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# Comparative study of Efficacy of curcumin oil and Levofloxacin against wound contamination by *Klebsiella Pneumoniae*

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**ABSTRACT**

The present study seen the antimicrobial resistance diversity phenotypes in (*Klebsiella pneumoniae*) is becoming important problem antibiotic management . identification of isolated bacteria from wound swabs, specific primers for 16S rDNA were used. 16S rDNA was amplified successfully and the sequence of it was identified as *Klebsiella pneumoniae* subsp. *pneumoniae* after BLAST with NR database of NCBI GenBank The clinical implications of extensively drug-Senstivity to (Levofloxacin),this evaluated the effected antibacterial activity of Curcumin oile MIC between (2.5,3 and 3.5µg/ml) while Levofloxacin MIC renege (0.5,1and 2mg/liter). Histopathology of *K. pneumonia* infected skin revealed that severe suppurative inflammation and abscess formation. Treatment of the infected skin with Levofloxacin reduces the lesion to some extent, while treatment of skin with curcuma decrease tissue destruction and enhanced the healing process. In conclusion, local treatment of skin wound infected with *K. pneumonia* using a curcuma lead to localization of the lesion and enhanced the healing process compared with Levofloxacin, which showed less therapeutic effect.

**Keywords** : *Klebsiella Pneumoniae*, curcumin oil , wound

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## INTRODUCTION

*Klebsiella Pneumoniae* is a facultative anaerobic, gram-negative, rod in shape bacterium that is generally found on human skin, mouth and in intestinal tract as a normal flora (1**).** In some cases due to the disruption of the normal flora *Klebsiella Pneumoniae* can produce severe bacterial infections such as pneumonia, bloodstream infections, wound infections, urinary tract infections (UTI), and meningitis (2).

The optimal treatment regimen for the *K. Pneumoniae* infections, have been reported through the usage of Combination therapies with carbapenems, polymyxins, aminoglycosides, tigecycline, and rifampin (3) and (4).Currently, due to the development of super-resistant strains, the usage of these antimicrobial agents is not effective. For this reason there is ongoing for new strategies, either by synthesis of new agents or through the investigation of natural therapeutics. (5) Several studies estimated the utility of 16S rRNA gene sequencing for clinical microbiology that permit identification of specific microbial taxa and their phylogenetic classification. The molecular markers, 16S rRNA, an~1500 base pair gene coding for a catalytic RNA that is part of the 30S ribosomal subunit (6)Plants are the oldest source of pharmacologically active compounds and have provided human kind with many medicinally useful compounds from centuries Plants are the oldest source of pharmacologically active compounds and have provided human kind with many medicinally useful compounds from centuries (**7**) The origin of many effective drugs is found in the traditional medicinal practices, and in view of this it is very important to undertake studies pertaining to screening of medicinal plants for their proclaimed biological activity (8) Temulawak (Curcuma xanthorrhiza) is a Zingi beraceae species which is empirically widely used as a traditional medicine especially its rhizome. Traditionally, it is widely used to treat kidney stones, fever, high-cholesterol level, joint pain, and hepatitis. Many research studies have been conducted on the active component of the temulawak rhizome for its antioxidant, antilipidemia, antibacterial, and antifungal activities (9)

The major pigment compound of Curcuma longa is Curcumin which has been shown to have potent anti-inflammatory activities with specific lipoxygenase and COX-2 inhibiting properties including cytokines (10) In spite of recent advances in treatment, burns are still a major threat to life due to infection, The local interruption of blood flow associated with burns makes prophylaxis of systemic infection difficult, and thus topical antimicrobial therapy is important in order to reduce the microbial accumulation in wounds, and hence reduce the chance of infection. (5).In the present study, a mouse burn wound model was used to evaluate the therapeutic potential of Temulawak (Curcuma xanthorrhiza) in the treatment of infection caused by *K. pneumoniae* .

