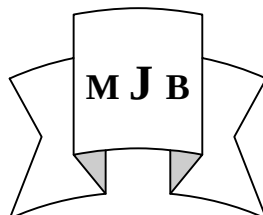


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The results of Amoxicillin-propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori*, *E. coli* and *K. pneumoniae*, while the results of clindamycin-propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori*, *K. pneumoniae*, and *E. aerogenes*. The results of Rifampin-propolis combination on the bacterial isolates were; synergistic to words on *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *S. pyogenes*, and *E. aerogenes*. Last but not least, the effect of Nistatin and propolis combination against *C. albicans* isolates indicated for additive effect.

خليط (نيستاتين و البروبوليس) على عزلات الخميرة ان لها تأثير مضاف .

Introduction

Propolis is a resinous substance collected by worker bees (*Apis mellifera*) from the bark of trees and leaves of plants. This salivary and enzymatic secretions-enriched material is used by bees to cover hive walls to ensure a hospital-clean environment. As a natural honeybee hive product, propolis extracts have been used both internally and externally for thousands of years as a healing agent in traditional medicine. Propolis shows a complex chemical composition. Its biological properties-such as antibacterial, antiviral, antifungal, among other activities, have attracted the researchers' interest [1].

Its biological properties may vary according to different plant sources. In Brazil, there are many plants that could be visited by bees as sources of propolis, whose chemical composition may differ depending on the geographic location [2].

In laboratory tests, studies have shown broad spectrum antimicrobial activity of various propolis extracts. Synergism with certain antibiotics has been demonstrated. Depending upon its composition, propolis may show powerful local antibiotic and antifungal properties. Many authors have demonstrated propolis antibacterial activity against *Enterococcus* spp, *Escherichia coli*, and *Staphylococcus aureus*. Reports have pointed out propolis efficient activity against Gram-positive bacteria and limited action against Gram-negative bacteria [3].

Different researchers [2,4] have reported that propolis antibacterial activity is attributed to a number of

phenolic compounds, mainly flavonoids, phenolic acids and their esters. Some prenylated coumaric acids were isolated from propolis in several countries [5] and [6]. Although numerous researchers have been reported the biological activities of propolis collected worldwide, information about Iraqi propolis are still absent. The aim of this evaluate the antimicrobial activity of the crud ethanolic extract of Iraqi propolis on some pathogenic bacterial isolates and *Candida* spp., Evaluate the antimicrobial activity of antibiotics on some pathogenic bacterial isolates and *Candida* spp. and Evaluate the antimicrobial activity of ethanol extracts of propolis- antibiotics combination on some pathogenic bacterial isolates and *Candida* spp.

Materials and Methods

Propolis samples

Propolis samples were collected from hives of honey bees of Al-Museiab, Iraqi during spring and summer seasons of 2010 and 2011.

Bacterial & yeast strains

Standard bacterial strain *E. coli* ATCC 25922 (USA, oxide) *S. typhi* TY21 (Central health lab, Baghdad). This bacterium was activated and cloned three successive times in nutrient agar and stored on nutrient agar slant at 4 °C. Local bacterial isolates represented by; *L. monocytogenes* (Kufa Univ./ College of science), *H. pylori* (Univ./ College of science), *E.aeruginosa*, *S. typhi*, *K. pneumoniae* *S. aureus*, *P. aeruginosa*, and *S. Pyogenes* (Babylon Univ./ College of Medicine).The identification of these organisms was

confirmed by using conventional biochemical tests [7].

Antibiotics susceptibility test of bacteria

The following antimicrobial discs (obtained from Bioanalyse, Trukia); Amoxicillin (AX, 25 mcg), Cefaxime (CFM, 5mcg), Clindamycin (DA,2mcg), Erythromycin (E, 15mcg), Naldixic acid (NA,10mcg), Refampin (RA, 5mcg), Vancomycin (VA, 30mcg), and Tetracycline (TE, 30mcg).

The following antimicrobial solution (obtained from oxide, USA and SDI, Iraq); Amoxicillin (100 mcg), Cefaxime(100mcg), Clindamycin(10mcg), Erythromycin(250mcg), Naldixic acid (500mcg), Refampin (250mcg), Vancomycin (500mcg), and Tetracycline (25mcg) for bacteria, and Nystatin (100000 I.U) for yeast. This method was detected according to [8].

Antifungal susceptibility testing

Preparation of antifungal concentration

This method was detected according to [9].

Preparation of antifungal discs and measurement of inhibition zone

Antifungal susceptibility was tested by using the paper disc agar diffusion method [10] and the zones of inhibition were subsequently measured in mm [11].

***In vitro* antibacterial and antifungal activities of crude propolis extract by the well diffusion method (NCCLS, 2002):**

This method was detected according to (NCCLS, 2002).

