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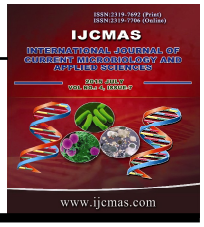


Original Research Article

Comparative Study of some Beta-Lactam Antibiotics Efficacy in Curing Resistant *Escherichia coli* Infection

Nageh EL-Mahdy[1], Tarek El-Banna[2], Ahmed Abd El-Aziz[3] and Yasmine Samy[4*]

[1]Professor of Pharmacology, Pharmacology Department, Vice Dean of Faculty of Pharmacy, Tanta University, Egypt
[2]Professor & Chairman of Pharmaceutical Microbiology Department, Faculty of Pharmacy, Tanta University, Egypt
[3]Professor of Pharmaceutical Microbiology Department, Faculty of Pharmacy, Tanta University, Egypt
[4]Pharmacist at General Mahalla Hospital, El-Mahalla El-Koubra, Egypt
**Corresponding author*

Keywords

Comparative, Efficacy, Beta-Lactam, Antibiotics, Resistant, *E. coli*

A B S T R A C T

Increased bacterial resistancy especially gram negative bacteria, impairs curing of some infections. On the other hand various studies showed that some antibiotics have efficacy in curing resistant bacterial infections. On account of that it was interesting to study efficacy of some beta lactam antibiotics (cephradine and ampicillin) and/or tenoxicam in curing wound infection caused by multiple antibiotic resistant (MAR) *Escherichia coli* (*E.coli*) and exploring other possible mechanisms irrespective of the antibacterial activity. This was performed by screening MAR strains of *E.coli in vitro* (agar dilution) and *in vivo* (bacterial infected rat wound and carrageenin acute inflammatory) models for assessing possible mechanisms of efficacy of tested antibiotics alone and in combination with standard nonsteroidal anti-inflammatory drug (tenoxicam), in curing resistant bacterial wound infection. This was evaluated by measuring immunological mediators and histopathological examination. This study revealed that *in vivo* cephradine and ampicillin alone or in combination with tenoxicam significantly improved healing of infected skin wounds with *E.coli* despite of resistancy *in vitro*, by improvement of immunological mediators involved in inflammatory reaction, oxidative stress and cytokines expression as response to bacterial infection. *In vitro* tenoxicam didn't have any antimicrobial activity alone nor in combination with tested antibiotics, but it was synergistically increasing efficacy of tested antibiotics when used in combination with it *in vivo*. Thus we concluded that immunomodulatory activity of cephradine and ampicillin through anti-inflammatory and antioxidant effects (beyond its antimicrobial activity) are the possible mechanisms by which these antibiotics have healed *E.coli* infections, despite its resistancy to those antibiotics. In addition to using these antibiotics in combination with tenoxicam synergistically increases their efficacy.

Introduction

Antibiotics considered the bulwark to combat bacterial infections, but unfortuntely the spread of antibiotic resistance compromises their clinical efficacy (Imperi *et al.,* 2014). Elevation of gram-negative bacteria multidrug resistance, presents a critical problem with increasing danger to human health. Limited therapeutic options have forced infectious disease clinicians and microbiologists to reappraise the clinical application of old antibiotics. So that recent clinical findings concerning with evaluation of efficacy, potential toxicities and combination therapy (Li *et al.,* 2006; Lee *et al.,* 2013).

On the other hand various studies have reported that some antibiotics have curing activity of infections caused by resistant bacteria despite of absence of susceptibility to those antibiotics, due to other mechanisms beyond their antimicrobial activity (Tsai *et al.,* 2009; Imperi *et al.,* 2014). For instance the macrolide showed beneficial efficacy in treatment of diffuse panbronchiolitis (DPB) patients with chronic bacterial infections with *Pseudomonas aeruginosa* of the lower respiratory tract, despite its lack of activity for *Pseudomonas* spp., which seems to be mainly related to suppression of the tissue response rather than an antimicrobial effect (Schultz *et al.,* 2001; Nagata *et al.,* 2004).

Consequently the potential benefits of downregulating immunomodulators entered the limelight, with the understanding that immune hyperactivation such as in sepsis, can have disastrous consequences (Labro, 2011). The immunomodulatory properties of various antibacterial agents were demonstrated *in vitro* and *in vivo*, such as fluoroquinolones and tetracyclines (Choi *et al.,* 2003; Wise 2007; Abelson *et al.,* 2008; Labro, 2012*)*. Thus the knowledge of the effects of antibiotics on the immune response allows researchers to see the drugs known for years in a new light (Kwiatkowska *et al.,* 2013). Concurrently wound infections are associated with increased morbidity and mortality irrespective of the cause of the wound (Alexander, 1994; Akinjogunla, *et al.,* 2009). Surgical wound infections were dominated compared to other wound infections (Nazeer *et al.,* 2014). *Escherichia coli* is one of the most commonly isolated microorganisms from wound infections (Bhadauria & Hariharan, 2013; Nazeer *et al.,* 2014). *E.coli* MAR mutants are resistant to a wide variety of antibiotics (Okusu *et al.,* 1996). In addition the bacterial biofilm is considered the major concern for clinicians in the treatment of infectious diseases, because of the resistance to a wide range of antibiotics (Kobayashi, 1995; Nagata *et al.,* 2004). Therefore, adjustment of the given dose of antibiotics is critical in removal of biofilm (El-Banna *et al.,* 2010). So that animal model of wound colonization which mimmicking wound infection is useful to study the wound infections and monitor the effect of therapeutic agents *in vivo* (Asada *et al.,* 2012). Thus this study aimed to investigate efficacy of cephradine and ampicillin alone and in combination with tenoxicam and evaluating the possible mechanisms of these antibiotics beyond their antimicrobial activity in curing resistant *E.coli* infected wound using *in vivo* rat model.

Materials and Methods

Antimicrobial agents: Cephradine (Squeeb, USA) and Ampicillin (Sigma, USA) were used.

Bacterial strains: A total of thirty clinical isolates of *E.coli* were obtained from

Microbiology Department**,** Faculty of Pharmacy, Tanta University, Egypt. Purity and identity of the clinical isolates were achieved by further identification, according to standard methods directed by (Koneman *et al.,* 2006).

Reference strain: *Escherichia coli* ATCC 25922 standard strain was obtained from NAMRU3, Cairo, Egypt.

Animals: A total of 136 adult male albino rats obtained from the animal house of the National Research Center (NRC), Egypt, weighing from 150-200 g were used in this study. They were fed standard pellet chow (El-Nasr Chemical Company, Cairo, Egypt) and allowed free access to water. Animals were housed in the same conditions for one week prior to the experiment for acclimatization and they were fasted for 12 hr before experimental procedures but allowed free access to water.

Kits: Enzyme Linked Immunosorbant Assay (ELISA) was utilized to examine the concentration of cytokines (Tong *et al.*, 2011). ELISA kit for tumor necrosis factor-alpha (TNF-α) (AssayMax, Ireland) and Interleukin-1 Beta (IL- 1β) (ELISA) kit (Cusabio Biotech, China) were used.

Drugs used in research: tenoxicam used as standard non steroidal anti-inflammatory drug (NSAID) and vitamin C used as standard antioxidant drug for comparison.

Chemicals: Bis-(3-carboxy-4-nitrophenyl) disulphide (Ellman's Reagent) (Sigma, U.S.A); Carrageenan sodium (Sigma,USA); Congo red (Sigma, USA); Crystal violet (Beecham, England); Dimethyl sulphoxide (DMSO) (BDH, England); Ethanol (Prolabo, France); Ferric chloride (BDH, England); Glacial acetic acid (Prolabo, France); Hydrochloric acid (HCL) (Prolabo, France); Hydrogen peroxide (Adwic, ARE); Hematoxylin and Eosin (H&E) (Sigma, USA); Iodine (BDH, England); Methyl red (Adwic, ARE); Monohydrogen disodium phosphate (BDH, England); n-Butanol (Prolabo, France); N-(1-naphthyl) ethylene-diamine Dihydrochloride (Sigma, U.S.A); Phenol (Sigma, USA); Potassium iodide (Prolabo, France); Reduced Glutathione (GSH) (BDH, England); Safranine (Beecham, England); Sodium chloride (Adwic, ARE); Sodium hydroxide (NaOH) (Sigma, USA); Sodium nitrite (El-Nasr, Egypt); Sulfanilamide (Winlab, England); Sulfosalicylic acid (Fluka Biochemica, Switzerland); 1,1,3,3- Tetramethoxy propane (Sigma, U.S.A); Thiobarbituric acid (Sigma, U.S.A); Thiopental sodium (Eipico, ARE); Trichloroacetic acid (Winlab, England); Vanadium (III) chloride (Sigma, U.S.A).

Methods

Screening of highly resistant strains of bacterial clinical isolates: This was performed through determination of MIC of the tested antibiotics against all isolates by agar dilution method according to the procedure described by Cursino *et al.,* (2005); CLSI (2010). MIC breakpoints for *E.coli* (CLSI, 2010): cephradine R ≥ 32 and ampicillin R ≥ 32.

