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# Antibacterial Activity of Propolis Extracted in Three Different Solvents and in Three Different pH values on some Pathogenic Bacteria

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**Abstract :** This research performed to investigate the effects of Al-Hillapropolis (Babylon province) aged for seven days, extracted by three different solvents (Ethanol, Aceton, Toluol) and with three different pH values(6, 7, 8) on (16) samples of pathogenic bacteria including (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsilla pneumonia*). Antibacterial activity of propolis tested for all the isolated bacteria. It was found that Ethanol and Aceton had maximum antibacterial action in pH (6 and 7) on Gram positive and Gram negative bacteria, while the last solvent used in this study(Toluol) had the highest effect compared with other solvents only on Gram negative bacteria and approximately in tested pH values(6,7, 8).
**Keywords:** Antibacterial Activity, Propolis, pH values, Pathogenic Bacteria.

## Introduction

The word propolis is derived from Greek, in which pro stands for "at the entrance to" and polis for "community" or "city," which means this natural product is used in hive defense. Propolis is a natural resinous mixture produced by honey bees from substances collected from parts of plants, buds, and exudates. Bees gather propolis from different plants, in the temperate climate zone and use it in the construction and repair of their hives for sealing openings, cracks and smoothing out the internal walls due to its waxy nature and mechanical properties, also they use it as a protective barrier against external invaders like snakes, lizards and so forth, or against weathering threats like wind and rain[1,2].

Recently, there are enormous increasing in focusing on the propolis by researches or by using as remedy around the world. This research aimed to evaluate the antibacterial activity of the Iraqi propolis (exactly Babylon province propolis) extracted in three solvents including (Ethanol, Acetone and Toluol) and in three different pH value (6,7 and 8) against some bacterial isolates.

## Materials and Methods

### Bacterial Samples:

A total of (16) Bacterial samples were included in this study. These bacteria were isolated from specimens like sputum, pus, urine, stool and blood of patients admitted to different wards in Marjan Hospital/ Hilla city/ Babylon Province / Iraq from May to June 2015, which were sent to the microbiology laboratory for

routine culture identification and sensitivity testing. All bacterial identification(including biochemical tests, culture and preserving of isolates were used according to[3,4].

**Propolis extracts preparations in three solvents and in three pH values:**

Propolis samples were collected from beehives of Hilla city (Babylon province) during winter and spring season of 2016. Preparation of propolis extracts performed by recombination of two methods [5]and [6] , three solvents were used to extract the active ingredients in Propolis: Ethanol ($C_2H_5OH$), Acetone ($(CH_3)_2CO$) and Toluol ($C6H_5CH_3$).Firstly, the propolis was cleaned to be free of wax, paint, wood, then crushed after freezing (cut into small pieces), and placed in clean container, Secondly, for any type of the solvent, mixing of solvent and water was done as (volume ratio 75:25) and poured into a mixer with a double container (used for cooling purposes), then the prepared solvent(ethanol or acetone or toluol) mixed with specific amount of propolis in dark brown bottle and left for 7 to 14 days at room temperature and in dark place for 2 weeks, the container was shaked 2 or 3 times per day and returned to warm dark place.

For both ethanol and acetone extraction, thirty grams of propolis were mixed with 100 ml of ethanol, the result of propolis extraction with ethanol was: 93 ml of filtrate with a specific weight of 0.845. The weight of the precipitate was 25 g, the temperature of the water bath was 65°C and the steaming time was 20 min, leading to propolis extract with the obtained weight 10 g, but when acetone was used for extraction of propolis, the result was 90 ml of filtrate with a specific weight of 0.842. The precipitate weight was 24.5g, the temperature of the water bath was 55°C, and the steaming time was 30 min. leading to 15 g weight of the propolis extract, while when the third solvent (toluol) was used for extraction of propolis, 37.5 g of propolis was added to125 ml of toluol that leading to97.2 ml of the filtrate with a specific weight of 0.865. The precipitate weight was 121g, the temperature of the water bath was 65°C, with the steaming time of 15 min. yielded 10 g. weight of the propolis extract by toluol. All three extracted propolis samples by(ethanol, acetone, toluol ) were needed to cooling which was done at 278 – 288K for 30 min and mixing at the speed of 20 m/sec. The resulting suspension was centrifuged three times in succession leading to the last extract which was clear liquid and dark brown in color. Subsequently the pH of the sterilized media (in three flasks) was regulated to 6, or 7, or 8, by the addition of either 0.1 mol of hydrochloric acid solution or 0.1 mol of sodium hydroxide solution, then each flask of the medium (Mueller-Hinton agar) that adjusted to the needed pH were divided into 16 plates depending on the number of isolated bacteria in present study.