Antibiotics and propolis susceptibility test bacteria and yeast

The solutions (antibiotic with propolis solution) were applied by using wells. The plates were incubated at 37 C° for overnight. The size of zone of inhibition was measured from edge of well to the edge of inhibition of growth.

Statistical analysis

Bonferroni test recommended by [12] was used for statistical analysis ($P \leq 0.05$) to show if there is any significant differences between results of propolis ethanolic extract and antibiotics.

Result and Discussion

***In vitro* antibacterial and antifungal activities of crud extract of propolis**

As a general rule extract is considered active against both bacteria and fungi when the inhibition zone is greater than 6 mm [13]. Antimicrobial activities of crud extract of Al - Museiab propolis (EEMP) at 10%, concentration against both bacteria and fungi were studied (Figure 1).The results of agar diffusion showed that most bacterial isolates were sensitive to EEMP *S. aureus* was higher sensitive to EEMP than other Gram positive and Gram negative bacteria followed by *L. monocytogenes* with inhibition zones of 25mm and 18mm respectively while standard strain *E. coli* was good sensitive to EEMP than other Gram negative bacteria with inhibition zones of 15mm. The inhibition zone for *S. pyogenes* was 14mm while the inhibition zones for each of *S. typhi* and *K. pneumoniae* were 12mm. The inhibition zone was 10 mm for each of *P. aeruginosa*, *H. pylori*, and *E. aeruigenes*. EEMP was not effective against *C. albicans*.

Statistical analysis showed no significant differences after treating

the MO with propolis ethanolic extract at 10% concentration of agar diffusion ($P \leq 0.05$). This result indicated that the active components of propolis are concentrated in this sample. This was in agreement with reports of several researchers which indicated that each propolis sample contain 80–100 chemical compounds with different concentrations[14, 15].

The present results on *S. aureus* are in agreement with those obtained by several authors who the inhibition zones obtained by propolis from Mongolia, Albania, Egypt and Brazil were 24, 21.8, 24.3, and 21.8 mm respectively [16]. These results are comparable with results obtained by [17] who found that the inhibition zone for Bulgarian propolis was 20 mm also with results obtained by [18] who found out that the inhibition zone of propolis from different geographical areas of Serbia ranged from 18 - 23 mm.

Biological and pharmaceutical activity of propolis contributed to the propolis contain active compound such as phenols, flavonoids and alkaloids that possessing antibacterial and antifungal activities against bacteria and fungi. This results were comparable with results obtain by several authors [19 ,20]. Ophori et al.(2010) [21] who reported that the

antimicrobial activity of propolis is as a result of the high content of flavonoids.

However, the variation might reflect the difference in the composition of the propolis, since the bacterial strain used was the same. The lower sensitivity (or resistance) of *E. coli* is in agreement with the findings by many researchers where this bacterium showed either very low sensitivity or total lack of sensitivity against propolis [16,22]. This emphasizes the fact that, gram negative bacteria are less sensitive than gram positive strains, which is in agreement with several reports [23, 22].

The most possible explanation for the low sensitivity of gram negative bacteria is that their outer membrane inhibits and/or retards the penetration of propolis [24]. Another possible reason is their possession of multi drug resistance (MDR) pumps, which extrude amphipathic toxins across the outer membrane [24].

Regarding anti-*L.monocytogenes*, the results in this study was in agreement with [25] who mention that the diameter of inhibition zone of ethanol extract against *L. monocytogenes* was 26mm-14mm and MIC was 0.25-11.75 mg/ml.

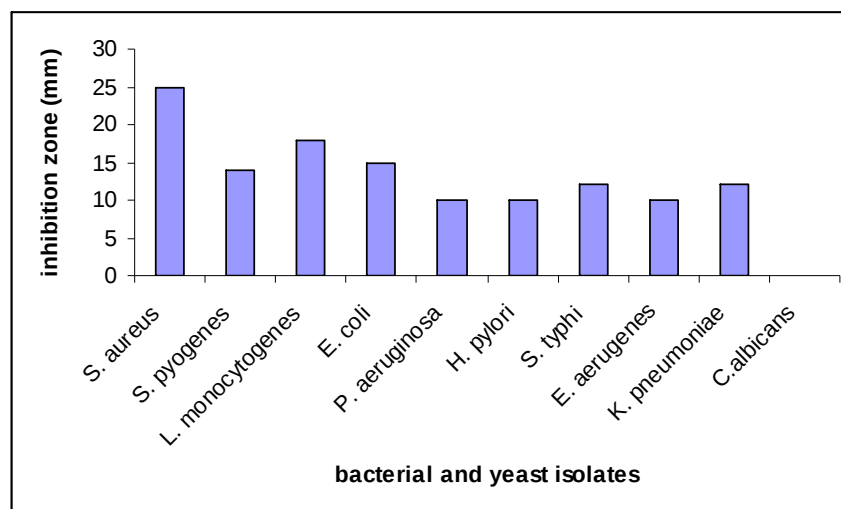


Figure 1 Effect of ethanol extracts of Al-Museiab crude propolis on the bacteria and fungi isolates in well diffusion test.