Investigation the effects of tenoxicam/antibiotics combinations against the selected MAR *E. coli* strain: This was achieved by determination of MIC of selected antimicrobial agents in the presence of serum plasma concentration of tenoxicam (30 μM) (Paino *et al.,* 2005), its ½ fold, its 10 fold and its 100 fold against corresponding concentrations of MIC, ½ MIC, ¼ MIC, 1/8 MIC of the tested antibiotics, on MAR *E.coli* strain. This was performed using agar dilution method

according to the procedure described by Cursino *et al.,* (2005); CLSI (2010). The fractional inhibitory concentration (FIC) was used to interpret the results of agar dilution method and calculated according to Makay *et al.,* (2000); Odds, (2003) as follows:

$$\text{FIC of antibiotic A} = \frac{\text{MIC of antibiotic A in combination with tenoxicam}}{\text{MIC of antibiotic A alone.}}$$

The interaction was recorded as synergism (S) when FIC $\leq$ 0.5, indifference (I) when FIC > 0.5 to 4, and antagonism (A) when FIC > 4.

Studying the efficacy of tested antibiotics, tenoxicam and their combinations on healing of resistant bacterial infected wound in animal model: This was achieved as described by Lau *et al.,* (2009).

A total of 56 adult male rats (about 150 gm/each) were used in this model, they were divided into 1 naiive; 1 infected control and 5 treatment groups, each group was composed of eight rats. one rat of each of the 7 groups was sacrified on day 3 of the experiment for the histopathological studies to confirm the formation of the bacterial biofilm within the wound bed.

Formation of wound in rat hind paw: On the day of wound induction (defined as day 0) each rat was anesthetized with an intraperitoneal injection (I.P) of 50 mg/kg thiopental sodium (Abd El-Aziz *et al.,* 2010). A rectangular pattern was marked on the dorsal surface of the foot using a flexible transparent plastic template, and then a layer of skin in full thickness with standard area of 2 mm x 5 mm was removed, Figure (1). The initial wound size was measured on day 1 (Lau *et al.,* 2009).

Bacterial inoculum and induction of wound infection: The bacterial inoculation was carried out one day after the wounding development to reduce mortality due to bacteremia in the bleeding phase (Asada *et al.,* 2012).

Overnight culture of highly resistant *E.coli* strain was used to inoculate the wound in rat hind paw. After bacterial inoculation, wounds were left for 48 hrs to allow biofilm formation (Davis *et al.,* 2008).

Antimicrobial therapy and dosage regimen: All tested antimicrobial agents and tenoxicam, each alone and in combinations were administered I.P. The 5 treated animal groups in addition to the two: naiive and infected control groups were handeled as follows:

I- Naïve group: It consists of normal rats not receiving any treatment.

II- Infected control group: Included animals infected with selected MAR *E.coli* strain and left 9 days post infection without treatment.

III- Tenoxicam treated group: This group of rats were treated with tenoxicam (standard NSAID), in a dose of (20 mg/kg) (Naziroğlu *et al.,* 2008).

IV- Cephradine treated group: animals treated with cephradine (1600 mg/kg) (Amacher *et al.,* 1991).

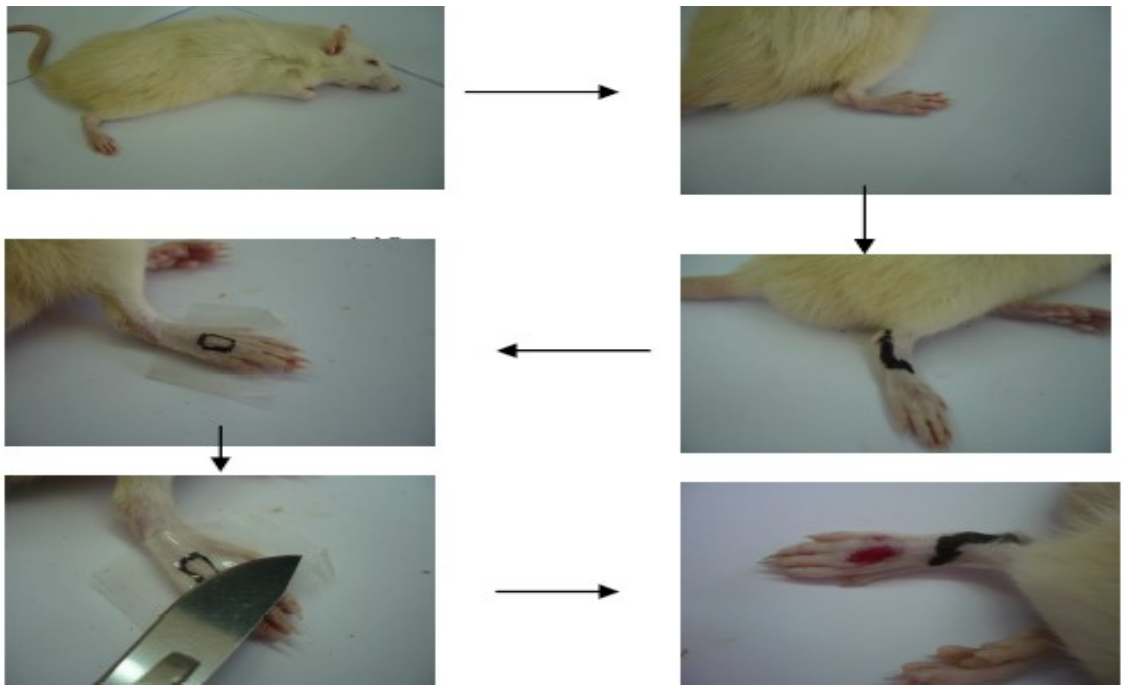
V- Cephradine/tenoxicam combination group: animals treated with cephradine (800 mg/kg)/tenoxicam (10 mg/kg) combination.

VI- Ampicillin group: animals were treated with ampicillin (1000 mg/kg) (Halpert *et al.,* 1985).

VII- Ampicillin /tenoxicam combination group: animals were treated with ampicillin (500 mg/kg)/tenoxicam (10 mg/kg) combination. The antibiotics were given to the treated groups in equally divided doses at 6- hours intervals and the tenoxicam was given once daily for 9 days.

Evaluation of wound healing: Observations of wound surface provided information on the gross extent of wound healing and improved wound healing by facilitating tissue regeneration (Lau *et al.,* 2009).

Figure.1 Steps of making wound on the dorsal surface of the rat hind paw



In this study we selected the one-point sampling method because of the relatively small size of wounds that can be induced in rats and to better avoid contamination with peri-wound flora (Abd El-Aziz *et al.*, 2010).

Digital photographs for the wound were taken on days 1, 3, 6 and 11. Wound area measurement were made by using the digital photographs of the wounds. Perior to picture taking, the rat hind paw was gently pinched manually, so that the wound site was positioned at the intersection of the vertical and horizontal grid lines on the graphic paper on to which vertical and horizontal rulers had been fixed (Lau *et al.*, 2008), which allowed us to enlarge the photograph in order to increase the accuracy of wound area measurement and the photographs were processed by using Microsoft Word where the wound area was traced and measured on the scale present in the same photograph (Abd El-Aziz *et al.*, 2010).

Assessment of possible mechanisms of tested antibiotics beyond their antimicrobial activity using acute inflammatory animal model

This was done through induction of acute inflammation in rats to evaluate anti inflammatory and anti-oxidant activity of tested antimicrobials beyond their antibacterial activity, using recommended doses of tested antibiotics in the model of carrageenan induced hind paw oedema in rats. Carrageenan-induced paw inflammation rat model was used for inflammation study and evaluation of anti inflammatory activity (Lau *et al.*, 2009; Tasleem *et al.*, 2014). Carrageenan is sulfated polysaccharide derived from marine algae (Wang *et al.*, 2012). This model was performed according to method adopted by Winter *et al.*, (1962).

Animal groups of carrageenin acute inflammatory model: A total of 80 rats (about 150 gm/each), were used in this

model. Antibiotics were administered I.P prior to carrageenin injection. Rats were allocated into the following groups:

i- Naïve group: Including normal animals without any treatment but were injected with (0.1 ml) of saline only into the subplantar region of the right hind paw of rats and serve as control group.

ii- Carrageenan control group: Rats were slightly anesthetized with ether and 0.1 ml of 1.5% (carrageenan sodium solution in 0.9 % saline) was injected S.C into the subplantar region of the right hind paw of rats. Thus oedema will be produced acutely into the right hind paw of the rat, and the left hind paw (used as self control), was injected with similar volume (0.1 ml) of saline only. Those rats did not receive any treatment.

iii- Tenoxicam treated group: Tenoxicam (20 mg/kg,) I.P (Naziroğlu *et al.,* 2008), was taken 30 minutes before carrageenan injection (Al-Arfaj *et al.,* 2003).

iv- Vitamin C treated group: Vitamin C has antioxidant and anti-infalmmatory activities. It was taken I.P in a dose of 500 mg/kg (Ikeda, *et al.,* 2004; Kanter, *et al.,* 2005) used for comparison.

v- Cephradine treated groups: It is divided into three subgroups according to cephradine concentrations (1600, 800, 400 mg/kg respectively) I.P.

vi- Ampicillin treated groups: It is divided into three subgroups according to ampicillin concentrations (1000, 500, 250 mg/kg respectively) I.P.