**Determination of Minimum Bactericidal Concentration (MBC) of Babylon propolis Samples in different solvents and in different pH :**

Determination of minimum bactericidal concentration (MBC) of Babylon propolis for the isolated strains of bacteria was performed in vitro by antibacterial activity testing using agar well diffusion assay according to NCCLS[7] **:**Loopfull growths from bacterial isolates were inoculated into nutrient broth incubated at 37 °C for 18 hours, the bacterial suspensions were diluted with normal saline and were adjusted the turbidity then compare with standard tube (McFarland number 0.5) to yield a uniform suspension containing 1.5×108 CFU/ ml. Cotton swab was dipped and streak into adjustment suspension the entire Mueller-Hinton agar that prepared with different pH values (6,7,8) for all 16 isolated bacteria on the surface of media and the plates were left for one 5 -15 minutes at room temperature to dry. Media were cut by cork borer into three wells (5mm diameter, the plates were performed in triplicates). Then the propolis samples extracted in 3 different solvents (ethanol, acetone and toluol) extracted in three pH(6,7,8) separately, all of these solutions in all pH values were tested by adding (20ì) of the specific propolis samples (The plates were performed in triplicates) in the holes made in the Mueller-Hinton agar. All plates of the tested bacterial isolates were then allowed to incubate at 37°C for overnight. After (24 h) of incubation, each tested propolis extracts in each tested pH was noted for zone of inhibition for all isolates. The diameters of the inhibition zone were measured by measuring scale in millimeter.

**Data Analysis:**

Statistical analysis was carried out using SPSS version 20. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as (Means ± SD). Two Way Analysis of Variance (ANOVA) was used to find the mean differences among continuous variables *P-value* of $\leq 0.05$ was considered as significant.

## Results and Discussion

In this research the effects of propolis extracted by three different solvents (Ethanol, Acetone and Toluol) searched on bacterial species, in acidic (pH=6), neutral (pH=7) and alkaline (pH=8) medium, The mean diameters of microbial growth inhibited by different concentrations of Propolis are shown in Table 1.

The 80% ethanol, 80% acetone and 80% toluol (negative control) did not show any inhibitory effects on the tested microorganisms. The results also showed that, the propolis extracted in each solvent and in each pH was more effective than standard ampicilline on all tested bacteria.

As a general rule, an extract is considered active against both bacteria and fungi if the zone of inhibition was greater than 6 mm[8]. The results of present research indicates some variations in the antibacterial activity of propolis and differences in the inhibition zones of it against tested bacteria, however, that may refer to the fact of chemical compositions and biological properties of propolis may vary according to different plant sources that bees could visit, geographic location and collecting time[9,10,11,12,13] and different constituents of propolis[14]. Propolis composition is different mainly due to collection season and the variability of plant species growing around the hive[15].

On the best of my knowledge, there are no international researches including my country (Iraq)to compare the results of my study with them about using of propolis extracted in different solvents and in different pH at the same time, there is only one available study in this matter, which mentioned earlier in preparation method of propolis[5] this study performed by faculty of stomatology, in Pančevo, Serbia to evaluate the propolis activity extracted by 5 different solvents and aged for 7 days on twelve species of bacteria in different pH. they tested propolis on different bacterial isolates but their results on *E.coli* and *S.aureus* were showed that inhibitory activity of all extracts of propolis on the growth of *E.coli* was distinct, and that propolis aged 7 days extracted by toluol and chloroform had stronger inhibitory effect than other extracts of propolis, in a slightly acidic environment (pH=6), while my result showed that when toluol used for extraction of propolis, *E.coli* were most sensitive than other tested bacteria at pH 7 only but when ethanol and aceton used *E.coli* were highest resistant tested bacteria in any pH and that not in agreement with my results.

On the other hand and with same mentioned study their results showed that all types of propolis had a uniform, statistically significant ($p<0.01$), inhibitory effect on growth of *S.aureus*, while the results of my research indicated that *S.aureus* were the most sensitive bacteria at pH 6 and 7 than other tested bacteria with ethanol and acetone extraction of propolis but this different when toluol used for extraction of propolis because *E.coli* were the most sensitive and that also not in agreement with my results, these differences may be refer to different origin of propolis and consequently the different chemical components that appear when propolis tested against bacteria, in addition different sources of bacteria from diseases.