However, this activity varies according to geographic regions, the pH of the culture medium [26].

The mechanism of antibacterial action of propolis has been the subject of only a few publications. [27] showed through electron microscopy and micro-calorimetric assays that ethanolic extracts propolis (EEP) interferes with the division of bacterial cell through the formation of pseudo-multicellular forms, cytoplasm disorganization or bacterial cytoplasm, cell membrane and cell wall collapse and inhibition of protein synthesis, leading to lysis of the bacteria [28] found that EEP and some of phenolic components affect the bioenergetical status of the membrane by inhibition of the membrane potential leading to increased permeability of the membrane to ions and to immobility of *B. subtilis*. A synergistic effect with conventional anti-mycotic drugs was also observed [29].

Antibiotic sensitivity test of bacteria and yeast

Comparative study was made to show the effect of the following eight antibiotics on bacteria; (Amoxicillin, Cefaxime, Vancomycin Tetracycline, Erythromycin, Clindamycin Nalidixic acid, and Rifampin) (Table 4).

The results revealed that Amoxicillin was effective against *S. aureus*, *E. coli*, and *S. typhi* isolates, while it was not effective against *P. aeruginosa*, *K. pneumoniae*, *S. pyogenes*, *L. monocytogenes*, *H. pylori*, and *E.aerugenes*. These results of high resistant to this antibiotic against *P. aeruginosa*, and *K. pneumoniae* in nearly compatible with that of [30] who found that 90% of *P. aeruginosa* isolates were resistant to Amoxicillin and [31] was found that 98.2% of *K. pneumoniae* were resistant to Amoxicillin.

On the other hand, *P. aeruginosa*, *K. pneumoniae*, *S. pyogenes*, *L. monocytogenes*, *H. pylori*, and *E.aerugenes* exhibited remarkable resistance to amoxicillin. This result was in agreement with result mentioned

by [32] who found that the primary mechanism for resistance to β -lactam is the enzymatic hydrolysis of the β -lactam ring, failure of antibiotic to penetrate to penicillin binding proteins (PBP) target site and low affinity binding of antibiotic to PBP also confers resistance to these antibiotic. Other mechanisms included decreased membrane permeability to words the antimicrobial agents.

Moreover, the results indicated that Cefaxime was effective only against *E. coli*. On the other hand, [33] had pointed out that (82.4%) of *S. aureus* strains were resistant to Cefaxime. In this study *P. aeruginosa* exhibited resistance to many antibiotics possibly due to the intrinsic or acquired in which these bacteria are highly inherently resistance and this arises from combination of unusually restricted outer membrane permeability and chromosomally encoded β -lactamase. These results were in agreement with result mentioned by [34].

The resistance result of *P. aeruginosa* to many antibiotics due to extracellular alginate of *P. aeruginosa* is impair diffusion of antibiotics and *P. aeruginosa* also produce more pigments, the most common pigments; pyocyanin and fluorescein are less susceptible to many antibiotics.

The resistance result of *K. pneumoniae* to cefotaxime is probably attributed to the fact that most clinical isolates of *K. pneumoniae* produce different plasmid and/or chromosomal mediated beta-lactamase enzymes. As plasmid mediated enzyme production is much more easily transferred to cells, there had been

increase in beta-lactamase resistance to third generation cephalosporins (i.e. cefotaxime). These enzymes are called "Extended Spectrum Beta-lactamses"(ESB) and these enzymes are currently inhibited by clavulanic acid. This result was in agreement with results obtained by [35] who stated that the new generations of β -lactam antibiotics are better to be used instead of ampicillin and amoxicillin like those containing β -lactamase inhibitors, namely clavulanic acid.

Regarding to vancomycin, it was effective against *S. aureus*, and *L. monocytogenes*. It inhibits early stages in cell wall peptidoglycan synthesis. In this study the result of *S. aureus* susceptibility was in agreement with (36) who clarified that the inhibition zone of vancomycin recorded 20mm against *S. aureus*. The results also revealed that tetracycline was effective against *S. aureus*, *L. monocytogenes*, *H. pylori* *E. coli*. [37] had mentioned that tetracycline is bacteriostic antibiotic, it is active against wide range of bacteria, tetracycline attaches to tRNA and prevent complexation of aminoacyl tRNA with m RNA of 30S unit.