Assessment of immunological mediators

In both animal models (*E.coli* infected wound and acute inflammation by carrageenin), the immunological mediators that resulted in response to the bacterial infection and acute inflammation were assessed, such as tumor necrosis factor factor-α (TNF-α), interleukin 1–β (IL-1β) (in rat serum using ELISA kits); nitric oxide (NO); glutathione (GSH) and malondialdhyde (MDA) (in rat hind paw tissue according to standard methods directed by (Miranda *et al.,* 2001; Ellman, 1959; Yoshioka *et al.,* 1979 respectively).

Histopathological examination

Histopathological Examination of rat hind paw sections through sectioning and staining with Hematoxylin and Eosin (H&E) was achieved using standard methods adopted by (Bancroft & Stevens, 1975).

In bacterial infected wound animal model, biopsy specimens were obtained 48 hours after inoculation and colonization to ensure the biofilm formation, and at the end of the treatment of each group in addition to naiive and infected control group for examination with light microscope. Specimens were placed in buffered 10 % formaldehyde for fixation and stained with H&E and Gram crystal violet (Kugelberg *et al.,* 2005; Davis, *et al.,* 2008; Abd El-Aziz, *et al.,* 2010). In Carrageenin hind paw oedema animal model, all treatment groups in addition to naiive and carrageenin control group in carrageenin model, the rat hind paw was removed, washed with saline, immediately fixed in 10% buffered formalin solution (pH 7.4) for 24 hrs and then routinely processed in ascending grades of alcohol, then xylene. The tissues were then embedded in paraffin wax, serially-sectioned to (3-5 mm) thickness, and stained with H&E. Finally, each stained tissue section was examined using a light microscope (Olympus BX 51, Olympus America, Melville, NY) and photographed with a digital camera (Olympus DP11) connected to the microscope.

Statistical analysis: Minitab computer software (version 16) was used to carry out the statistical analysis. Results were expressed as the mean ± Standard Error of mean (S.E.M) and analysed using Student t-test.

Results and Discussion

Tested *E.coli* isolates expressed high level of resistance to the studied antibiotics, their MICs values were (60 %) > 1024 mg/ml; (40 %) 1024 mg/ml for cephradine but (80 %) 1024 mg/ml and (20 %) 512 mg/ml for ampicillin.. The assessment of antimicrobial activity of tenoxicam revealed that tenoxicam has not antimicrobial activity against any tested isolates nor the reference strain. Thus from these *in vitro* experiments, it was noted worthy that combinations of tested antibiotics with tenoxicam, were indifferent (FIC =1) from using those antibiotics agents alone against MAR *E.coli* isolates.

The efficacy of tested antibiotics, tenoxicam and their combinations on healing of resistant bacterial infected wound in animal model: The wound healing effects of cephradine, ampicillin and/or tenoxicam of rat hind paw infected with resistant *E.coli* strain, are shown in the following figure (2) of the wound area photos were taken on days 1, 3, 6 and 11.

It was obvious that cephradine, ampicillin each alone and in combination with tenoxicam or tenoxicam treated groups, there were greater reduction in the ulcer area than was observed in untreated infected control group. The average ulcer area in the untreated infected control group decreased from 14 mm^2 on day 1 to 6 mm^2 on day 11. On the other hand the average ulcer area in treatment groups of cephradine alone and with tenoxicam decreased from 12 mm^2 on day 1 to 0 mm^2 (complete healing) on day 7. while ampicillin alone and/or tenoxicam required 8 days for complete healing.

The effect of tested antibiotics and/or tenoxicam on the inflammation of rat hind paw infected with MAR *E. coli* strain: Obtained data revealed that rats in infected control group, showed a significant ($p < 0.001$) increase (252.1 %) in rat hind weight of paw compared to naïve group, while treated rats with cephradine and cephradine/tenoxicam combination resulted in a significant ($p < 0.001$) reduction (60.1 and 60.2 % respectively) in hind paw weight as compared to the infected control group. Treatment of rats with tenoxicam resulted in a significant ($p < 0.001$) reduction (43.7%) in hind paw weight as compared to the infected control but treatment of rats with ampicillin and ampicillin/tenoxicam combination resulted in a significant ($p < 0.001$) reduction (48.4 and 54 % respectively) in hind paw weight as compared to the infected control (Figure 3).

The effect of tested antibiotics and/or tenoxicam on cytokines content in serum of rats infected with MAR *E.coli* strain:

Studied cytokines showed that IL-1β content in rat serum significantly ($p < 0.001$) increased (1603.4 %) in infected control group as compared to naïve group; treatment of rats with cephradine alone and in combination with tenoxicam resulted in a significant ($p < 0.001$) reduction (92.1 and 93.96 % respectively) in IL-1β content in rat serum as compared to the infected control group. While treatment of rats with tenoxicam alone resulted in a significant ($p < 0.001$) reduction (50.3 %) in IL- IL-1β content in rat serum as compared to the infected control group. Treatment of rats with ampicillin and ampicillin/tenoxicam combination resulted in a significant ($p < 0.001$) reduction (90.85 and 91.7 % respectively) in TNF-α content in rat serum as compared to the infected control group, figure 4 (A). TNF-α content in serum of rats of infected control group showed a significant ($p < 0.001$) increase (1174.8 %) as compared to naïve group. Treatment of rats with cephradine and cephradine/tenoxicam combination resulted

in a significant ($p < 0.001$) reduction (90.13 and 88.1 % respectively) in TNF- α content in rat serum as compared to the infected control. Treatment of rats with ampicillin and ampicillin/tenoxicam combination resulted in a significant ($p < 0.001$) reduction (87.2 and 90.6 % respectively) in TNF-α content in rat serum as compared to the infected control. Also treatment of rats with tenoxicam resulted in a significant ($p < 0.05$) reduction (40 %) in TNF-α content in rat serum as compared to the infected control, Figure 4 (B).

The effect of tested antibiotics and/or tenoxicam on other immunological mediators in rats infected with MAR *E.coli* strain:

NO content of rat hind paw significantly increased in infected control group rats by (412.2 %) as compared to naïve group, but cephradine and cephradine/tenoxicam combination treated groups have shown a significant ($p < 0.001$) reduction (71.3 and 80 % respectively) in hind paw NO content as compared to the infected control; while ampicillin and ampicillin/tenoxicam combination treated groups have shown a significant ($p < 0.001$) reduction (57 and 58 % respectively) in hind paw NO content as compared to the infected control; also tenoxicam treated rats resulted in a significant ($p < 0.01$) reduction (48.8 %) in hind paw NO content as compared to the infected control Figure 5 (A).

GSH content of rat hind paw in infected control group showed a significant ($p < 0.001$) decrease (92.8 %) as compared to naïve group. Treatment of rats with cephradine and cephradine/tenoxicam combination resulted in a significant ($p < 0.001$) increase (372 and 383.8 % respectively) in hind paw GSH content as compared to the infected control group; while treatment of rats with ampicillin and ampicillin/tenoxicam combination resulted in a significant ($p < 0.01$) increase (310.6 and 323.5 % respectively) in hind paw GSH content as compared to the infected control group on the other hand treatment of rats with tenoxicam resulted in a significant ($p < 0.001$) increase (394.4 %) in hind paw GSH content as compared to the infected control group Figure 5 (B).

MDA content of rat hind paw in infected control group showed a significant ($p < 0.001$) increase (527.9 %) as compared to naïve group. Treatment of rats with cephradine and cephradine/tenoxicam combination resulted in a significant ($p < 0.001$) reduction (80.3 and 80.5 % respectively) in hind paw MDA content as compared to the infected control. Treatment of rats with ampicillin and ampicillin /tenoxicam resulted in a significant ($p < 0.001$) reduction (71.6 and 74.7 % respectively) in hind paw MDA content as compared to the infected control; Treatment of rats with tenoxicam resulted in a significant ($p < 0.001$) reduction (63.2 %) in hind paw MDA content as compared to the infected control Figure 5 (C).

Acute inflammatory model (Carrageenin induced hind paw oedema in rats)

This model was performed to illustrate the possible mechanisms beyond the antimicrobial activity of tested antibiotics, by which they healed infected wounds despite resistance of *E.coli* strain used in infection, through assessing the anti-inflammatory and anti-oxidant activities of different doses for those antibiotics. tenoxicam and vitamin c were used as standard anti-inflammatory and antioxidant respectively for comparison. The results of tested immunological parameters in this model were shown in Table (1).