The size of the inhibition zones depends on the diffusibility of the test substance in the agar, which is under the influence of molecular weight, negative charge, composition of samples, and the thickness and pH of the agar culture medium[16] .

Data from numerous studies concerning antibacterial properties of propolis support the fact that propolis is active mainly against Gram-positive bacteria and either displays much lower activity against the Gram-negative ones or is inactive at all[17,18,19,20,21,22,23,24,25]. Propolis affects cytoplasmic membrane, inhibits bacterial motility and enzyme activity, exhibits bacteriostatic activity against different bacterial genera and can be bactericidal in high concentrations[26]. A possible explanation for propolis action mechanism may be due to the fact that one or some of its constituents caused a significant inhibition of bacterial motility, besides ion permeability alteration on the inner bacterial membrane[27,28,29].

In this study, when ethanol extraction of propolis was applied on all isolates, *S.aureus*were the most sensitive bacteria at pH 6 and 7 than other tested bacteria (Table 3 & Figure 1), followed by *K. pneumonia* (Table 5 & Figure 3) which were sensitive for this extract only in pH 7. In the extraction of propolis by aceton, *S.aureus* were also the most sensitive bacteria than others but at pH

7 only, followed by *P. aeroginosa* (Table 6 & Figure 4) which were sensitive for the acetone extract in pH 6 and 7 more than in pH 8, while when toluol used for extraction of propolis *E.coli* (Table 4 & Figure 2)were the most sensitive than other tested bacteria at pH 7 only, followed by *K. pneumonia* which had a proximate sensitivity for toluol extract in all pH ranges applied in this study.

**Table(1):The mean of the Inhibition Zones Diameter of propolis against isolated Bacteria(mm).**

| NO | Bacteria | pH | Isolate NO. | Ethanol | Acetone | Toluol | Ampicilline |
|---|---|---|---|---|---|---|---|
| **1** | *S. aureus* | 6 | 1 | 23 | 7 | 5 | 3.0 |
| | | | 2 | 19 | 9 | 6 | 3.0 |
| **2** | *S. aureus* | 7 | 1 | 15 | 12 | 3 | 3.1 |
| | | | 2 | 23 | 16 | 5 | 3.3 |
| **3** | *S. aureus* | 8 | 1 | 12 | 7 | 7 | 3.0 |
| | | | 2 | 22 | 10 | 9 | 3.1 |
| **4** | *E.coli* | 6 | 1 | 6 | Nill | 7 | - |
| | | | 2 | 15 | Nill | 12 | - |
| | | | 3 | 7 | Nill | 9 | - |
| **5** | *E.coli* | 7 | 1 | 20 | 1 | 12 | - |
| | | | 2 | 10 | 4 | 12 | - |
| | | | 3 | 8 | 2 | 19 | - |
| **6** | *E.coli* | 8 | 1 | 7 | 5 | 11 | - |
| | | | 2 | 9 | 6 | 10 | - |
| | | | 3 | 4 | 4 | 12 | - |
| **7** | *K. pneumonia* | 6 | 1 | 7 | 9 | 11 | - |
| | | | 2 | 11 | 5 | 6 | - |
| | | | 3 | 5 | 4 | 6 | - |
| **8** | *K. pneumonia* | 7 | 1 | 14 | 4 | 10 | - |
| | | | 2 | 12 | 6 | 7 | - |
| | | | 3 | 12 | 6 | 9 | - |
| **9** | *K. pneumonia* | 8 | 1 | 16 | 2 | 6 | - |
| | | | 2 | 9 | 3 | 10 | - |
| | | | 3 | 11 | 2 | 8 | - |
| **10** | *P. aeroginosa* | 6 | 1 | 6 | 10 | 6 | - |
| | | | 2 | 4 | 10 | 3 | - |
| **11** | *P. aeroginosa* | 7 | 1 | 16 | 9 | 7 | - |
| | | | 2 | 7 | 11 | 6 | - |
| **12** | *P. aeroginosa* | 8 | 1 | 17 | 4 | 2 | - |
| | | | 2 | 12 | 10 | 5 | - |

**Table (2) : Mean difference of *S.aureus* isolates by pH and media**

| Solvent / PH | Ethanol | Acetone | Toluol |
|---|---|---|---|
| **6** | 2.10 ± 0.28 B,C | 0.80 ± 0.14 A | 0.55 ± 0.07 A |
| **7** | 2.40 ± 0.14 B,C | 1.40 ± 0.28 A,C | 0.40 ± 0.14 A,B |
| **8** | 1.70 ± 0.71 | 0.85± 0.21 | 0.80 ± 0.14 |