Regarding to erythromycin and clindamycin, they were effective against only *S. aureus*, and *L. monocytogenes*. This result of bacterial sensitivity was closely compatible in agreement with the results obtained by [38] who had found that (5%) of *Streptococcus* were resistance to erythromycin, and [39] who mentioned that (32.6%) of *S. aureus* were resistance to erythromycin. The resistance to erythromycin due to an alteration

(methylation) of the rRNA receptor, which is under control of a transmissible plasmid [40]. This result was similar to the result observed by [30] who found that all of the *P. aeruginosa* were resistant to erythromycin.

Moreover, the results revealed that naldixic acid was effective against *K. pneumoniae*, *L.monocytogenes*, *P. aeruginosa* and *E. coli*. [15] observed that the naldixic acid and clindamycin had antibacterial activity against slandered strain of *E. coli* with. It is the drug of choice against *S. aureus* due to blocking of DNA gyrase. *P. aeruginosa* possesses active efflux pump system acts as wide transporters for a whole range of antibiotics that coupled with the narrow porin channels in the outer membrane of this organism restrict diffusion of many antimicrobial agents into the cell.

Regarding to the results of refampin, which was found to be effective against *L. monocytogenes*, and *H. pylori*, it binds strongly to DNA-dependent RNA polymerase and thus inhibits RNA synthesis in bacteria. The difference in the level of susceptibility to certain drugs could be attributed to the frequency at which that individual drug has been used in the hospital during the period of study. Furthermore, the antibiotic resistance could be transferred from one bacterial strain to another by transposable gene (Transposes), which could be transposed from the chromosome to the proper plasmid [41].

Results also showed that Nistatin had no activity on yeast isolates. Antifungal sensitivity test of *C.albicans* was completely resistance

to nistatin drugs which may due admensentraion of nistatin to the patients for a long time [42]. In conclusion the antibacterial and antifungal activities of propolis extracts were more effective than antimicrobial agent due to the affinity of antimicrobial agent and reaction with cell component and have specific receptors on bacterial cell wall or specific carrier into the cell for stopped the enzymes and coenzymes or interference with bioactivities such as inhibition of protein and nucleic acid synthesis [43].

Antimicrobial activity of antibiotics and propolis combinations

The antibacterial activities of crude propolis extracts and antibiotics were mentioned above. The activities of propolis combination with antibiotics on the bacterial and yeast isolates were studies. The results of Amoxicillin–propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori*, *E. coli* and *K. pneumoniae*, additive to words *S. aureus*, *S. pyogenes*, *L. monocytogenes* and *E. aerugenes*, and indifference to words *S. typhi* (Figure 2). Statistical analysis showed significant differences between effect of propolis and Amoxicillin–propolis combination on bacterial isolates but there was no significant differences between Amoxicillin and Amoxicillin –propolis combination at level ($P \leq 0.05$).

A synergistic effect of different propolis samples with commercial antibiotics was reported worldwide [19]; and also bacteria resistant to these drugs were susceptible to propolis [44]. [45] stated that the antimicrobial activities of propolis

against bacteria especially against bacteria resistant for random using of antibiotics. Propolis had antimicrobial activities against only pathogenic bacteria with it had not any side effect.

Regarding to the results of Cefaxim and Cefaxim-propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori*, *E. coli*, *K. pneumoniae* *L. monocytogenes* and *E. aerogenes*, additive to words *S. typhi* and *S. pyogenes*, and antagonism to words *S. aureus* (Figure 3). Statistical analysis showed significant differences between effect of propolis and Cefaxim–propolis combination on bacterial isolates but there were no significant differences between Cefaxim and Cefaxim –propolis combination at level ($P \leq 0.05$).

[46] stated that the propolis inhibits bacterial growth by preventing cell division, thus resulting in the formation of pseudo-multicellular Streptococci. In addition, propolis disorganized the cytoplasmic membrane and the cell wall, causing a partial bacteriolysis and inhibition of protein synthesis. It was evidenced that the mechanism of action of propolis on bacterial cell is complex and a simple analogy cannot be made to the mode of action of any classic antibiotics components [47].

[48] reported that the ethanolic extract of propolis had marked synergistic effects against *S. typhi* with some antibiotics (amoxicillin and cefalexin) acting on the cell wall. This result is of great importance in medicine, since the dose, the side effects and the cost of antibiotics can be reduced. In this work, propolis

seemed to aid β -lactamic antibiotics in PBP inhibition, which could explain the synergistic effects. Besides, propolis could also diminish the resistance of the bacterial cell wall to antibiotics, promoting β -lactamics action on PBP. Propolis could be used as an alternative therapy for the resistant strain treatment.