Table.1 Efficacy of tested antibiotics and/or tenoxicam on studied parameters in acute inflammatory carrageenin model in relation to carrageenin control

Group	Inflammation of hind paw (gm)	IL-1β content (pg/ml)	TNF-α content in rat serum (ng/l)	NO content of rat hind paw (µm/gm)	GSH content of rat hind paw (µm/gm tissue)	MDA content of rat hind paw (µm/gm tissue)
Naiive	0.845± 0.058	0.537± 0.066	5.15± 0.54	19.99± 2.4	481± 37	47.6± 5.1
Carrageenin control	1.927± 0.074 *	6.32± 0.52 *	48.08± 2.5 *	68.8± 8.4 **	128.5± 18 *	179.8± 12 *
Tenoxicam	0.953± 0.036 #	0.647± 0.066 #	7.76± 1.1 #	22.74± 2.6 ##	431± 43 #	52.2± 4.8 #
Vitamin C					464± 52 #	49.8± 5.2 #
Cephradine (400 mg/kg)	0.964± 0.09 #	0.717± 0.086 #	6.78± 0.7 #	27.3± 3.1 ##	369± 50 ##	69.2± 8.8 #
Cephradine (800 mg/kg)	0.887± 0.095 #	0.606± 0.064 #	6.23± 0.72 #	24.32± 1.4 ##	417± 57 ##	58.15± 3 #
Cephradine (1600 mg/kg)	0.848± 0.1 #	0.547± 0.049 #	3.77± 0.43 #	20.28± 2.7 ##	439± 61 ##	50.97± 2.8 #
Ampicillin (250 mg/kg)	1.119± 0.12#	0.932± 0.11***#	7.4± 0.89 #	37.4± 5.1 ***##	311± 42 ***##	93.3± 11**#
Ampicillin (500 mg/kg)	1.064± 0.11 #	0.69± 0.059 #	7.15± 0.88 #	30.5± 4.2 ##	349± 49 ##	69.8± 9.2 #
Ampicillin (1000 mg/kg)	0.993± 0.16 #	0.512± 0.065 #	5.7± 0.45 #	27.25± 3.1 ##	383± 50 ##	60.6± 8.1 #

Data are presented as mean (± S.E.M; n = 8/group). * Significant from naïve at P < 0.001,** Significant from naïve at P < 0.01, ***Significant from naïve at P < 0.05, # Significant from carrageenin control at P < 0.001, ## Significant from carrageenin control at P < 0.01

Figure.2 Effects of the treatment with cephradine, ampicillin each alone and/or tenoxicam on wound healing of rat hind paw infected with MAR *E.coli*

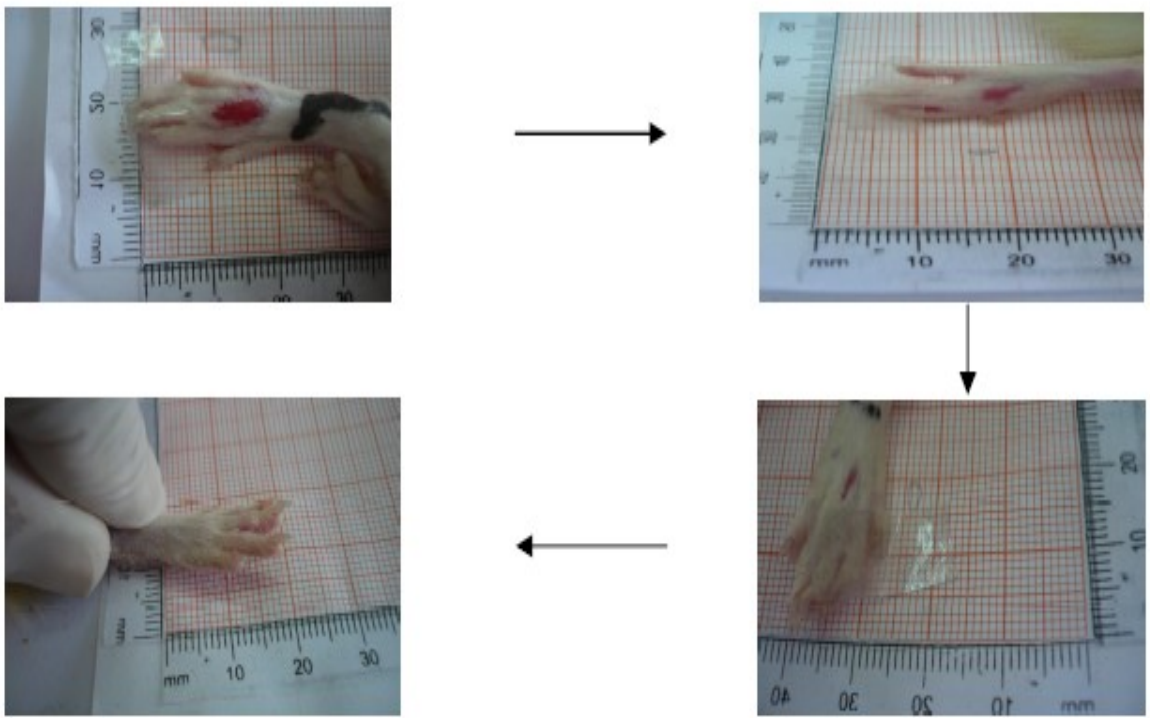
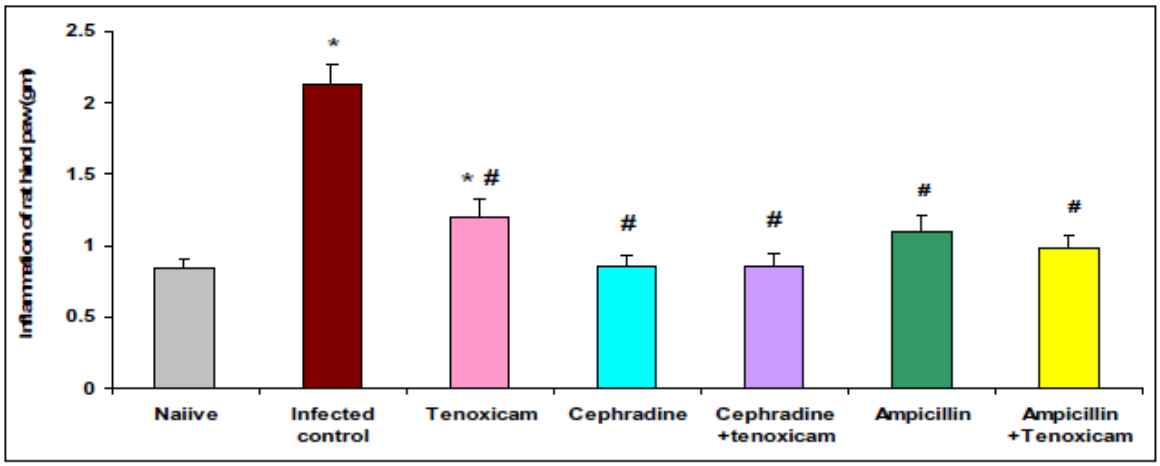


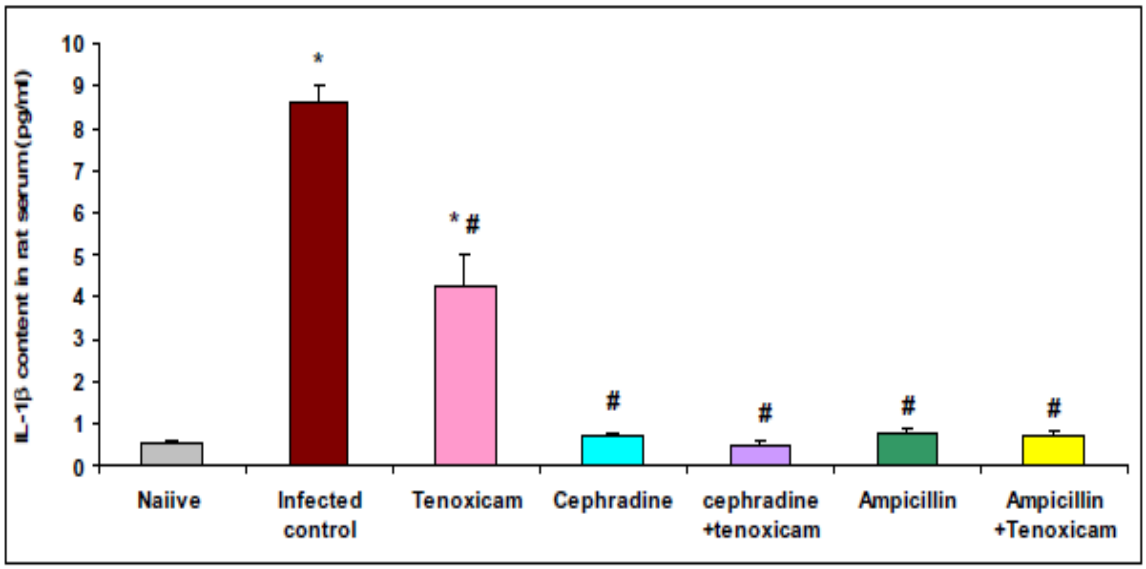
Figure.3 Effect of cephradine (1600 mg/kg); tenoxicam (20 mg/kg); cephradine (800 mg/kg)/tenoxicam (10 mg/kg) combination; ampicillin (1000 mg/kg) and ampicillin (500 mg/kg)/tenoxicam (10 mg/kg) combination on inflammation of rat hind paw infected with resistant *E.coli*



* Significant from naïve at P < 0.001 for infected control and at P < 0.05 for tenoxicam group.
# Significant from infected control at P < 0.001. The results are means ± S.E.M.