## MATERIALS AND METHODS

A total of 10 samples collected from AL Kadhimiya Teaching Hospital represented by wound swabs and screening depended antibiotic resistance test **.** Isolation and identification of *Klebsiella pneumoniae* Several different media were used for culture such as (Nutrient agar, MacConkey agar and Blood agar) (11).

**Antibiotic sensitivity test *Klebsiella pneumoniae*:**

The sensitivity test of *K. pneumoniae* to antibiotics was determined according to the method (12) Using 5 different type antibiotics (Azithromycin, Clindamycin, Erythromycin, Levofloxacin and Metronidazole).and selection mulit resistance antibiotic and act MIC against wound contamination by *Klebsiella Pneumoniae.*

**Molecular assay:**

**DNA extraction**

Genomic DNA was extracted from the detected bacterial isolates according to the protocol of Wizard Genomic DNA Purification Kit, Promega. Quantus Florometer was used to detect the concentration of extracted DNA.

**Primers Selection**

The set of primers 27F (AGAGTTTGATCTTGGCTCAG) and 1492R (TACGGTTACCTTGTTACGACTT) was used for amplification of 16s rRNA for identification of bacteria at gene level (13).

**Polymerase chain reaction**

The PCR mixture was set up in a total volume of 25 µl included 12.5µl of GoTag Green Master Mix (Promega, USA), 1 µM of each primer and 2 ng/µl of template DNA then the rest volume was completed with nuclease free water. PCR protocol involved an initial denaturation for 5 min at 95ºC; 35 cycles of denaturation for 30 sec at 95ºC, annealing for 30 sec at 60ºC, extension for 1min at 72ºC then final extension for 7 min at 72ºC. After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. 5µl of PCR product was separate in 1% agarose gel electrophoresis stained with ethidum bromide and visualized on a UV transilluminator, the size of amplified products were compared with the 100pb DNA ladder to determine the exact size of these products.

**Sanger sequencing**

PCR products were sending for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. Consensus sequence of 16s rRNA gene were generated from forward and reverse sequence data using genious software. DNA sequencing data was analyzed using BLAST with NR database of NCBI GenBank.

**Plant Material:**

The Rhizomes fresh of *C. longa* from the wet market. The rhizomes were put into smaller pieces and air-dried then extracted using solvent extraction. Samples weighing (300gm) were transferred into a glass bottle and halve liter of ethanol (100%)was added. The shaken to mix up the materials and placed in room temperature at dark. (14).

**Extract purification:**

By Whitman (No.1) filter paper using sample extract was filtrated, the clear solution was collected in an bottle. This process helped to remove the (powder plant ). In order to obtain high concentration of the sample, by using rotary evaporator further process had to be done. (15).

**Checkerboard micro dilution assay MICs:**

Determined were using broth micro dilution method according to M07-A9 guideline (16). Diluted two-fold in fresh medium Overnight bacterial cultures were and incubated at 37°C until they reached exponential growth phase.

Two fold serial dilutions of an antibiotic Levofloxacin (0.5,1, 2) mg/liter were tested in combination with sub-inhibitory concentration of Curcumin (2.5,3,3.5 µg/ml) in MHA. The inoculum (5µL) containing 1 x 107CFU/mL of test strain was added to wells of microtiter plate.then incubation 37°C.

**Antibacterial Activity of Curcumin oil :**

The Curcumin oil were Two serial dilution were prepared by using (DMSO) on Nutrient agar was mixed together. Specifically (0.1ml) of the organism inoculated (*Klebsiella Pneumoniae*) was striking on MHA . Agar Well Diffusion Method using each concentration Curcumin oil and incubated at $37^0$C for 24 hrs, of incubation to determine the (MIC), that showed concentration is the lowest no growths shows . (17)

**Animals**

Twenty five albino male mice, weighing 23±2 grams. The mice were kept in plastic cages and fed on standard pellet and provided by water *ad libitum*. The temperature ranged between 20C and 25C. This study was conducted under the approval of the animal ethics committee of Baghdad University.