On the other hand, the results of vancomycin -propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori*, *E. coli*, *K. pneumonia* and *S. pyogenes*, additive to words *E. aerogenes* and *L. monocytogenes*, indifference to words *S. typhi*, and antagonism to words *S. aureus* (Figure 4). Statistical analysis showed significant differences between effect of propolis and vancomycin –propolis combination on bacterial isolates but there were no significant differences between vancomycin and vancomycin –propolis combination at level ($P \leq 0.05$).

Furthermore, the results of Clindamycin-propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori*, *K. pneumonia*, and *E. aerogenes*, additive to words *S. typhi*, *L. monocytogenes* and *S. pyogenes*, indifference to words *E. coli*, and antagonism to words *S. aureus* (Figure 5). Statistical analysis showed significant differences between effect of propolis and Clindamycin –propolis combination on bacterial isolates and there were significant differences between Clindamycin and Clindamycin –propolis combination at level ($P \leq 0.05$).

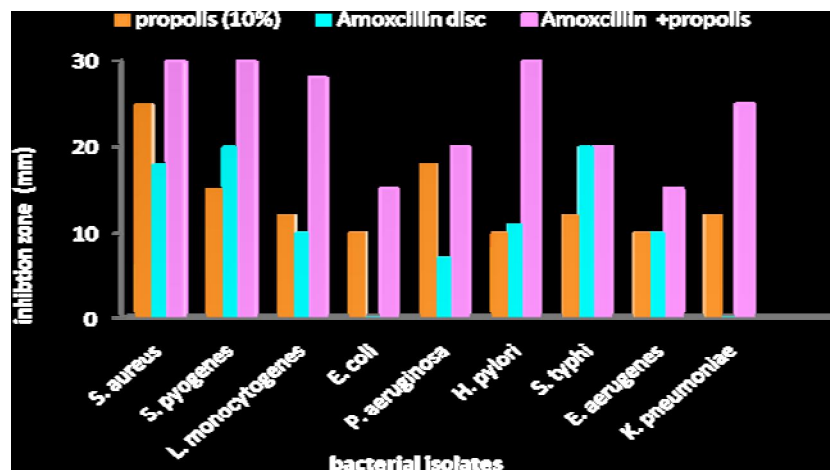


Figure 2 Effect of ethanol extracts of propolis, Amoxicillin and Amoxicillin – propolis combination on the bacterial isolates.

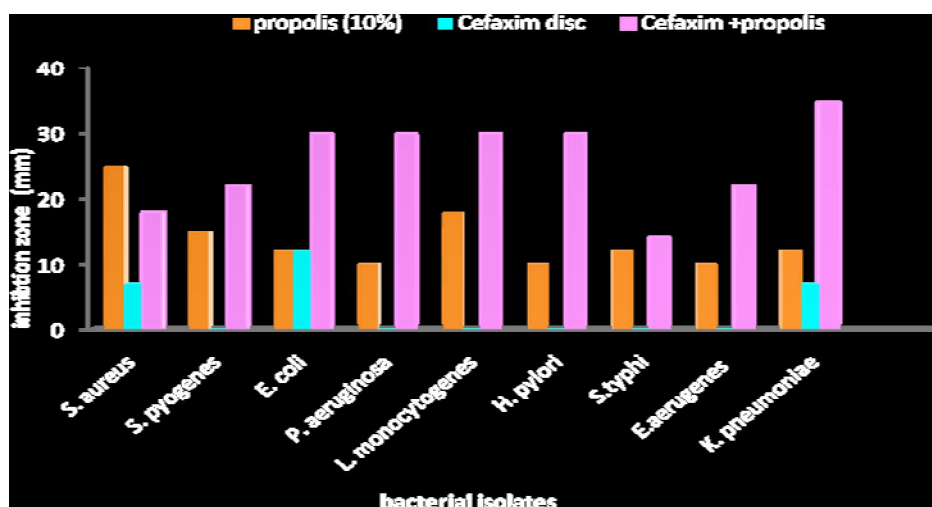


Figure 3 Effect of ethanol extracts of propolis, Cefaxim and Cefaxim-propolis combination on the bacterial isolates.

Moreover, the results of erythromycin-propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, *H. pylori* and *K. pneumoniae*, additive to words *S. typhi*, *E. coli*, and *E. aerugenes*, and antagonism to words *S. aureus*, *L. monocytogenes* and *S. pyogenes*

(Figure 6) Statistical analysis showed no significant differences after between effect of propolis and erythromycin–propolis combination on bacterial isolates but there was significant differences between erythromycin and erythromycin–

propolis combination at level ($P \leq 0.05$)