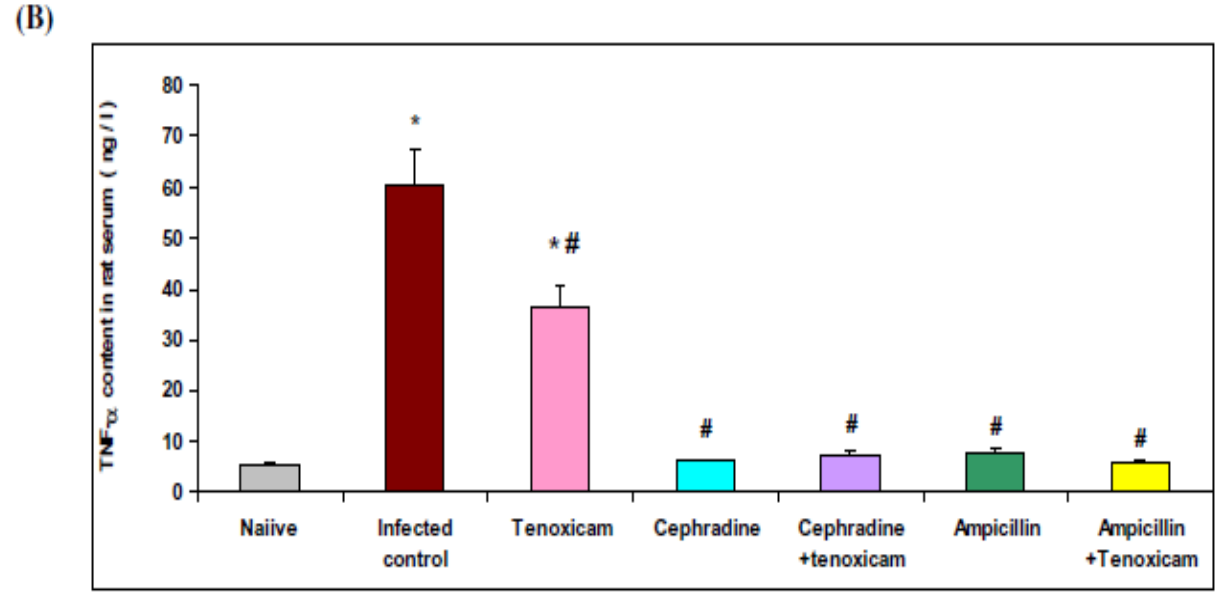
Figure 4: (A) Effects of cephradine (1600 mg/kg), tenoxicam (20 mg/kg), cephradine (800 mg/kg)/tenoxicam (10 mg/kg) combination, ampicillin (1000 mg/kg) and ampicillin (500 mg/kg)/tenoxicam (10 mg/kg) combination on IL-1β content in serum of rats infected with MAR *E.coli*.

(A)



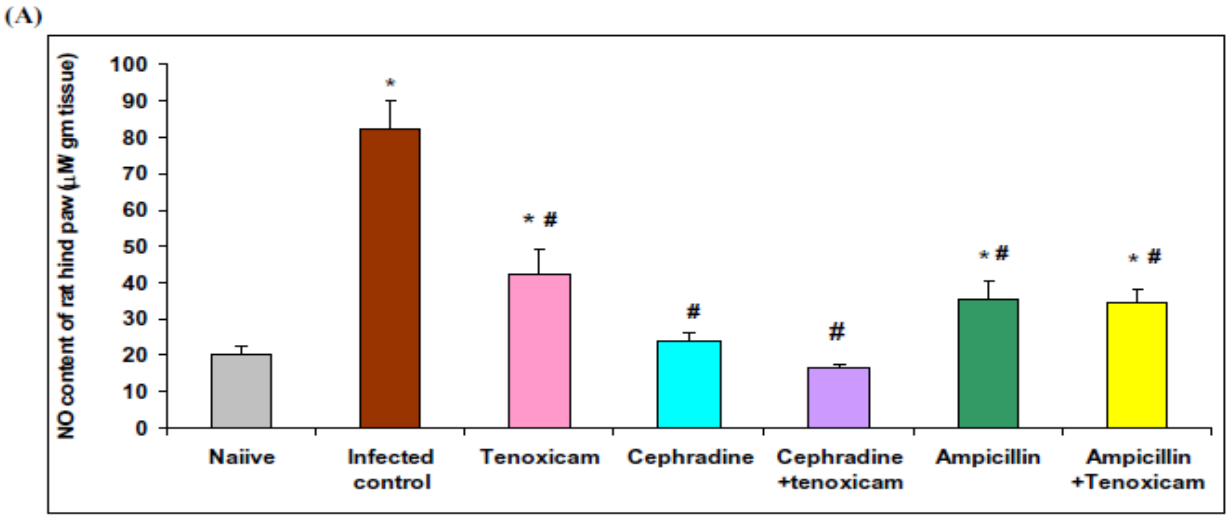
* Significant from naïve at P < 0.001 and at P < 0.01 for tenoxicam.
# Significant from infected control at P < 0.001. The results are means ± S.E.M.

Figure.4(B) Effects of cephradine (1600 mg/kg), tenoxicam (20 mg/kg), cephradine (800 mg/kg)/tenoxicam (10 mg/kg) combination, ampicillin (1000 mg/kg) and ampicillin (500 mg/kg)/tenoxicam (10 mg/kg) combination on TNF- α content in serum of rat infected with MAR *E.coli.*



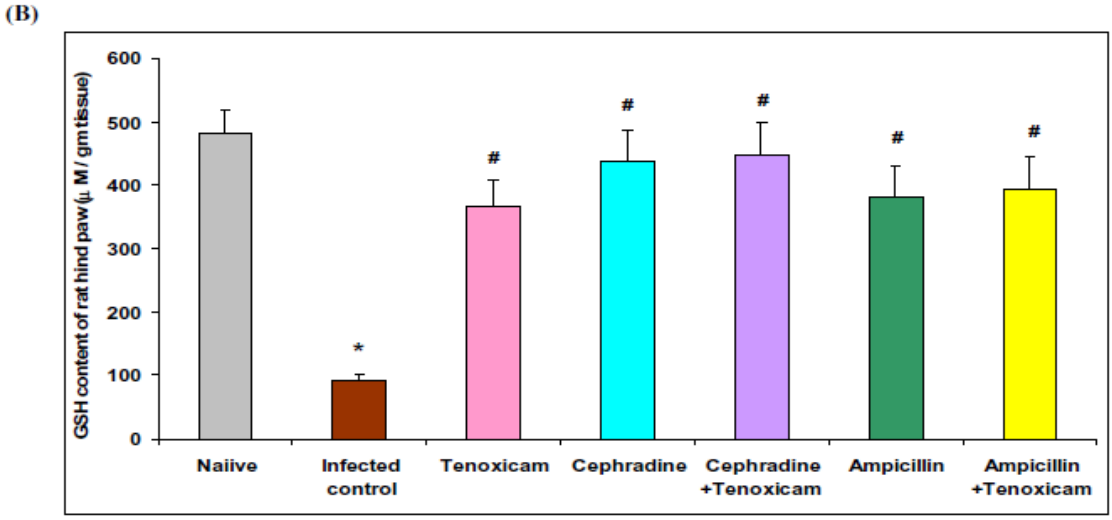
* Significantly different from naiive at P < 0.001.
# Significantly different from infected control at P < 0.001, tenoxicam at P < 0.05. The results are means ± S.E.M.

Figure.5 The results are means ± S.E.M. (A): Effects of cephradine (1600 mg/kg), tenoxicam (20 mg/kg) and cephradine (800mg/kg)/ tenoxicam (10 mg/kg) combination, ampicillin (1000 mg/kg) and ampicillin (500 mg/kg)/ tenoxicam (10 mg/kg) combination on NO content of rat hind paw infected with resistant *E.coli*.



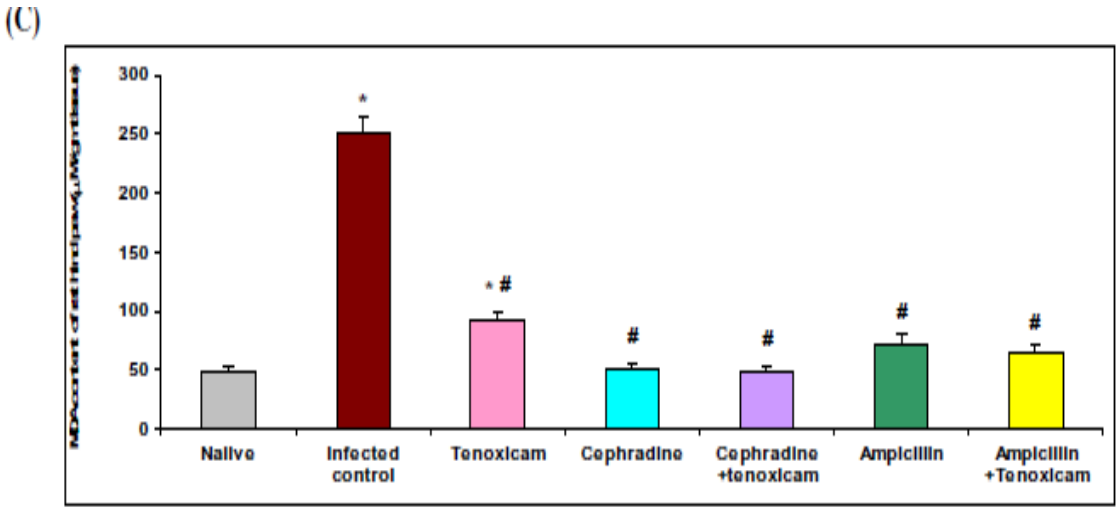
* Significant from naïve at P < 0.001 for all groups but for ampicillin/tenoxicam combination group significant at P < 0.01 and for tenoxicam, ampicillin groups significant at P < 0.05.
# Significant from infected control at P < 0.001 for all groups, but for tenoxicam at P < 0.01.

