ANTIBACTERIAL ACTIVITIES OF IRAQI PROPOLIS AND ITS ACTIVE COMPONENTS EXTRACTS ON SOME BACTERIAL ISOLATES (IN VITRO STUDY)

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Abstract

Background: Propolis (bee glue) is a resinous product that honeybees collect from living plants and use in construction and adaptation of their hives. It is extensively used in folk medicine, and a number of investigations have shown that propolis possesses antibacterial, antiviral and antifungal properties. Aims: In this paper evaluated the antibacterial activity of propolis and its active component against ten bacterial pathogens.

Methods: The antibacterial effects of propolis and active component of propolis against 5 Gram-positive and 5 Gram-negative bacterial isolates, including Staphylococcus aureus marxa, Staphylococcus epidermidis, Streptococcus mutans, Enterococcus faecalis, Streptococcus pneumoniae, , Pseudomonas fluorescense, Escherchia coli, Proteus mirabilis, Proteus vulgaris, and Acinetobacter, all of them were studied.

Results: Antimicrobial activities of crude ethanol extract of Kerbalaa propolis (EEKP) and active components of propolis at 25% concentration against bacterial isolates were studied. The results of agar diffusion at 25% concentration showed that most bacterial isolates were sensitive to EEKP. S. pneumoniae was highly sensitive to EEKP than other Gram positive and Gram negative bacteria with inhibition zones was 30mm. all bacterial isolates were highly sensitive to active component of propolis. Showing the maximum zone of inhibition of 30 mm at the 25% concentration

Conclusions: In summary, the propolis and its active component extract showed a wide spectrum activity and appears to satisfy all of the criteria for antibacterial agents. These
results suggest that propolis can be used to protect food and reduce the risk of contamination from pathogenic microorganisms.

**Keywords:** propolis, active component of propolis, and antibacterial activity.

**INTRODUCTION**

Propolis (bee glue) is a resinous product that honeybees collect from living plants and use in construction and adaptation of their hives ([1]). It is extensively used in folk medicine, and a number of investigations have shown that propolis possesses antibacterial, antiviral and antifungal properties ([61]).

Several reports have been published on the scientifically confirmed antimicrobial properties of propolis ([62, 52, 27]), seem useful for their therapeutic potential. Preliminary scientific studies show propolis have in vitro antibacterial ([63]) and antifungal ([64]) activity (with active constituents including flavonoids like galangin ([65]) and hydroxycinnamic acids like caffeic acid ([66]). Most studies has been shown that there were variations in the antimicrobial activity of propolis according to its origin ([67, 68]).

Although numerous researchers have been reported the antibacterial activities of propolis collected worldwide, information about Iraqi propolis are still little. In this paper evaluated the antibacterial activity of propolis and active component of propolis against ten bacterial pathogens.

**METHODS**

Propolis samples and preparation of Ethanolic extract of propolis

Propolis samples were collected from hives of honey bees of Kerbalaa, Iraqi during all seasons of 2013. It were cleaned, free of wax, paint, wood, cut into small pieces, and placed in clean container. Ten gram of propolis were mixed with 100 ml of ethanol 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. The liquid was filtered through Whatman No. 1 filter paper and the water was evaporated by oven at 45°C, then the extract was weighed and stored in dark clean container for further usage. Ethanolic extract was dissolved by Dimethyl Sulfoxide (DMSO), sterilized by filtration (using Millipore 0.45 filter paper), and the requisite dilutions were prepared ([7]).
Bacterial Isolates
A total of 5 Gram negative, 5 Gram positive bacteria (isolated from clinical samples) were used in this study. The bacterial isolates represented by: *S. aureus*, *S. epidermidis*, *St. mutanus*, *St. pneumoniae*, *E. feacalis*, *P. fluoresence*, *E. coli*, *Proteus mirabilis*, *P. vulgaris*, and *Acinetobacter*. These bacteria were activated and cloned three successive times in nutrient agar and stored on nutrient agar slant at 4 °C. The identification of these organisms was confirmed by using conventional biochemical tests.\(^8\)

*In vitro* Antibacterial activity testing using Agar well diffusion assay\(^{60}\)
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. Adjust the turbidity and compare with standard tube (McFarland number 0.5) to yield a uniform suspension containing 1.5×10\(^8\) CFU / ml. Cotton swab was dipped and streak into adjustment suspension the entire Mueller-Hinton agar (for all tested bacteria) surface of plates and the plates were left for one 5 -15 minutes at room temperature to dry. Media were cut into four wells (5mm diameter) by cork borer and add 20µ of the propolis extracts solutions or active component of propolis (The plates were performed in triplicates). All plate of the tested organisms was then allowed to incubate at 37°C for overnight. After 24 h of incubation, each extract was noted for zone of inhibition for all isolates. The diameters of the zone of inhibitions were measured by measuring scale in millimeter (mm).