Regarding to, the results of Tetracycline-propolis combination on the bacterial isolates were; synergistic to words *P. aeruginosa*, and *K. pneumoniae*, additive to words *S. typhi*, *S. pyogenes*, *E. coli*, *L. monocytogenes* and *E. aerogenes*, and antagonism to words *S. aureus* and *H. pylori* (Figure 7). Statistical analysis showed no significant differences between effect of propolis and tetracycline –propolis combination on bacterial isolates but there were significant differences between tetracycline and tetracycline–propolis combination at level ($P \leq 0.05$)

On the other hand, the results of naldixic acid-propolis combination on the bacterial isolates were; synergistic to words *H. pylori* and *K. pneumoniae*, additive to words *S. typhi*, *S. pyogenes*, *L.monocytogenes* and *E. aerogenes*, and antagonism to words *S. aureus*, *P. aeruginosa*, *E.coli* (Figure 8). atistical analysis showed significant differences between effect of propolis and naldixic acid –propolis combination on bacterial isolates and there was significant differences between naldixic acid and naldixic acid –propolis combination at level ($P \leq 0.05$).

[49] asserted that the synergism activities between ciprofloxacin and propolis in the treatment of experimental *S. aureus* keratitis, which found the combination of ciprofloxacin and propolis had better therapeutic effects than either agent alone.

Moreover, the results of Rifampin-propolis combination on the bacterial

isolates were; synergistic to words on *P. aeruginosa*, *E. coli*, *K. pneumoniae*, *S. pyogenes*, and *E. aerogenes*, additive to words *S .aureus*, *H. pylori*, and *L.monocytogenes*, and indifference to words *S. typhi* (Figure 9). Statistical analysis showed significant differences between effect of propolis and Rifampin –propolis combination on bacterial isolates and there were significant differences between Rifampin and Rifampin –propolis combination at level ($P \leq 0.05$).

In this study the results of antibiotics and propolis combinations against bacterial isolates were in agreement with [50] who clarified that the inhibition zone of tetracycline, vancomycin, ofloxacin, cephalothin, ampicillin and erythromycin against *S. aureus* 24, 20, 28, 34, 22 and 23mm respectively, while the inhibitions zones of these antibiotics combination with propolis against *S. aureus* were 27, 22, 29, 37, 29, 37, 25 and 24mm respectively. This study determined the potential medical use of propolis in combination with certain antimicrobial drugs on *S. aureus*. Since bacteria may be resistant to several antimicrobial drugs, the synergism reported here is of relevance and propolis may constitute alternative for treating this pathogen. Although the properties of propolis have been the subject of several investigations, it is difficult to compare the results of different studies, due to the different composition and different methods used for the evaluation of propolis antibacterial activities.

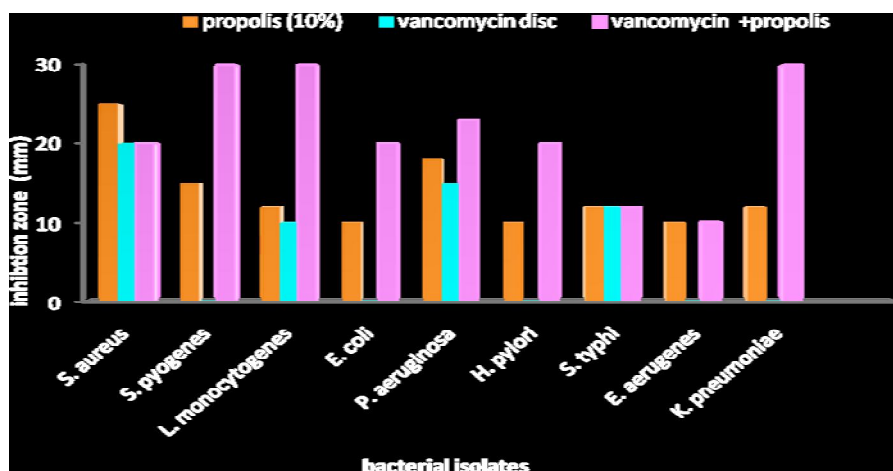


Figure 4 Effect of ethanol extracts of propolis, Vancomycin and Vancomycin - propolis combination on the bacterial isolates.