**Experimental design:**

The mice were divided into 5 equal groups (n=5 for each group), the mice in the 1st, 2nd, 3rd and 4th groups were anesthetized intraperitoneally with a combination of xylazine (5 mg/kg) and ketamine (75 mg/kg), then the hair of the right flank was shaved (3×2 cm) using electrical shaver and the remaining hair was shaved using disposable hand shaver. The shaved area was cleaned by soap and sterile D.W. and disinfected by alcohol 70%, after that skin wound was induced using sterile lancet in which 3 parallel line of superficial skin wound was made and the injured skin in the 2nd, 3rd, and 4th group were contaminated with *K. pneumonia* using one drop (0.1 ml) from the bacterial suspension $1\times10^6$ cfu/ml. The 5th group considered as control negative group.

The mice in the 1st group were considered as positive control group and the injured skin did not contaminated with *K. pneumonia* and did not received any treatment, also the *K. pneumonia* infected skin in the 2nd group did not recived any treatment too. The mice of the 3rd group were treated locally with Levofloxacin (MIC=0.5 mg/liter) after 2 hr post infection and treatment repeated every 12 hr for 3 days. The 4th group treated with curcuma (3.5 µg/ml) same way as in the 3rd group.

**Histopathological Study:**

All mice were euthanized after Three day post infection and samples (1×2 cm) of injured skin were taken and fixed immediately in 10% formalin solution for 48 hrs, then the samples were processed routinely and sectioned by microtome (thickness 4-6 micron) and the slides stained by Hematoxyline and Eosin stain (18).

## RESULT AND DISCUSSION

*K.pneumoniae* demonstrated that usually have well- (polysaccharide capsule) ,which give their colonies their characteristics sever mucoid appearance abundant (growth, large colonies, and a golden yellow color). The colonies were larger at 48 hrs. on MacConkey agar grew on the test medium more mucoid. *K. pneumoniae*, the colony size was significantly smaller, and the colony color was different from (Muller Hinton Agar) then other medium (Figure 1).

These results were coinciding with pervious results in Iraq (19)

Result show *K. pneumoniae* sensitive to Levofloxacin then Clindamycin, and resistance Azithromycin, Erythromycin and Metronidazol (Fig 2 ) and (Fig 3)

The result agree with (20) the antibiotics showed the highest MIC values for the selected isolates were for amoxicillin and chloramphenicol (16 µg/ml).

On the basis of these results, we suggest that (Levofloxacin) was observed that the MIC values of different antibiotics for *K. pneumoniae* isolated from patients' sputum were found as follows: ampicillin (256 µg/ml), and chloramphenicol (4- 128 µg/ml ). (21) who hows the four groups antibiotic erythromycin, azithromycin and clindamycin *K. pneumoniae* resistant and levofloxacin sensitive.

**Conformation using Vitek 2 ANC System:**

The probability of the isolates by vitek 2 ANC system was twenty-one isolates were (98%) as determined by VITEK 2, we evaluated the true susceptibility and mechanism of resistance. Results coincides with the new Vitek 2 ANC were correctly identified at the genus level. (22)

In this study, the genomic DNA of bacterial isolate was successfully extracted and the extracted DNA has an appropriate quality to perform PCR (10 ng/µl). 16S rDNA was amplified by PCR using specific primers that give a distinct amplicon pattern with a size of 1500 bp when analyzed in gel electrophoresis (Fig 4 ).

The 16S rRNA sequence of our local isolates was identified as *Klebsiella pneumoniae* subsp. *pneumoniae* after BLAST with NR database of NCBI GenBank that showed 100% identity to the strain BR21 (chromosome, complete genome; accession no. CP018885.1).