**Different small letters means significant ($p \leq 0.05$) results between media**

**Different capital letters means significant ($p \leq 0.05$) results between pH**

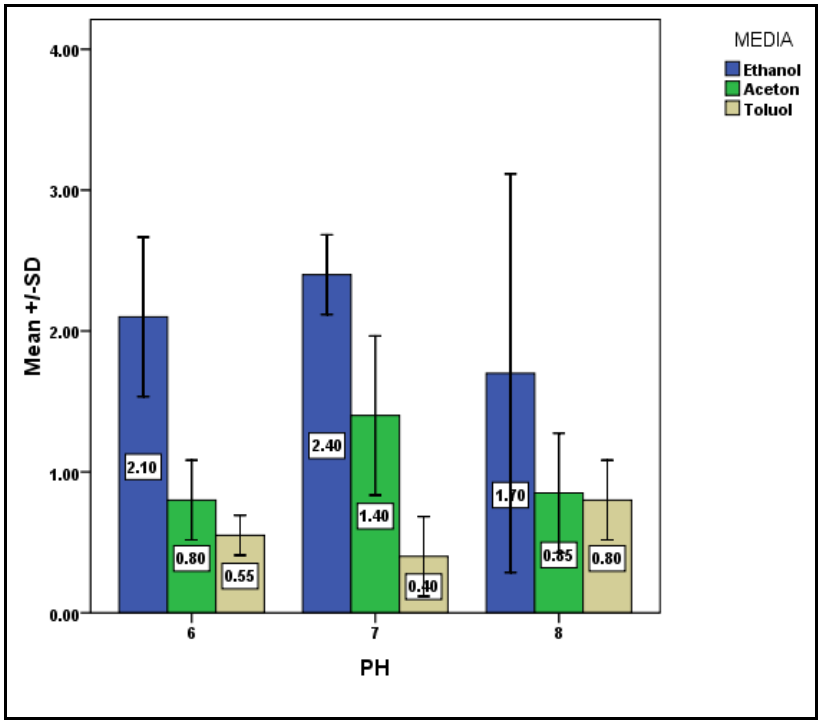


**Figure (1): Mean difference of Inhibition Zones for *S.aureus* by pH and media**

**Table (3): Mean difference of *E.coli*i solates by pH and media**

| Solvent / PH | Ethanol | Aceton | Toluol |
|---|---|---|---|
| **6** | 0.93 ± 0.49<br>b | 0.0 ± 0.0<br>a,c C | 0.93 ± 0.25<br>b |
| **7** | 1.27 ± 0.64 | 0.23 ± 0.15 | 1.43 ± 0.40 |
| **8** | 0.67 ± 0.25 | 0.50± 0.10<br>c A | 1.10 ± 0.10<br>b |

**Different small letters means significant ($p \leq 0.05$) results between media**

**Different capital letters means significant ($p \leq 0.05$) results between PH**

**Table (4): Mean difference of *K.pneumonia* isolates by pH and media**

| Solvent / PH | Ethanol | Aceton | Toluol |
|---|---|---|---|
| **6** | 0.77 ± 0.30 | 0.60 ± 0.26 | 0.76 ± 0.29 |
| **7** | 1.27 ± 0.11<br>b,c | 0.53 ± 0.11<br>a | 0.87 ± 0.15<br>a |
| **8** | 1.20 ± 0.36<br>b | 0.23± 0.06<br>a | 0.80 ± 0.20 |