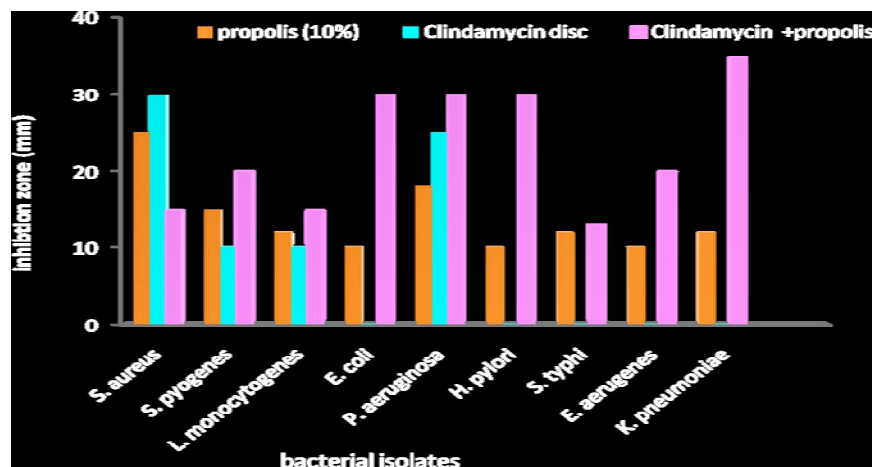


Figure 5 Effect of ethanol extracts of propolis, Clindamycin and Clindamycin - propolis combination on the bacterial isolates.

The effects that can be achieved with combinations of antimicrobial drugs vary with different combinations and are specific for each strain of microorganism. Thus no combination is uniformly synergistic. Combined therapy should not be used indiscriminately; every effort should be made to employ the single antibiotic of choice. In resistant infections, detailed laboratory study can at times

define synergistic drug combinations that may be essential to eradicate the microorganisms.

In this study, *Pseudomonas* resistant to antibacterial drug was sensitive to propolis extract compounds. The activity may be possibly because these microorganisms may acquire plasmids encoding extended spectrum B-lactamase (ESBL) that can confer

resistance to nearly all cephalosporin including cefotaxime and ceftazidime [51]. Therefore propolis compounds

may be effective in bacterial plasmid and may be inhibit reproductive process.

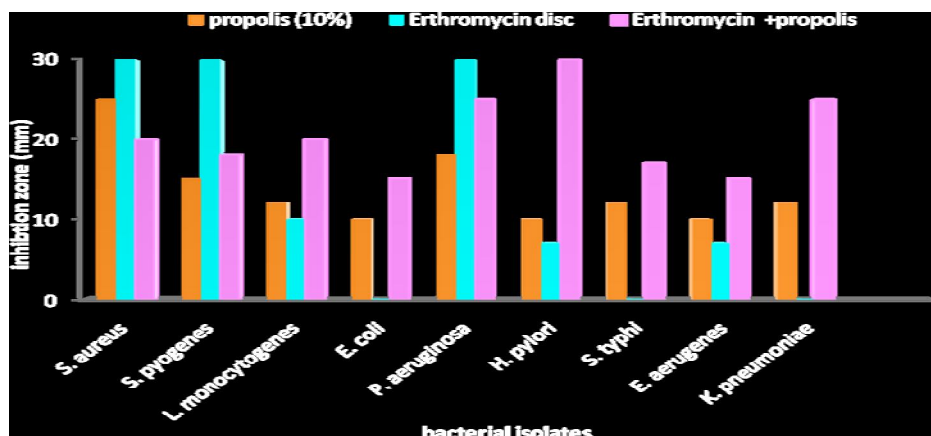


Figure 6 Effect of ethanol extracts of propolis, Erythromycin and Erythromycin - propolis combination on the bacterial isolates.

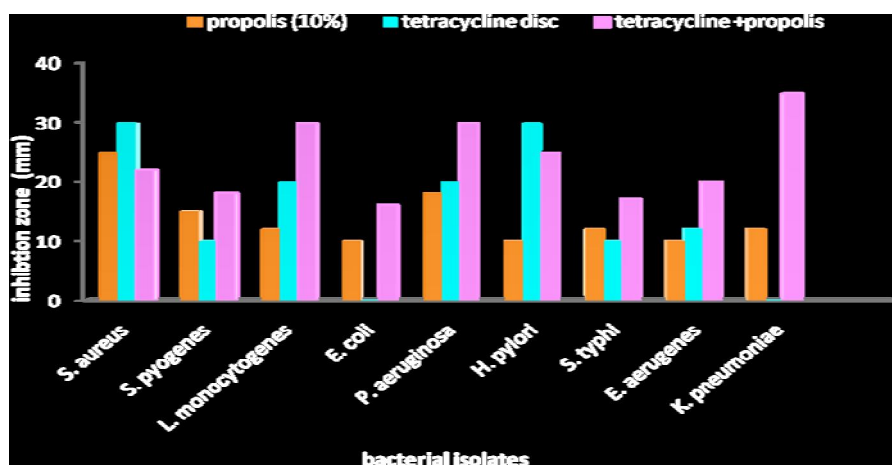


Figure 7 Effect of ethanol extracts of propolis, Tetracycline and Tetracycline - propolis combination on the bacterial isolates.