The traditional methods for detection of *K. pnemouniae* is mainly taken from biological specimens and body fluid but these methods take a long time and are not very accurate (23). Among thousands genes within a bacterial genome, the 16S rRNA gene has served as the initial key for phylogeny based identification when compared against 16S rRNA gene sequence GenBank databases (24).

Moreover, devoted 16S databases that include near full length sequences for a large number of strains and their taxonomic placements exist could be used for the comparison with the sequence from an unknown strain (13).

**Curcumin Extraction:**

(Figure 5 ) by solvent ethanol was statistically,amongst the solvents tested (25).

Curcumin oil extraction on *K. pneumoniae* (MIC) Figure ( 6) , the results indicated as Curcumin oil increases observed concentration the broth less turbid becoming an absence of growth was also showed the (MIC) of Cleanser on *K. pneumoniae* **.**Result agree with (26*)* shows The aqueous extract of (*C. longa* ) exhibited antimicrobial activity against (*Escherichiacoli* **,** *Krebsilla pneumoniae*) .The result obtain from (27) shows appeared to indicate the (ethanol turmeric extract) has high potential to inhibit some pathogenic bacteria.

The MIC agree with (28) in which curcumin (2.5-50 mg/mL) inhibited only *Staph. aureus*, T*yphi, Salmonella serv. Typhimurium, Salmonella serv, Ent. aerogenes, Kleb. pneumonia, Er. carotovora* and *Cit. frundii* were resistant to turmeric extract ethanol . Simler result (29) shows Patients treated with (levofloxacin ) MICs were elevated for burn infections caused by gram-negative organisms .

The skin in the first group (injury only) showed regeneration of the epithelial layer of the skin under a cellular debris and inflammatory cells (Figure 7a), also mild infiltration of neutrophils in the dermis layer, other section showed hyperplasia of the epidermis layer in the incision site (Figure 7b).

In the skin of mice in the 2$^{nd}$ group (injury+infection) the lesion extend deep and all of skin layers were included (epidermis, dermis and subcutaneous tissue) whit necrosis and severe infiltration of inflammatory cells mainly neutrophils (Figure 8 a), also other section showed abscess under the muscular layer (Figure 8b)
The skin in the 3$^{rd}$ group (treated with antibiotic) showed mild regeneration of the epithelial cells of the epidermis, while there is infiltration of neutrophils in dermis (Figure 9a) in addition there is mild regeneration of the epidermal cells in the edge of the incision site under cellular debris and infiltration of collagenous fiber in dermis (Figure 9b).

The 4$^{th}$ group (treated with curcuma) showed early signs of healing in which newly thin layer of regenerated epithelial cells of the epidermis from both sides of the incision extend under the necrotic tissue of the dermal layer and separate it from the healthy tissue (Figure 10a), while other sections showed a thick complete regenerated epidermal epithelia under the cellular debris (Figure 10b) and restoration of the fibrous connective tissue in the dermis layer.

The results of *in vivo* in the 1$^{st}$group (wound only) characterized by the influx of inflammatory cellular infiltrate consisting of PMNs and macrophages under a fibrin plug and this represent the first phase of normal proceed of wound healing. The result agreed with normal wound healing process after 27 hr which descried by (30) And (31). In addition, complete epithelial regeneration of the epidermis layer during 72 hrs revealed normal healing process as described by (31)

In the 2nd group (infection only), sever tissue necrosis, heavy neutrophils infiltration and abscess formation may be due the ability of *k. pneumonia* to evade and survive, rather than actively suppress, many components of the immune system and grow at many sites in hosts, the bacterium achieved this through many virulence factors such as capsule which protect it against the host immune response through multiple mechanisms which include inhibiting phagocytosis by immune cells, preventing activation of the early immune response, and abrogating lysis by complement and antimicrobial peptides.(32). In addition *K. pneumonia* produced more than one siderophore which may be a means of optimizing successful colonization of different tissues and/or avoiding neutralization of one siderophore by the host (33)and (34)

When the infected skin treated with Levofloxacin (3rd group) the tissue showed signs of healing, although these signs are not strong enough to rebuild the skin (after 72 hr of infection) when compared with other similar experiment on other bacteria (31), but it represent slow progress which indicate the high virulence of the bacterium or it ability to resist the effect of the antibiotic, since outer membrane proteins of *K pneumonia* are likely contribute to the integrity and selective impermeability of the cell membrane in an LPS- and capsule-independent manner and along these lines also strengthen *K. pneumoniae* against anionic detergents and certain antibiotics.(35).