(B): Effects of cephradine (1600 mg/kg), tenoxicam (20 mg/kg), cephradine (800 mg/kg)/tenoxicam (10 mg/kg) combination, ampicillin (1000 mg/kg) and ampicillin (500 mg/kg)/tenoxicam (10 mg/kg) combination on GSH of rat hind paw infected with MAR *E.coli.*



* Significant from naïve at P < 0.001.
# Significant from infected control at P < 0.001 for all groups but at P < 0.01 for ampicillin and ampicillin/tenoxicam combination.

(C): Effects of cephradine (1600 mg/kg), tenoxicam (20 mg/kg) and cephradine (800 mg/kg)/tenoxicam (10 mg/kg) combination, ampicillin (1000 mg/kg) and ampicillin (500 mg/kg)/tenoxicam (10 mg/kg) combination on MDA of rat hind paw infected with MAR *E.coli*.



* Significant from naïve at P < 0.001.
# Significant from infected control at P < 0.001.

Histopathological studies: These studies were performed to confirm effects of tested antibiotics on immunological markers of both *in vivo* models, which revealed that all animals of naiive group showed normal cutaneous structure regarding epidermis and dermal connective tissue structures, normal skin appendages (sweat glands) figure 6(a).

Infected animal model: After 48 hours of wound infection with *E.coli* before treatment (to allow formation of bacterial biofilm on

wound of rat hind paw), there were signs of acute inflammation manifested as severe oedema, prominent capillary diltation and prevascular inflammatory cellular infiltration associated with severe dense interstitial acute inflammatory cellular infiltration down in deep dermis figure 6(b). Infected group without treatment showed mild relief of acute signs (decrease in oedema and density of acute inflammatory infiltration with early fibrosis) figure 6(c). Tenoxicam treated group showed decrease of inflammatory reaction signs figure 6(d).

Cephradine treated group: all animals showed evidences of healing (decrease inflammatory cellular infiltration) with mild oedema, figure 6(e).

Combination of cephradine/ tenoxicam treated group: this group showed healing with mild fibrosis, figure 6(f). Ampicillin and combination of ampicillin/tenoxicam treated groups where all animals showed healing with mild dermal fibrosis, figure 6(g).

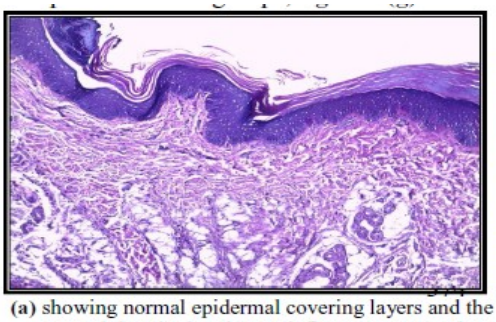
Carrageenin acute inflammatory (noninfected) animal model:

Carrageenin control group**,** all studied animals showed severe dermal dense acute inflammatory cellular infiltration (dilated, engorged capillaries with prominent endothelial swelling with oedema) figure 6(h).

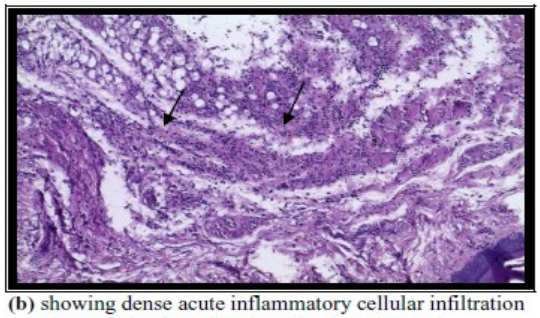
In both of tenoxicam and vitamin C groups (used for comparison as standard (NSAID) and standard antioxidant respectively), studied animals showed apparently normal sections as naïve group with early inflammatory changes manifested as mild oedema with dilated, congested capillaries and normal covering epidermis figure 6(i).

Cephradine treated groups, where all animals showed evidences of healing (decrease inflammatory cellular infiltration) with mild oedema, figure 6(j).
Ampicillin treated groups**,** figure 6(g).

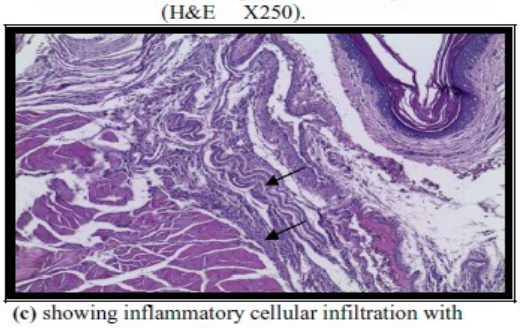
Figure.6 Histopathological studies



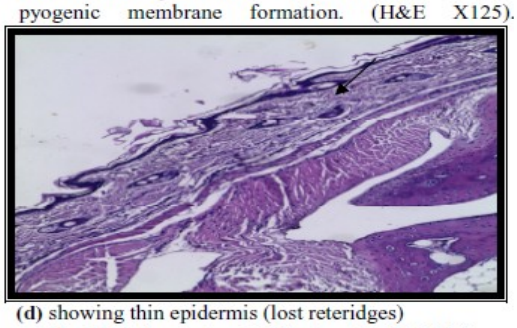
(a) showing normal epidermal covering layers and the normal dermis showing no inflammatory reactions. (H&E X250).



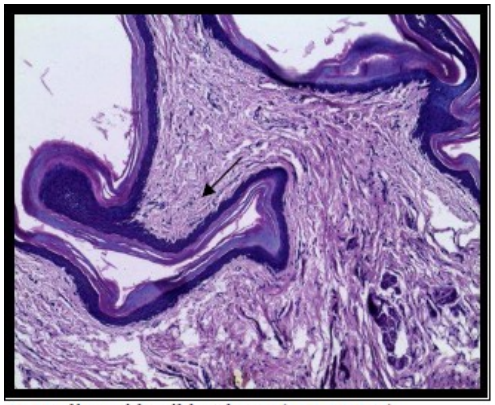
(b) showing dense acute inflammatory cellular infiltration down to the deep dermis with ulceration and pyogenic membrane formation. (H&E X125).



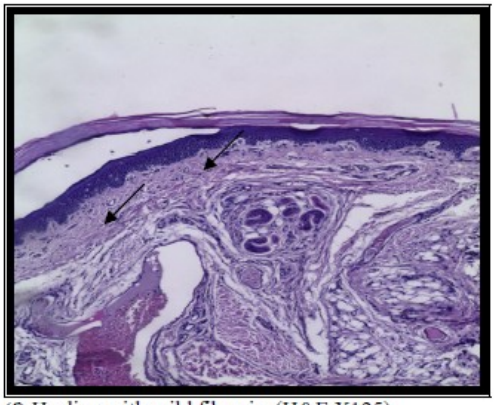
(c) showing inflammatory cellular infiltration with presence of pus cells and early collagen fibrous formation with mild oedema. (H&E X125).



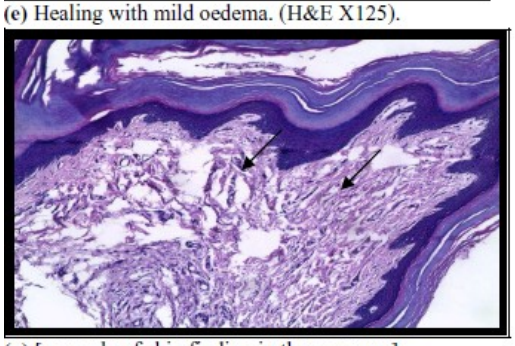
(d) showing thin epidermis (lost reteridges) with mild inflammatory infiltration. (H&E X125).



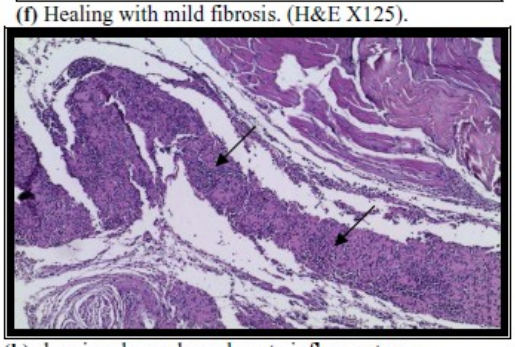
(e) Healing with mild oedema. (H&E X125).



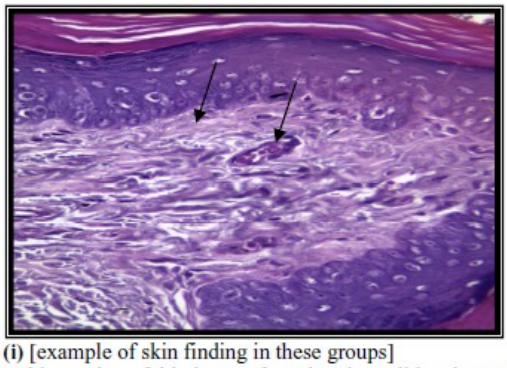
(f) Healing with mild fibrosis. (H&E X125).



