

Synergistic Effect of Propolis Extract and Antibiotics on Multi-Resist *Klebsiella Pneumoniae* Strain Isolated fom Wound

Noor M.Taher

Biology Department, Science College, University of Anbar, Ramadi, Anbar, Iraq

Abstract

This study has been done to evaluate the *in vitro* activity of synergistic effect between ethanolic extracts of Propolis and 12 different antibiotic drugs against multi-resistant *Klebsiella pneumoniae* isolated from infected wounds; this evaluation was done by Agar well-diffusion method and Disc diffusion method.

The results of this study showed that the isolation bacteria were resisted to most antibiotics which used in this experiment; especially that drugs containing β -lactam ring. Results of this study showed that alcoholic extracts of Propolis increase the inhibition zones of all antibiotics; and the sensitivity of bacteria increase by direct correlation with the increase in the concentration of alcoholic extract of Propolis.

Keywords: Synergistic effect, Propolis, Antibiotics, Multi-resistance, *Klebsiella pneumoniae*

1. Introduction

Natural products are an auspicious source for the discovery of new pharmaceuticals products. In the last decades, numerous works dealing with Propolis structure and biological properties have been published, revealing the interest of researchers on this bee product and its potential for the development of new drugs as well (Liberio *et al.*, 2009), in order to solve the continuous problem of bacterial resistance to antibiotics (Normark & Normark, 2002), where natural products used for these purposes since ancient times are the main target (Silver, 1990).

Among these natural products, propolis has been considered a good candidate as adjuvant in the treatment and prevention for various infectious diseases. The word Propolis is derived from the Greek word *pro* = before and *polis* = city (Ivančajić *et al.*, 2010). Due to its waxy nature and mechanical properties, bees use propolis in the construction and repair of their hives for sealing openings and cracks and smooth out the internal walls and seal off inside the hive any dead animals or insects which are too large to be carried out and perhaps most important of all, to mix small quantities of propolis with wax to seal brood cells (Ramos & Miranda, 2007). Humanity has recognized this Propolis significant activity at very early times, for its magnitude purposes, especially as a medicinal substance because of its antimicrobial properties.

Propolis was used specially in antiquity, in Egypt. There some thousand years BC, propolis was very well known to the priests who had monopolized medicine and chemistry. The fact that propolis was also known to Ancient Greek, their writings refer to this substance as a cure for suppurating wounds whereas history reviles during the 12th century Europe prescribe Propolis for treatment of mouth and throat infections as well as dental caries, and in the Second Global War it was used in several Soviet clinics for tuberculosis treatment, due to the observed decline of lung problems and appetite recovery. In the Balkan states it was one of the most frequently used remedies, applied to treat wounds and burns, sore throat and stomach ulcer (Haddadin *et al.*; 2008, Ramos & Miranda, 2007; Fokt *et al.*; 2010).

Propolis a complex resinous mixture, collected by Honey bees from exudates and buds of the plants and mixed with pollen and wax and bee enzymes. Its color varies from yellowish-green to dark brown depending on its source and age (Greenaway *et al.*, 1990) and The chemical composition and The biological activities of propolis (antibacterial, antiviral, antifungal etc.) varies depending on the diversity of plants and geographic locations from which bees collect it (Kumar *et al.*, 2008).

As mentioned above, propolis is a complex mixture made from plant-derived and bee-released compounds. The percentage of the various substances is variable and depends upon the place and time of collection (Bankova, 2005; Yang *et al.*, 2007), but, in general, raw propolis is composed of around 50% resins, 30% waxes, 10% essential oils, 5% pollen and 5% of various organic compounds (Haščik *et al.*, 2012). More than 300 components were identified in different samples and new ones are still being recognized during chemical characterization of new types of propolis (Tazawa *et al.*, 1998).

Many analytical methods have been used for separation and identification of propolis constituents and the substances recognized belong to the following groups of chemically similar compounds: flavonoids; benzoic acids and derivatives; benzaldehyde derivatives; cinnamyl alcohol, and cinnamic acid and its derivatives; other acids and respective derivatives; alcohols, ketones, phenols and heteroaromatic compounds; terpene and sesquiterpene alcohols and their derivatives; sesquiterpene and triterpene hydrocarbons; aliphatic hydrocarbons; minerals; sterols and steroid hydrocarbons; sugars and amino acids (Fokt *et al.*; 2010). Several composites are possibly present in all propolis samples and contribute to its biochemical properties. These magnificent components contribute propolis a numerous therapeutic properties, such as antibacterial, antiinflammatory, and antifungal, antiprotozoal and antiviral activities (Lofty, 2006).

Until these days, Infectious diseases still represent an essential cause of morbidity and mortality among humans, especially in developing countries. Even though pharmaceutical companies have produced a number of new antibacterial drugs in the last years, resistance to these drugs by bacteria has increased and has now become a global concern. In general, bacteria have the genetic aptitude to transfer and acquire resistance to drugs used as therapeutic agents (Nascimento *et al.* 2000).