The 4th group (treated with curcuma) showed butter healing sings compared with antibiotic treatment, since curcuma decrease neutrophils infiltration and decrease tissue damage (36).

Also Akbik et al (2014) Provided a nice review to view the ability of topical application of curcumin to reduce inflammation and oxidation, and enhance granulation tissue formation, collagen deposition, tissue remodeling and wound contraction during skin healing. (37)


**Figure 1: Colonies of *K. pneumoniae* on . A: Blood Agar , B: Muller Hinton Agar, C: MacConkey agar under anaerobic condition at 37˚C was done at (48) hrs.**

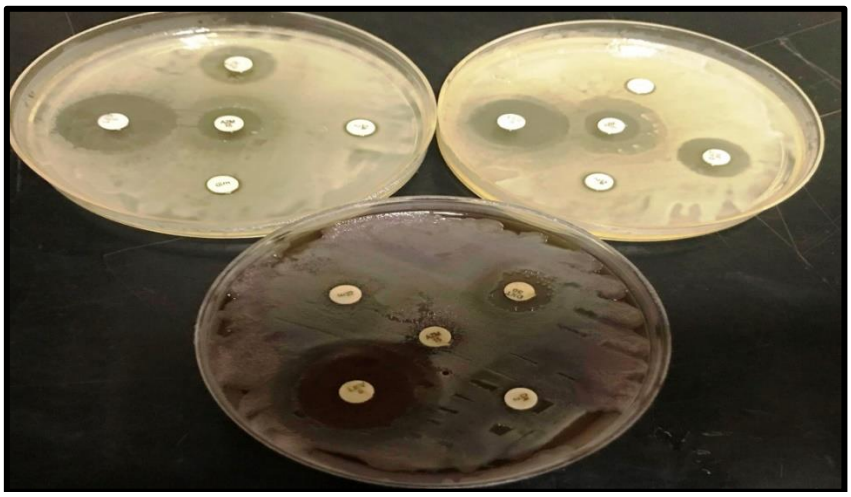

**Figure 2: Antibiotic susceptibility test of isolates most resistance then other *K. pneumoniae* on Muller-Hinton agar plates at 37 ºC for 24 hours**

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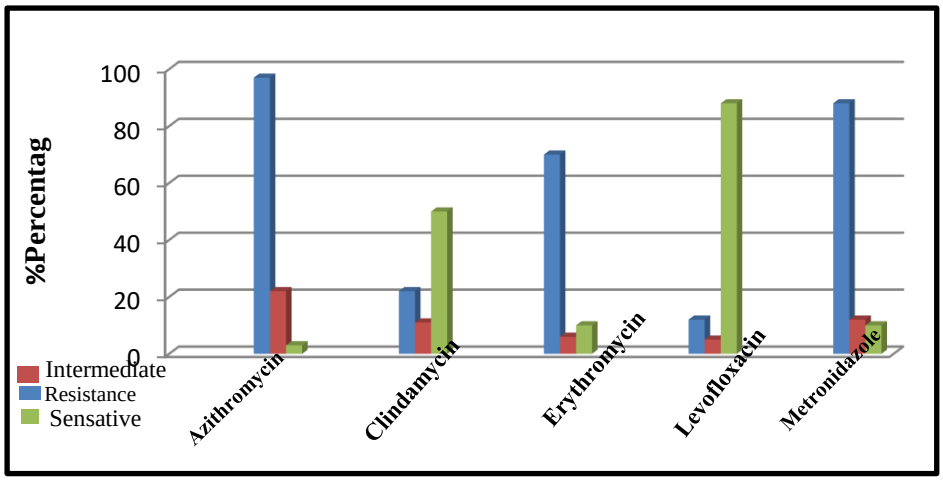


