جامعة الإسكندرية
ALEXANDRIA
UNIVERSITY
Faculty of Science

Faculty of Science

Department of Botany and Microbiology

# Immobilization of Egyptian Propolis Active Fraction(s) on Nanoparticles as Therapy against MDR *Klebsiella pneumoniae*

**A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy**

**In**

**Microbiology**

**Presented by**

**Bassma Hassan Mohammed Elwakil**

**B.Sc. Microbiology/Chemistry, Faculty of Science, Alexandria 2010**

**M.Sc. Microbiology, Faculty of Science, Alexandria 2014**

**2018**

# 5. SUMMARY

*Klebsiella pneumoniae* is an opportunistic Gram-negative pathogen that may cause hospital-acquired infections. After the emerging of extended spectrum beta lactamases (ESBLs), carbapenemase enzyme has burst as a critical threat. The emergence of KPC-type carbapenemases in *K. pneumoniae* serves to highlight the important therapeutic role of polymyxins such as colistin which has a cidal effect against MDR Gram-negative bacteria. The use of polymyxins has been associated with the emergence of bacterial resistance. Combination therapy of polymyxins with natural products may be the answer to improve the efficacy, the bioavailability and to prevent the emergence of further resistance. Natural products with bioactive ingredients will help to overcome the issue of drug resistance in bacteria. Propolis (bee glue) is a resinous mixture collected by honey bees from living plants and use in construction and adaptation of their hives and it is a good candidate for combination therapy due to its antibacterial activity. Nanotechnology provides the platform to modify the key features of different materials and drug delivering. Nanoparticles can improve the bioavailability, increasing half-life and delivering the drug to its site of action so it will be a suitable solution to deliver the combined drug efficiently.

**The results obtained in this current work are summarized as follow:**

1. In order to evaluate the incidence of ESBL & CRKP K. pneumoniae in ICU's at Al-Shatby Pediatric Hospital, Alexandria, Egypt; a survey study was conducted during 7 months. The most commonly isolated pathogen in all the examined samples was *Klebsiella* sp. representing 51.35% (95 out of the total 185 isolated pathogens) and were distributed as 5.27, 25.26 and 69.47% in urine, BAL and blood samples, respectively.
2. *K. pneumoniae* was identified using Vitek 2 and RapID™ one system.
3. The resistance prevalence among the isolated strains of *K. pneumoniae* towards 21 known antibiotics. All the isolates were resistant to aminopenicillins, 1st generation cephalosporins, aminoglycosides (tobramycin), ureidopenicillins, trimethoprim-sulfamethoxazole and monobactams. The highest susceptibility percentage was noticed with phenicols (53.6%).
4. In a trial to detect the ESBL producing *K. pneumoniae*, 27 and 30 out of 95 isolated *K. pneumoniae* (28.4 and 31.5%) were ESBL producers using DDST and CLSI confirmatory test, respectively.
5. In the present study, 14 out of 30 (46.6%) ESBL positive *K. pneumoniae* were considered as AmpC β-lactamase producing bacteria.
6. In a trial to detect carpabenemase production by the bacterium under investigation a modified carbapenem inactivation method (mCIM) was evaluated. It was concluded that all the 30 isolates under test were carpabenemase producers.
7. Nine out of 30 *K. pneumoniae* strains were CRKP positive using modified Hodge test (MHT).
8. Propolis samples used in the present study were collected from different geographic areas namely: Alexandria, Tanta, Menoufia and Siwa Oasis were harvested away from the entrance of the beehive. Alexandria, Menoufia and Siwa Oasis propolis

were dark brown and Tanta propolis was light brown. Propolis classification showed variation according to origin due to the difference in the dominant pollen grains.

9. Scanning electron microscope of the propolis samples showed the morphology of the propolis samples, it was revealed that no strange materials were detected.
10. The qualitative chemical analysis for the tested propolis samples showed that all the tested samples contained flavonoids and terpenoids while tannins was not found in Alexandria propolis and glycosides were absent in Menoufia propolis.
11. Propolis samples were prepared and analyzed using GC/MS. It was revealed that the common components present in the propolis extracts were heptacosane, octacosanol and pinocembrin.
12. Siwa ethanolic extract proved to be the most active extract against all the tested *K. pneumoniae* using disc-diffusion method.
13. Siwa Oasis propolis extract proved to have MIC values ranged from 6.25 to 100 µg/ml against the tested *K. pneumoniae* strains and had a bacteriocidal effect against the ESBL & CRKP *Klebsiella pneumoniae* strains with an MIC index < 4.
14. Determination of bacterial time-kill curve showed that the bacterial growth was sharply decreased between 4 and 6 hrs.
15. Analysis of SEM micrographs was used to observe any morphological alterations in EEP treated bacterial cell, it was revealed that Siwa Oasis's propolis extract interacted with the outer membrane of the cell wall and induced structural disruption.
16. Transmission electron microscopy was used to evaluate the effect of EEP (Siwa Oasis) against the bacterial cells under test (ESBL & CRKP *Klebsiella pneumoniae* 1). It was observed that the extract was precipitated and adsorbed on the cell surface leading to cell deformation followed by cell membrane disruption leading to release of cellular contents and became as a ghost cell.
17. Column chromatography was used for Siwa Oasis propolis extract fractionation. Each fraction obtained from CC was further separated using TLC for the major components detection.
18. The collected fractions from CC were further subjected to TLC analysis. Eluted bands (6) were tested for their antibacterial activity using disc-diffusion method and MIC. Data revealed that fraction with $R_f$ 1.45 showed an inhibitory effect against the growth of *K. pneumoniae* under test (strain K. 1). The active fraction was further identified using GC/MS analysis. Data revealed that the major detected components of the active fractions were plamitic acid (PA) and phenol,2,4-Bis(1,1-dimethylethyl).
19. A survey study was conducted to test the synergistic effect of ethanolic extract of Siwa Oasis propolis with different antibiotics, colistin combined with the propolis extract showed a synergistic activity.
20. Different nanoparticles were prepared namely: Chitosan/propolis, Chitosan/colistin and Chitosan/propolis/colistin and tested for their antibacterial activity against *K. pneumoniae* stain 1, Chitosan/propolis/colistin was proved to be the most promising novel nano formula and named as nCCp-BeeZ formula.

21. MIC value of the nCCp-BeeZ formula was 6.2 µg/ml and it showed a bactericidal effect against the tested bacterium.

22. Determination of bacterial time-kill curve showed a significant reduction in the bacterial growth after 2 hrs of incubation with the most promising formula with a significant stability.

23. Transmission and scanning electron microscopy (TEM & SEM) were done for ESBL & CRKP *Klebsiella pneumonia* (strain K.1) treated with nCCp-BeeZ formula, both showed leakage of the intracellular components.

24. Characterization of the nCCp-BeeZ formula was done using different techniques. FT-IR and XRD were used to confirm the nanoparticles formation and the increased amorphous nature of nanochitosan after crosslinking with sodium tripolyphosphate.

25. The prepared nano formula had a mean diameter of 48.3 nm, PDI value of 0.67 and negative Zeta potential of -43.6 mV after cremophore addition with entrapment efficiency of 75%.

26. The new formula under test was able for gradualy release of the loaded drug by time.

27. Morphological examination of the nCCP-BeeZ was performed using TEM and SEM revealed the presence of well identified vesicles that exist in a dispersed form and narrow size distribution.

28. *In vivo* investigation (rat model) was applied in order to evaluate the antibacterial activity as well as the toxicity profile of nCCP-BeeZ formula. It was proved that nCCP-BeeZ formula showed high antibacterial effect against the bacterial growth under test with no toxic effect.