Klebsiella pneumoniae is renowned as one of the major causes of infections in humans occurring in both the community and the hospital and it have become a major nosocomial pathogen (Ghotaslou *et al.*, 2007). Therefore, the importance of identifying new active antimicrobial agents cannot be exaggerated.

The synergistic effect from the combination of antibiotics with natural product extracts against resistant bacteria leads to new ways for the treatment of infectious diseases. The increasing and indiscriminate antibiotics have led to the development of bacterial resistance to antibiotics (Hemaiswarya *et al.*; 2008).

The concept of synergistic between natural products and antibiotic drugs open an unusual method to reuse of antibiotic in different way and has been recently reported (Nascimento *et al.*, 2000; Aqil *et al.*, 2005; Junior *et al.*, 2005; Betoni *et al.*, 2006; Esimon *et al.*, 2006; Ibezim *et al.*, 2006; Ali *et al.*, 2007; Horiuchi *et al.*, 2007; Halawani & Shohayeb, 2011; Ahmed *et al.*, 2010; Lacmata *et al.*, 2012) In this *in vitro* study, we evaluated the possible synergism between ethanolic extracts of propolis and certain known antibiotic which used in bacterial infection treat against multi-resistance *Klebsiella pneumoniae*.

2. Materials and Methods

2.1. Sample of Propolis

Propolis samples were collected from country provinces in Anbar, Iraq. These samples were collected in March and July of 2013, in sterile glass container away from sunlight.

2.2 Extraction and sample preparation

Thirty gram (30gm) of sample was cut into small pieces and extracted at room temperature with 100 ml of 70% ethanol. The solution was shaken daily and left at room temperature for 7 days, then the solution was filtered through Wattman paper No. 1 and placed in dark sample bottles, Alcoholic extract was evaporated under vacuum at 50° C until dryness.

2.3. Collection and transport of clinical samples

The study was performed on 21 patients who suffer from wound infections from Ramadi University Hospital. Samples were collected and transported to laboratory according to Chessbrough (1984). By using a sterile cotton swab; samples were collected from wound, immersed in a container of transport medium. On collecting samples from wounds, special precaution should be taken to prevent contaminating specimen with commensal organisms from the skin.

2.4. Identification of bacterial isolates

One bacterial isolate of *Klebsiella pneumoniae* is collected from the specimens; *Klebsiella pneumoniae* was isolated from the bacteriological examination of purulent secretion taken from the wound. We obtained gram-stained slides which we examined microscopically.

Purulent secretion was cultivated on Nutrient Broth (37°C, 24 h), and then transferred on selective media (blood-agar, MacConkey agar) and subsequently on multitest media (TSI, Simmons Citrate, SIM, Urea Agar base, MR-VP). For the final identification we used vitek AutoMicrobic for identification of gram-negative system.

2.5. Antibiotic sensitivity test

The antimicrobial susceptibility was determined by Kirby and Bauer (1966) disk diffusion method in accordance with the Clinical and Laboratory Standards using commercially available antimicrobial discs (Bioanalyse). Following antibiotics were used: Ampicillin (10µg), Piperacillin (75µg), Ceftazidime (30µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Gentamicin (15µg), Chloramphenicol (30µg), Amikacin (30µg), Cefepime (30µg), Ticarcillin (75µg), Amoxicillin (25µg), Amoxicillin/clavulanic acid (30µg). *Escherichia coli* (ATCC 25922) was used as control strain for antibiotic sensitivity test. Then, the results were compared tables of World Health Organization.

2.6. Susceptibility of bacterial isolates to propolis extract

Antibacterial activity of propolis was measured using a well diffusion method. Briefly, Petri plates containing 20 ml of Mueller-Hinton agar were inoculated with 18-24 h culture of the bacterial strains at concentration of 10^6 cell/ml. Wells (6 mm diameter) were punched in the agar and filled with 30 µl of propolis extract (100 and

200 mg/ml). The plates were incubated at 37 °C for 24 h. The antibacterial activity was calculated by measuring the diameter of the area in which bacterial growth was inhibited around the well.

2.7. Evaluation of synergistic effect

Klebsiella pneumoniae was grown in brain heart liquid medium at 37°C, after 24 h of growth the bacteria at concentration of 10^6 cell/ml was inoculated on surface of Mueller-Hinton agar plate. Then the antibiotic disks saturated with 10 µl propolis extract were placed on surface of each inoculated plates. The plates were incubated at 37°C for 18-24 h; the diameters of clear zones were measured and compared with that of the antibiotic alone (Betoni et al., 2006).