**Different small letters means significant ($p \leq 0.05$) results between media**

**Different capital letters means significant ($p \leq 0.05$) results between PH**

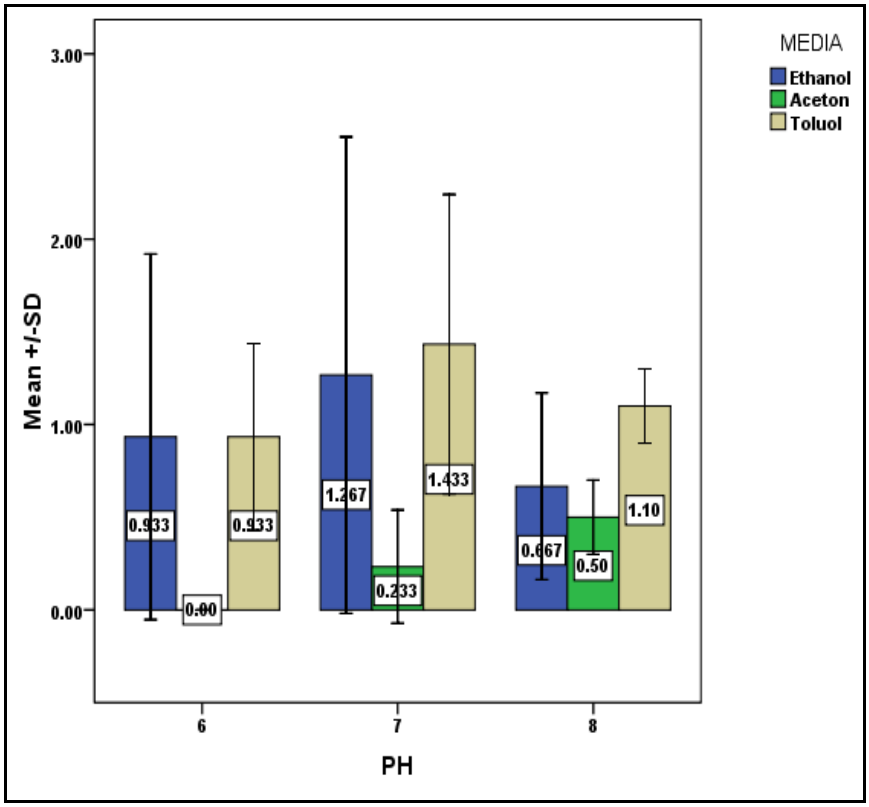


**Figure (2): Mean difference of Inhibition Zones for *E.coli* by PH and media**

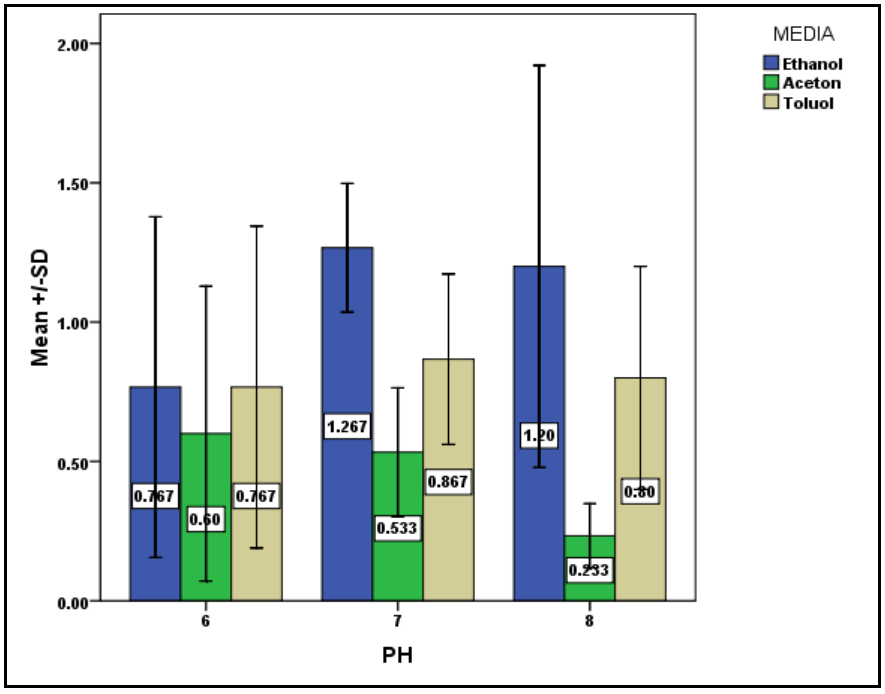


**Figure (3): Mean difference of Inhibition Zones for *K.pneumonia* by pH and media**

**Table(5): Mean difference of *P. aeroginosa* isolates by PH and media**

| Solvent / PH | Ethanol | Aceton | Toluol |
|---|---|---|---|
| **6** | 0.15 ± 0.14 | 1.00 ± 0.0 c | 0.45 ± 0.21 |
| **7** | 1.15 ± 0.64 | 1.00 ± 0.14 c | 0.65 ± 0.07 |
| **8** | 1.45 ± 0.35 | 0.25± 0.21 a,b | 0.35 ± 0.21 |

