2.80×10^3 cell/μL, respectively Table 2. As shown in the Table 2, 7 days after infection the total WBC, NE, Ly, and bacterial number increased in group infected with bacteria or infected and treated with pyrazoline comparing with the control. Twelve days after infection the all immunological indicators in the blood were increased for infected mice, while for infected and treated group was the same as day seven. Sixteen days from infection all immunological parameters for infected mice were increased, while for infected and treated group decreased, moreover the remaining treated mice with bacteria alone 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 E. coli alone.

Pyrazoline administration 7 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 after 12 days from infection, while in infected and treated remain 166 CFU/mL, 16 days after infection the blood of mice treated with pyrazoline and infected with *E. coli*, the number was retracted and reached to zero CFU/mL, in infected treatments only were 500 CFU/mL.

DISCUSSION

In this study, we investigated the effects of a synthesized chemical material pyrazoline in mice. Pyrazoline is a chemical material prepared by the Department of Chemistry, college of Education/Scientific departments, Salahaddin University Erbil, Iraq, and has been reported during production to be effective against some pathogenic bacterial. The assayed doses represented 5×10^6 CFU/mL since the pyrazoline 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 infected orally with 5×10^6 CFU/mL, *E. coli*, and treated with pyrazoline have clear effects in prophylaxis and in treatments of acute bacterial infections. Prophylactic use of pyrazoline treatment in particular brought the results. After 7 days from infection, the symptoms of disease appeared on infected mice, such as swallowing, and become weak. The total WBC count raised from 4.5×10^3 to 12.4×10^3 cell/μL and 10.8 CFU/mL, for infected with bacteria only and infected and treated with pyrazoline, respectively (Table 2), the type of

Neutrophil raised from 3.05×10^3 to 10.97×10^3 cell/ μL. and 9.32 cell/μL for infected with bacteria only and infected and treated with pyrasoline, respectively. The type of WBC_s used as immunological parameters to determine the case of infection (Provan *et al*., 2004). While normal range was 4.2×10^3 cell/μL (Hoffman *et al*., 2000; 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 (Henderson and Oyston, 2003; Evnest and Stendahl, 2006), so during infection with bacteria the range of nutrophils increased compared to the control. While basophil and eosinophil have a role in immunity, eosinophil is increasing in cancer and parasitic infections (Bain and Flower, 1996). After 12 days from infection the TWBC count elevated to 13.0 in infected mice with bacteria only and 13.5, after 16 days from infection, while for infected and treated with pyrazoline the TWBC decreased to 10.4 then 10 after16 days from infection. However, pyrazoline treated infected mice decreased the number of bacteria in the blood of mice compared to the infected with *E. coli* and untreated or uninfected mice. The number of viable bacterial cells in the blood of infected mice with *E. coli* only were increased from 166 CFU/mL at day 7 to 500 CFU/mL at day 12 from infection, and the number remain 500 CFU/mL at 16 days after infection, while in infected mice with *E. coli* and treated with MIC concentration of pyrasoline the number of viable bacteria in the blood not exceeding 500 CFU/mL and remain 166 at day 12 from infection, then decreased to zero at 16 days. All remaining mice infected with bacteria or infected and treated with pyrazoline died at day 20, may be due to severe infection or due to effect of pyrazoline. The present study forms the basis for further investigations to clarify the mechanisms involved in this action and evaluation of toxicity of these compounds are warranted.

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