3. Result and Discussion

The need to combat bacterial resistance to antibiotics is an increasing global concern. *Klebsiella pneumoniae* is one of the Gram-negative bacteria as shown in table 1, that have been shown to exhibit resistance to an extensive range of commonly available antibiotics. Therefore, new chemotherapeutic agents and new methods are urgently needed to combat such multiantibiotic-resistant bacteria. Combined antibiotic therapy has been shown to delay the emergency of bacteria resistance and may also produce desirable synergistic effects in the treatment of bacterial infection.

Our results in this report indicated that *Klebsiella pneumoniae* showed a great sensitivity to the alcoholic extract of propolis with average inhibition zone 12.6 mm at concentration of 100 mg / ml and 16 mm at concentration 200 mg /ml, which indicates that the antimicrobial activity of propolis extract increase with a proportional increase of propolis concentration. The antibacterial activity propolis due to its high content of flavonoids which vary in the quantity and formulae depending on the area which combines the propolis (Ophori & Wemabu; 2010).

Moreover; the antimicrobial action of propolis is recognized, however the mechanisms of how this effect works are still unknown. Some components present in the propolis extracts have been described, like flavonoids and caffeic, benzoic, and cinnamic acids. These probably act someplace on the membrane or the bacterial wall, causing purposeful and structural damage (Scazzocchio *et al.*, 2006; Kosalec *et al.*, 2005).

On the other hand; the antibiotic sensitivity test results showed the decline efficiency of routinely available antibiotics (used in the hospital) against highly frequencies pathogenic bacteria, *Klebsiella pneumoniae* was shown huge degree of resistance to Ampicillin, Piperacillin, Cefazidime, Ceftriaxone, Ciprofloxacin, Gentamicin, Chloramphenicol, Cefepime, Ticarcillin, Amoxicillin and Amoxicillin/clavulanic acid whereas, the isolate was sensitive to Amikacin only.

As shown in Table 1. *Klebsiella pneumoniae* as nosocomial pathogen which is resistant to the most available antibiotics especially to β - lactame group. In the same sense, Elhag *et al.* (1999); Orrett & Shurland (2000) noted that the resistant occur because of the widespread and misuse of antibiotic, especially in the developing countries, the resistance profile of microorganisms has been altered significantly, also there is could be attributed to several mechanisms, mostly related to antibiotics overuse.

Recently, the use of single antibiotic does not produce the desired or the effective inhibitory effects and to overcome this, combination between antibiotics and natural product often exercise their synergistic effect which surpasses their individual performance. Drug synergism between known antibiotics and bioactive product extracts is a novel concept, and could be beneficial (synergistic or additive interaction), however when treat the isolation intersecting extract of propolis with antibiotics, the results showed, there is great variation in the sensitivity of *Klebsiella pneumoniae* to antibiotics independently, on the one hand and change this sensitivity when the interference of antibiotics with the extract.

A perusal of Table 2 clearly indicates that Amoxicillin for example couldn't cause any inhibition, but when combined with propolis extract surpassed both these values of inhibitory zone and synergistically resulted in the formation of 21 and 25 mm inhibition zone. The case was similar with the combination of other antibiotics with propolis extract.

The ability of natural extracts to potentiate antibiotics has not been well illuminated. It is speculated that inhibition of drug efflux, and alternative mechanisms of action could be responsible for the synergistic interactions between the extracts and antibiotics (Lewis & Ausubel, 2006; Zhao *et al.*, 2001).

The preponderance of investigates on the interaction between natural extracts and antibiotics have been focused on the identification and isolation of potential resistance modifiers from such natural sources which are considered to be positive results. However, it is likely that such combinations could produce antagonistic interactions that most studies have considered irrelevant and therefore ignored (Sibanda & Okoh, 2007).

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Table (1) Identification of *Klebsiella pneumoniae*

| Test | Result |
|-----------------------------|--------|
| IMVC | --++ |
| TSI | A/A |
| Gelatinase | - |
| Urease | + |
| Nitrate Reduction | + |
| H ₂ S production | - |
| Starch analysis | - |
| Lactose ferment | + |
| Oxidase | - |
| Catalase | + |
| Motility | - |

Table (2) synergistic effect of alcoholic extract of Propolis and antibiotics on *Klebsiella pneumoniae*

| Antibiotic | Antibiotic alone | Propolis extract and antibiotic | |
|---------------------------|------------------|---------------------------------|-----------|
| | | 100 mg/ml | 200 mg/dl |
| Ampicillin | 0 | 17 | 19 |
| Ceftazidime | 10 | 30 | 31 |
| Amoxillin\clavulanic acid | 0 | 22 | 30 |
| Ciprofloxacin | 0 | 18 | 20 |
| Chloramphenicol | 0 | 19 | 23 |
| Ceftriaxone | 0 | 34 | 36 |
| Amikacin | 17 | 23 | 25 |
| Gentamicin | 0 | 22 | 24 |
| Ticarcillin | 0 | 29 | 30 |
| Piperacillin | 0 | 32 | 34 |
| Amoxillin | 0 | 21 | 25 |
| Cefepime | 0 | 16 | 19 |