**Figure 3: Antibiotic susceptibility test of *K. pneumoniae***

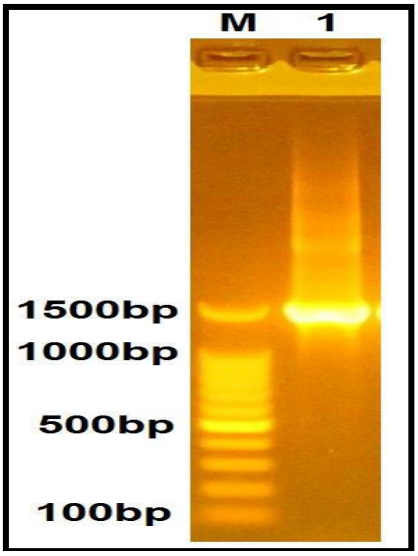


**Figure 4: Agarose gel (1%) electrophoresis (100v/mAmp for 90min) of amplified *16s rRNA* (1500pb) from bacterial DNA stained with ethidium bromide. Lane M. 100 bp DNA ladder, Lane 1. Unknown bacterial isolates.**

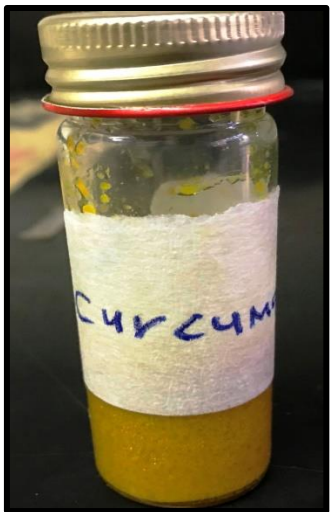

**Figure 5: Curcumin extraction**

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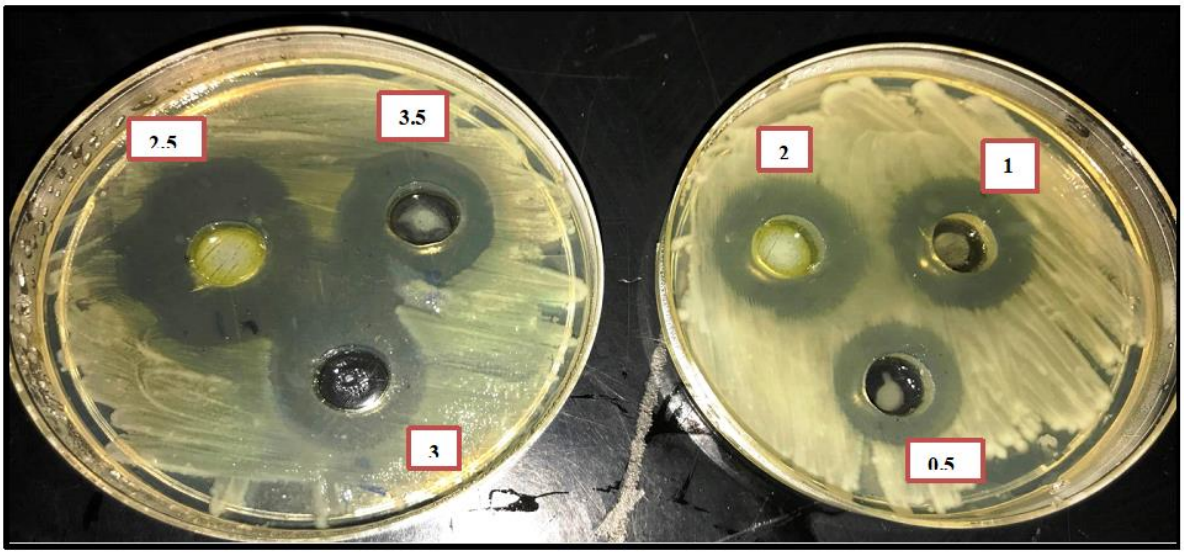