**Different small letters means significant ($p \leq 0.05$) results between media**

**Different capital letters means significant ($p \leq 0.05$) results between PH**

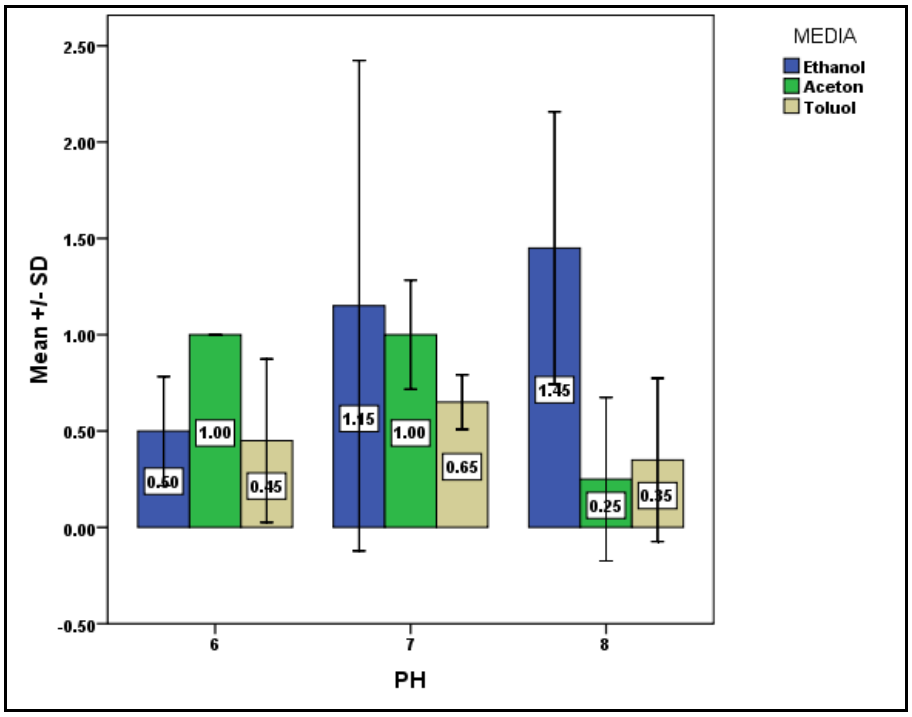


**Figure (4): Mean difference of Inhibition Zones for *P. aeroginosa* by PH and media**

Thus, it is obvious in this study that when ethanol extraction of propolis was applied on all isolates, *S.aureus*were the most sensitive bacteria at pH 6 and 7 than other tested bacteria, followed by *K. pneumonia* which were sensitive for this extract only in pH 7.

In the extraction of propolis by aceton, *S. aureus* were also the most sensitive bacteria than others but at pH 7 only, followed by *P.aeroginosa* which were sensitive for the acetone extract in pH 6 and 7 more than in pH 8, while when toluol used for extraction of propolis *E.coli* were the most sensitive than other tested bacteria at pH 7 only, followed by *K. pneumonia* which had a proximate sensitivity for toluol extract in all pH ranges applied in this study.

Overall, Gram positive strains are more sensitive than Gram negative strains when ethanol and acetone used as propolis solvent in any pH applied, but with toluol extraction there was inverse situation because Gram negative bacteria were predominantly sensitive isolates more than Gram positive ones in any pH applied, but that don't agree with the fact that Gram negative bacteria are less sensitive to propolis than Gram positive strains[30-33], this may justify that pH value of extraction could effect on bacterial response to the bioactivity of propolis or because *S.aureus* isolates were multi resistant and may be these isolates are MRSA, another probable explanation may be the propolis sensitive Gram positive bacteria represent bacteria other than *S.aureus*.

## Conclusions

The results of this study indicate that Iraqi propolis (Babylonian propolis) have antimicrobial activity with different solvents and in different pH. Application of various extracts to propolis and changing pH lead to wide range of antibacterial activity of propolis.

## Recommendations

Further evaluation of the bioactivity of propolis in vitro and in vivo from various geographical regions prepared in different solvents and in different pH maybe recommended for further studies and test it on multiple pathogenic bacteria and detect the ideal pH for the antimicrobial activity of propolis chemical compounds separately against different pathogenic bacterial species.

As bacteria have powerful and highly resistance to the most useful antibiotics therefore we need trying other substituent treatment, Testing propolis instead of chemical drugs may help in discovery novel antibiotics to reduce pain without or with low side effect and less toxic than drugs.

However, there are numerous questions yet to be answered concerning antibacterial properties of Iraqi propolis and additional researches are required for clarification.

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