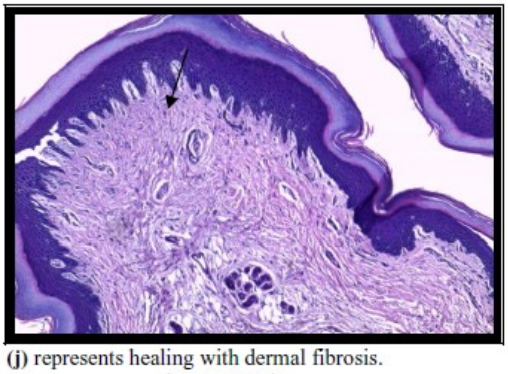
(g) [example of skin finding in these groups] showing healing and mild fibrosis with dermal oedema. (H&E X125).



(h) showing dense dermal acute inflammatory cellular infiltration. (H&E X125).



(i) [example of skin finding in these groups] skin section of hind paw of rat showing mild oedematous spaces and dilated engorged capillaries. (H&E X250).



(j) represents healing with dermal fibrosis. (H&E X125).

Antibiotics were used as first line drugs for the treatment of bacterial infections, but the widespread resistance to these agents combined with the shortage of novel antimicrobial compounds developed by the pharmaceutical industry results in an urgent need for new strategies to combat bacterial infections (Fernebro, 2011). Therefore it is worthy reminding the clinician that it is necessary to be far more selective, when an antibiotic is prescribed, it should be the one with the narrower spectrum of activity (Alanis, 2005). This to be urgently established to conserve antibiotics for the future, through optimization of dosing regimens and global harmonization of breakpoints that will achieve the microbiological and clinical outcome desired and would add efficacy to antibiotic therapy (Mouton *et al.,* 2011).

In contrast the focus was directed to the

clinical aspects such as the efficiency of antibiotics in clearing infections and pathogens that are resistant to antibiotic treatment (Aminov, 2009), where it has been reported that some antibiotics have curing activity of resistant bacterial infections although absence of suscebetibility to those antibiotics, due to other mechanisms beyond their antimicrobial activity (Nagata *et al.,* 2004 and Tsai *et al.,* 2009). Several efforts in re-evaluation of older antimicrobial agents have been established and further understanding and investigation of their mechanisms of actions was suggested (Mouton *et al.,* 2011; Garonzik *et al.,* 2011; Velkov *et al.,* 2013).

On account of that, it was interesting to investigate the possible mechanisms beyond the antibacterial activity of some beta-lactam antibiotics that contribute to curing resistant *E.coli* infection. The present study focused on assessment of the efficacy of cephradine and ampicillin (narrow spectrum and old antibiotics) in healing *E.coli* infected wounds. As the shortage in discovering new antibiotics concuurently with wide spread of resistant bacterial infections, paid attention to re-evaluation of old antibiotics using highest safest dose of them or using them in combination with other drugs.

This work included *in vitro* and *in vivo* studies. *In vitro* study includes screening of resistancy of *E.coli* to tested antibiotics, where it was confirmed that all of these bacterial strains were resistant to cephradine and ampicillin either alone or in combination with tenoxicam, as standard NSAID. The addition of tenoxicam to cephradine or ampicillin, has indifferent effect on the MICs of those antibiotics on the tested bacterial strains. Thus data revealed that tenoxicam alone lacked antimicrobial activity against those tested isolates. *In vivo* study has included wound infection in rat hind paw animal model, using selected one of highly MAR strains of *E.coli*, revealed that cephradine and ampicillin each alone and in combination with tenoxicam, were effective in treatment of those wounds despite of resistancy *in vitro*. This pay attention to the increased efficacy of some antibiotics when used by lower dose in combination with NSAIDs.

These results were coincided with Viehmann and coworkers who had reported that combination therapy of an antibiotic (enrofloxacine-arginine) and NSAID (ketoprofen) had a superior therapeutic effect compared to a single antibiotic treatment in nursery piglets experimentally infected with *Haemophilus parasuis* (Viehmann *et al.,* 2013). Also combination therapy of administering subantimicrobial dose doxycycline plus low-dose NSAID (two host modulating drugs) to chronic periodontitis patients, synergistically enhancing clinical efficacy of doxycycline (Lee *et al.,* 2004).

Early stimulation of the acute inflammatory response may be beneficial in infections, but its resolution is crucial to avoid excessive injury to structural tissue. Inhibition of unresolved inflammation, either by antibiotics or specially anti-inflammatory agents, is needed (Parnham, 2005; Rubin & Tamaoki 2005; Aminov, 2013).

The relevance of the immunomodulatory actions of some antibiotics for their therapeutic efficacy in various diseases is now generally admitted, with growing number of supportive experimental and clinical studies, (Pasquale & Tan, 2005; Labro, 2011). Various classes of antibacterial agents were demonstrated *in vitro* and *invivo* immunomodulatory properties, such as tetracyclines, quinolones and cephalosporins may have beneficial immunomodulatory effect (Choi, *et al.,*

2003; Nau & Tauber, 2008; Zhang & Ward, 2008; Labro, 2012).

E.coli is one of most commonly isolated microorganisms from wound infections (Taiwo *et al.,* 2002; Nazeer *et al.,* 2014). *E.coli* was used as a model system to explore intrinsic mechanisms of MAR which is a major cause of clinical failure in treating bacterial infections (Duo *et al.,* 2008). Where it had been reported that several *E.coli* strains have intrinsic resistance to various antimicrobial agents with a wide range of activities such as gentamicin, trimethoprim, mecillinam and β-lactam antibiotics (Greenway & England, 1999; Moore *et al.,* 2013; Carone *et al.,* 2014); some strains express the beta-lactamase causing ampicillin resistance (Chakrabarti *et al.,* 2014).
Bacterial lipopolysaccharide (LPS), the cell wall component of all gram-negative bacteria including *E.coli*, causes the systemic inflammatory response syndrome. It triggers the synthesis and release of cytokines, NO, and ROS (Zhang *et al.,* 2000; Cimen *et al.,* 2005).

The present study revealed that cytokines expression; inflammatory reaction and oxidative stress were considered as possible pathways. It was found significant down regulation of hind paw inflammation, some immunological mediators (Il-1β, TNF-α, NO, MDA) and increased GSH by both cephradine and ampicillin each alone and in combination with tenoxicam. On account of that, it was necessary to confirm that obtained results from bacterial infected model were beyond the antibacterial activity of tested antibiotics. This was achieved by using carrageenan induced acute inflammation rat model, through examining the effects of different concentrations of tested antibiotics on the investigated immunological mediators. Where carrageenan-induced inflammation (paw oedema) animal model was adopted for the quantification of the inflammatory response (Salvemini *et al.,* 1996; Lam & Ng, 2003). Tenoxicam has antioxidant effects (Ozgocmen *et al.,* 2005; Naziroğlu *et al.,* 2008). So it could be used as control for evaluation of anti-infalmmatory efficacy of tested antibiotics.

Hind paw inflammation in this study was evaluated in both *E.coli* infected rat model and carrageenan-induced paw oedema rat model, the inflammation was significantly reduced by both cephradine and ampicillin.

IL-1β in *E.coli* infected animal model of the present study significantly decreased by cephradine and ampicillin nearly with about ninety percent. Similarly, when using combination of those antibiotics and tenoxicam revealed synergestic effect *in vivo* irrespective of its indifferent effect *in vitro*. IL-1β in carrageenin acute inflammatory model significantly decreased by cephradine and ampicillin in a dose dependent manner.

TNF-α and Il-1β produced acutely in large amounts, are extremely potent inflammatory molecules: they are the primary cytokines that mediate acute inflammation induced in animals by intradermal injection of bacterial LPS and two of the primary mediators of septic shock (Feghali & Wright, 1997). Those results of examined antibiotics on IL-1β were in line with previous reports where, It was demonstrated that reduction in endogenous IL-1 activity improves host defense against various infections while suppressing the inflammatory response (Boelens, *et al.,* 2000; Schultz, *et al.,* 2002).

TNF-α in *E.coli* infected animal model of the present study significantly reduced by both ampicllin and cephradine alone and in

combination with tenoxicam by approximately ninety percent, which revealed synergestic effect *in vivo* irrespective of indifference *in vitro*. TNF-α in carrageenin acute inflammatory model significantly decreased by cephradine and ampicillin in a dose dependent manner.

Some investigations have revealed that *E.coli* stimulate the release of the pro-inflammatory cytokines such as TNF-α (Rolhion *et al.,* 2007; Subramanian, 2008). Also Carrageenan-induced acute inflammation in rats causes increase the proinflammatory cytokine TNF- α, that are activated in certain inflammatory conditions (Gonzalez *et al.,* 2011; Katsori *et al.,* 2011). While β-lactam antibiotics have potent immunomodulatory properties, including inflammation controlling in various diseases, e.g, ceftriaxone confers significant neuroprotection and reduction of proinflammatory cytokines level (Wei *et al.,* 2012; Melzer *et al.,* 2008; Verma *et al.,* 2010). Tenoxicam had an inhibitory effect upon the production of TNF- α *in vitro* (Syggelos *et al.,* 2007). Also immunomodulating effects of tetracyclines, consisting in the inhibition of T- cell activity and a decrease in levels of proinflammatory cytokines IL-2, TNF, IL-1 (Golub *et al.,* 1983; Kloppenburg *et al.,* 1995; Guz & Bugla-Płaskońska, 2007). Some quinolones antibiotics reducesd the synthesis of TNF-α in monocytes stimulated with LPS (Kahn *et al.,* 1998).