**Figure 6: Comparsion between MIC A) Curcumin oil (2.5,3,3.5 µg/ml) B) Levofloxacin (0.5,1, 2) mg/liter agnist *K. pneumoniae* on Muller-Hinton agar plates at 37 ºC for 24 hours**

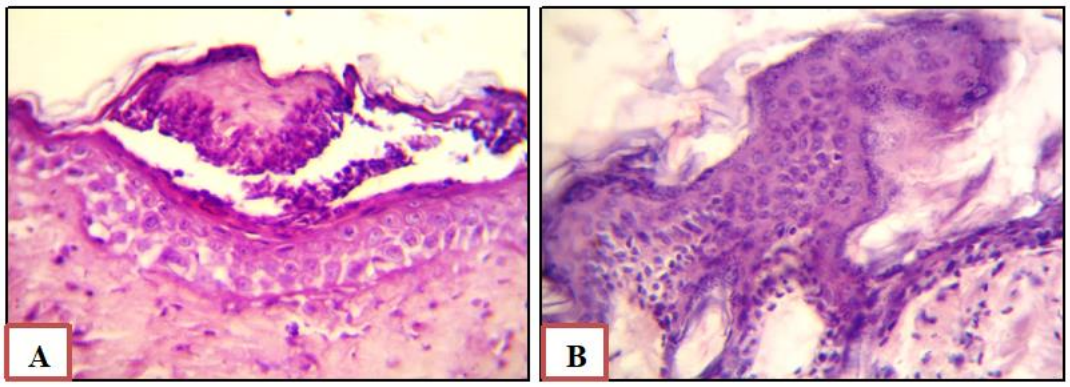


**Figure 7: Histological section in the skin of the 1st group (H & E stain; ×400): (a) regeneration of the epithelial layer of the skin under a cellular debris and inflammatory cells; (b) hyperplasia of the epidermis layer in the incision site.**

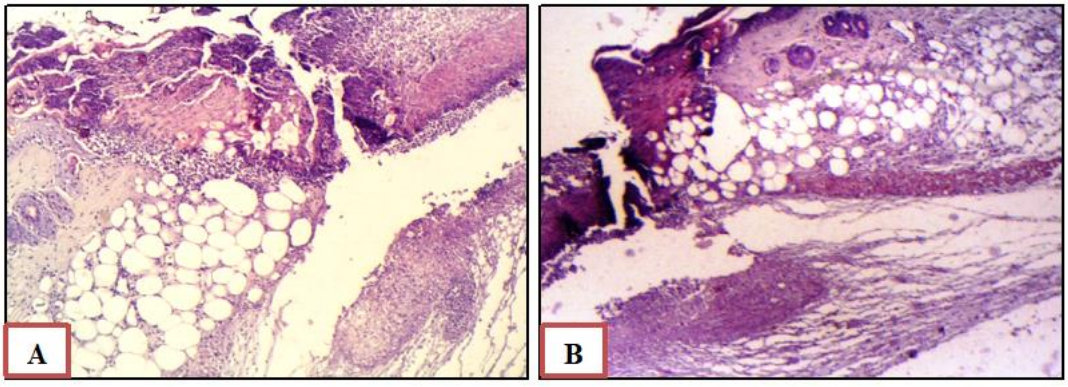


**Figure 8: Histological section in the skin of the 2nd group (H & E stain; ×100): (a) necrosis and severe infiltration of inflammatory cells mainly neutrophils; (b) abscess under the muscular layer.**