The effect of Nistatin and propolis combination against *C. albicans* isolates indicated for additive effect (Figure 10).The resistance of *C. albicans* to Nistatin which was in accordance to the result observed by [52] and [53] who found that the synergism effect between active components of propolis with antibiotics

against yeast. [54] asserted that the synergism activities between Nistatin and propolis in the treatment of oral candidiasis. The efficacy of propolis in oral candidiasis treatment is of great interest for public health in Brazil. Statistical analysis showed no significant differences between effect of propolis and Nistatin-propolis

combination on yeast isolates and there were no significant differences

between Nistatin and Nistatin–propolis combination at level ($P \leq 0.05$).

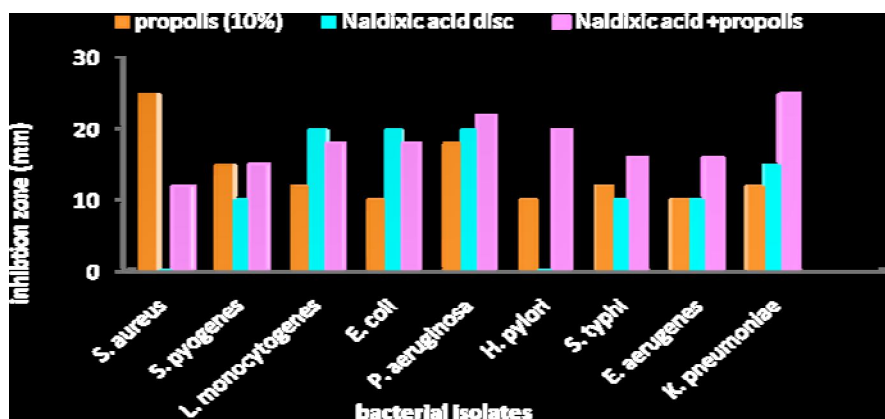


Figure 8 Effect of ethanol extracts of propolis, Nalidixic acid and Nalidixic acid - propolis combination on the bacterial isolates.

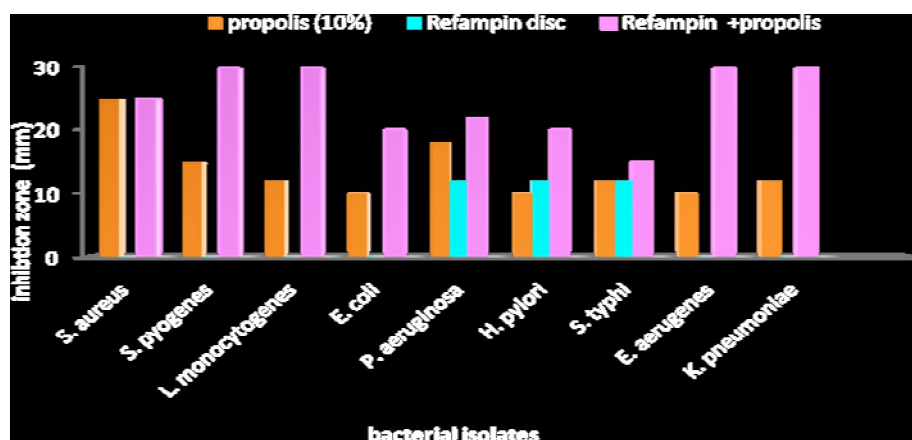


Figure 9 Effect of ethanol extracts of propolis, Refampin and Refampin - propolis combination on the bacterial isolates.

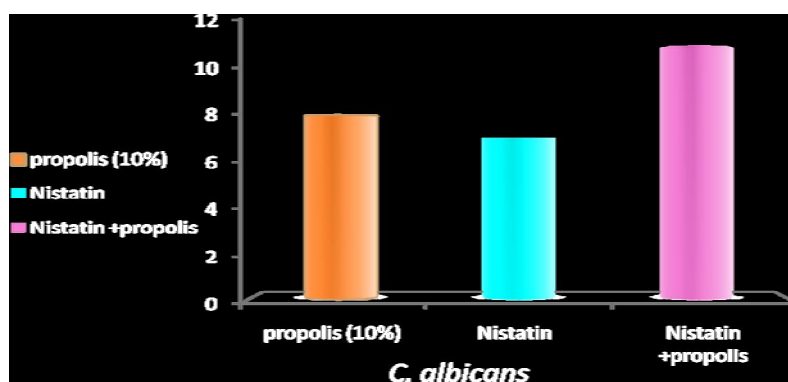


Figure 10 Effect of ethanol extracts of propolis, Nistatin and Nistatin - propolis combination on yeast isolates.

Conclusion

Thus it was concluded that *S. aureus* was more sensitive to EEP than other bacteria, whereas *S. typhi* was lesser sensitive for extracts. Cefaxim-propolis combination has most effect against bacterial and yeast isolates, while tetracycline-propolis combination has lesser effect. The study of other bioactivity of propolis constituents *in vitro* and *in vivo* must be recommended for further studies.

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