Mice deficient for TNF-α receptor showed enhanced clearance of *Pseudomonas aeruginosa* from the lungs during sub-acute pneumonia (Skerrett *et al.,* 1999). Consquently suppression of cytokine production by antibiotics may therefore be of great clinical benefit especially to patients who are immunocompromised or who have severe infection (Hotchkiss & Karl, 2003).

NO is a potent inflammatory mediator and an important cellular signaling molecule produced in many inflammatory and infectious conditions (Hou *et al.,* 1999; Meki *et al.,* 2009).

In low concentrations NO protecting against cell death, whereas higher concentrations are cytotoxic (Joshi *et al.,* 1999).

NO in infected animal model of the present study significantly decreased by cephradine and ampicillin. Also combination of those antibiotics with tenoxicam synergistically reduced NO by approximately eighty percent for cephradine/tenoxicam combination and sixty percent for ampicillin/tenoxicam combination. NO in carrageenan induced acute inflammatiory model was modulated to reach its normal levels as naïve in a dose dependent manner by cephradine and ampicillin in a dose dependent manner.

One of the anti-inflammatory activities is through inhibition of NO production (Maruyama *et al.,* 2010). Where NO appears to be involved in the acute inflammatory response following the intraplantar injection of carrageenan into the rat hind paw (Ialenti *et al.,* 1992). Ampicillin significantly inhibited the increase in mucosal inducible NO synthase activity resulted from injury in rat jejunum provoked by indomethacin as well as the enterobacterial numbers invaded in the mucosa and inhibited the occurrence of intestinal lesions (Whittle *et al.,* 1995; Konaka *et al.,* 1999). Tenoxicam-treated patients showed a significant decrease in nitrite levels (Ozgocmen *et al.,* 2005). Thus inhibition of NO production could be a marker of the anti-inflammatory activity of cephradine and ampicillin. Microbicidal effects of phagocytes are mediated by the generation of reactive oxygen species (ROS) and reactive nitrogen species but they can also lead to tissue injury (Forman & Torres,

2002; Henrotin, *et al.,* 2003). The production of ROS at the site of inflammation is proposed to be a major cause of the cell and tissue damage associated with many inflammatory diseases. When ROS exceeds cellular antioxidants it is called oxidative stress, leading to lipid peroxidation which may produce injury by compromising the integrity of membranes which can be detected by malondialdhyde (MDA) (Gupta, 2004; Ullevig *et al.,* 2013; Heeba *et al.,* 2014).

Reduced glutathione (GSH) is an efficient antioxidant and is considered to be one of the most important scavengers of ROS (Gupta *et al.,* 2004; Zitka *et al.,* 2012). So that it was very valuable to assess the anti-oxidant activity of tested β-lactam antibiotics through evaluating their efficacy on both GSH and MDA. MDA in the *E.coli* infected animal model in this study significantly decreased by ampicillin and cephradine alone/and in combination with tenoxicam showing synergestic activity *in vivo*. Similarly in carrageenan-induced acute inflammatiory model, MDA was reduced in a dose dependent manner by cephradine and ampicillin. It reached its normal levels as naïve and by comparison with vitamin c as standard antioxidant.

The removal of oxygen-derived free radicals significantly inhibited the paw oedema (Salvemini *et al.,* 1996). Vitamin c (Ascorbic acid) supplementation increase levels of antioxidants and decrease lipid peroxidation (LPO) (Rennie et al, 2003; Block *et al.,* 2008). Also tenoxicam could decrease MDA levels (Ozgocmen *et al.,* 2005). On the other hand GSH in the *E.coli* infected animal model of this study significantly increased by cephradine and ampicillin by approximately three hundreds eighty and three hundreds twenty percent respectively. Similarly, when using combination between these antibiotics and tenoxicam, revealed synergestic activity *in vivo* irrespective of its indifference *in vitro*. Similarly GSH in carrageenan-induced acute inflammatiory model was increased by cephradine and ampicillin to reach nearly its normal levels as naïve and as vitamin c, in a concentration dependent manner.

Histopathological investigations revealed that after wound infection with *E.coli*, there were signs of acute inflammation manifested as severe oedema, cellular inflammatory infiltration and pyogenic membrane, then associated with mild relief of acute signs and early fibrosis within several days. While in carrageenin acute inflammatory model rats had shown oedema with dense dermal acute inflammatory cellular infiltration. Both animal models had showed evidences of healing with mild oedema by using cephradine and ampicillin alone in combination with tenoxicam, that resemble to high grade those sections obtained from tenoxicam or vitamin c treated groups showing apparently normal sections as control group with minor inflammatory changes.

In addition various previous studies were in compliance with the present obtained results of cephradine and ampicillin in healing MAR *E.coli* wound infection *in vivo,* despite of resistancy *in vitro*. The biological functions of antibiotics are not limited to killing bacteria, they can be effective in treatment of a broad spectrum of diseases and pathological conditions other than those of infectious etiology and, in this capacity, may find widespread applications beyond the intended antimicrobial use. The host modulating properties of macrolides, tetracyclines, and β-lactams were observed (Aminov, 2013). Patients with pneumococal pneumonia treated with β-lactam improved

outcome through dampening systemic cytokine levels, where researchers confirmed that an excess of proinflammatory cytokines is associated with poor prognosis (Padrones *et al.*, 2010). Tetracyclines and macrolides not only help to clear infections but also prevent generally excessive immune responses to infections characteristic for modern humans (Aminov, 2013). Tetracycline family of antibiotics is one of the best-studied examples of non-antimicrobial effects of antibiotics on the host, for example minocycline displays anti-inflammatory, neuroprotective, selective down-regulation of proinflammatory cytokines, anti apoptotic properties and antioxidant activity (Lai & Todd, 2006; Soczynska *et al.*, 2012; Aminov, 2013; Garrido-Mesa *et al.*, 2013).

Macrolides influencing the production of cytokines, they have a dampening effect on the proinflammatory response by multiple anti-inflammatory mechanisms such as NO inhibition. Having such an obvious effect on the various aspects of the immune system, macrolides seem to be exceptionally suited for the treatment of chronic inflammatory diseases (Tamaoki, 2004; Nau & Tauber, 2008 and Altenburg *et al.*, 2011). Azithromycin had showed significant lowering of the expression of inflammatory factors (TNF-α, IL-6, and IL-1β) in cell culture supernatant and mouse serum of animal sepsis model experiments and raise the survival rates significantly higher, which suggested its function in immunological regulation and provided reliable data for new clinical applications (Tong *et al.*, 2011).

Recent data suggest that several antibiotics such as tetracyclines and cephalosporins may have a beneficial immunomodulatory or neuroprotective effect on neuroimmunological and neurodegenerative diseases (Tauber & Nau, 2008).

In addition incidental observations that some non-infectious diseases, including inflammatory disorders, may be improved by antibacterials have bolstered interest in the immunomodulatory activity of this class of drugs (Labro, 2011). Also With understanding that host defenses can become detrimental for the host himself, interest has moved to the inhibitory (anti-inflammatory) potential of antibacterials (Labro, 2012). Therfore the knowledge of the effects of antibiotics on the immune response, the synthesis and secretion of proinflammatory cytokines allows for them new applications beyond the infection treatment (Kwiatkowska et al,. 2013). Although, the predictive efficacy of antibacterial agents is still evaluated in terms of MICs, MBCs, and pharmacokinetics, much evidence derived from clinical studies underlines the need for synergy between the host defence system and these drugs to obtain optimal therapeutic efficacy. Recently, the concept of biological response modifier-antibiotics has come under the limelight (Labro, 1994). Therefore, evaluation of the efficacy of antibacterial agents requires citeria other than those defined *in vitro*, such as minimum inhibitory cocentrations (MICs) or minimum bactericidal concentration (MBCs), the last years have seen a renewal of interest in another approach to the successful resolution of infection: the achievement of synergestic interaction between therapeutic agents and the host defense system (Mandell & Coleman, 2001; Labro, 2011). Consquently those evidences could explain our results obtained from the efficacy of cephradine and ampicillin in the healing of infected wounds with resistant *E.coli in vivo*, despite lack of activity *in vitro*. This efficacy has been found to be mediated via immuomodulatory, anti-inflammatory and anti-oxidant activities (beyond their antibacterial activities), which also lead to

synergestic effects when used in combination with tenoxicam.

The overall conclusion that can be drawn from these investigations is that some members of β–lactam antibiotics (cephradine and ampicillin) significantly have efficacy for healing MAR *E.coli* infected wounds in rat hind paw, irrespective of *in vitro* resistancy to it. That could be explained through other possible mechanisms beyond their antibacterial activity, such as immunomodulatory, anti-inflammatory and anti-oxidant activities on rat hind paw inflammation and investigated immunological mediators (IL-1β; TNF-α; NO, MDA, GSH) released in response to *E.coli* infection. Combination of antibiotics with NSAIDs increases the efficacy of tested antibiotics in curing resistant bacterial infection, where it synergistically giving the same effect as the high dose of tested antibiotics alone. Accordingly considering the bacterial infection, it is necessary to better understanding the interaction between immune system and antibacterial agents.

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