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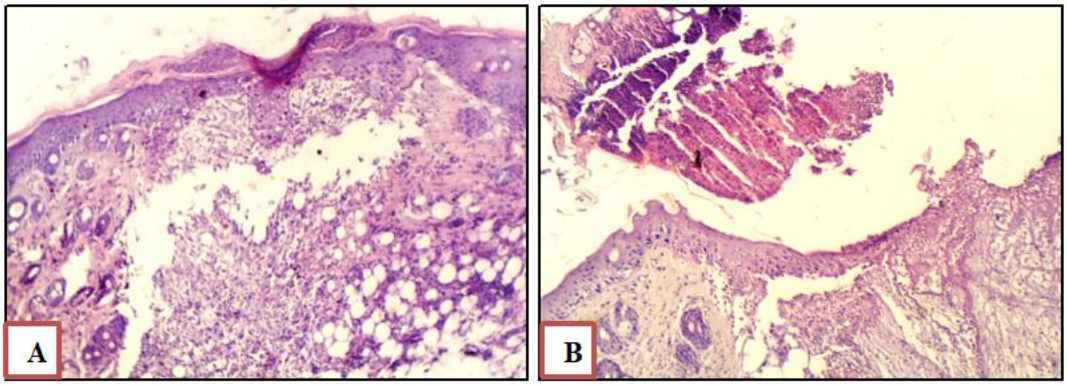


**Figure 9: Histological section in the skin of the 3rd group (H & E stain; ×100): (a) mild regeneration of the epithelial cells of the epidermis and infiltration of neutrophils in dermis; (b) collagenous fiber in dermis.**

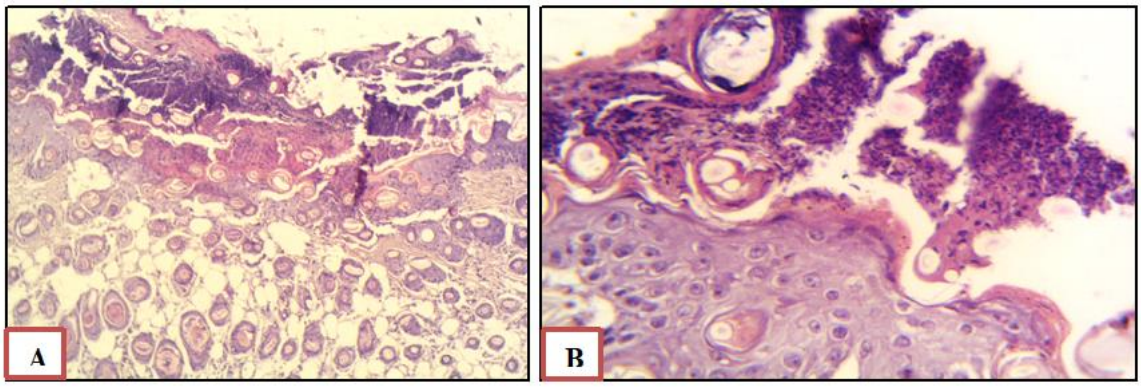


**Figure 10: Histological section in the skin of the 4th group (H & E stain): (a) the new regenerated epithelial from both sides of the incision extend separate the necrotic debris from the healthy tissue (×100); (b) thick complete regenerated epidermal epithelia under the cellular debris (×400).**

## CONCLUSION

The increase in *K. pneumoniae* resistance not only are strains resistant linked to lack or worsening of clinical response to treatment, but the pathogenicity of *K. pneumoniae* has increased over recent years, was very sensitive to curcumin oil more then Levofloxacin *Klebsiella Pneumoniae* by well diffusion assay method. Amplification and sequencing of 16s rDNA is a sensitive, specific, accurate and fast technique for detection of the bacterial isolate at species level and subsp. Treatment of *K. pneumonia* contaminated wound with curcuma limited the skin damage and enhances healing process compared with Levofloxacin treatment which showed limited improvement.

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