Statistical analysis
Bonferroni test recommended by \(^9\) was used for statistical analysis (P ≤ 0.05) to show if there is any significant differences between results of agar diffusion methods of propolis ethanolic extract.

RESULTS AND DISCUSSION
*In vitro* antibacterial activity of crude extracts of propolis and its active components:
When the inhibition zone of propolis extract against both bacterial groups was greater than 6 mm is considered active.\(^{10}\)

Antimicrobial activities of crude ethanol extract of Kerbalaa propolis (EEKP) and active components of propolis at 25% concentration against bacterial isolates were studied. Antibacterial activities of crude ethanolic extract of propolis against bacteria are shown in (figure 1 and figure 2). The results of agar diffusion at 25% concentration showed that most
bacterial isolates were sensitive to EEKP. *S. pneumoniae* was highly sensitive to EEKP than other Gram positive and Gram negative bacteria with inhibition zones was 30mm, while *P. fluorescense* was highly sensitive to EEKP than other Gram negative bacteria with inhibition zones of 25 mm.

Statistical analysis showed no significant differences after treating the microorganisms with propolis ethanolic extract at 25% concentrations of agar diffusion (P ≤ 0.05).

![figure (1) Antibacterial activity of 25% concentrations of propolis on Gram positive bacteria.](image1)

![figure (2) Antibacterial activity of 25% concentrations of propolis on Gram negative bacteria.](image2)

This result indicated that the active components of propolis were concentrated in the sample. This was in agreement with reports of several papers which indicated that each propolis
sample contained 80 to 100 chemical compounds with different concentrations \(^{(3,11,12)}\). The present results on *S. aureus* were in agreement with those obtained by several authors who found that the inhibition zones obtained by propolis from Mongolia, Albania, Egypt and Brazil were 24, 21.8, 24.3, and 21 mm respectively \(^{(13)}\). These results are comparable with results obtained by \(^{(14)}\) who found that the inhibition zone for Bulgarian propolis was 20 mm, also with results obtained by \(^{(15)}\) who found out that the inhibition zone of propolis form different geographical areas of Serbia ranged from 18 to 23 mm. These differences in antibacterial activity of propolis from the different regions in the world supported the commonly reported statements in literature which indicated that sensitivity of microbes and differences in chemical composition of propolis are greatly affected by variations in geographical origins \(^{(16,2)}\). Furthermore, Biological and pharmaceutical activity of propolis may contribute to the fact that propolis contains active compounds such as phenols, flavonoids and alkaloids that possess antibacterial and antifungal activities against bacteria and fungi. These results were comparable with results obtained by several authors \(^{(17)}\).

Moreover, this difference of the inhibition zones of propolis was related to the different constituents of propolis collected from different geographical regions \(^{(18)}\). Several researchers \(^{(13,15,19)}\) reported that there was no effect of propolis from different geographical regions on *E. coli*.

This again might reflect the fact that chemical composition of propolis differs greatly from one region to another \(^{(15)}\). 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 *Acinetobacter* and *E. coli* to propolis, was in agreement with the findings obtained by many researchers who revealed that this bacterium showed either very low sensitivity or total lack of sensitivity against propolis \(^{(19)}\). This emphasizes the fact that, Gram negative bacteria are less sensitive than Gram positive strains, which is in agreement with several previous reports \(^{(16,19,20)}\).

The most possible explanation for the low sensitivity of Gram negative bacteria to propolis extract is that, their outer membrane inhibits and/or retards the penetration of propolis \(^{(21)}\). Another possible reason is their possession of multi drug resistance (MDR) pumps, which extrude amphipathic toxins across the outer membrane \(^{(21)}\). Several authors \(^{(4,11)}\) have reported that propolis antibacterial activity is attributed to a number of phenolic compounds,
mainly flavonoids, phenolic acids and their esters and some prenylated-coumaric acids were isolated from propolis in several countries \((15)\).

The antibacterial activity of volatile compounds and diterpenes from Brazilian propolis was identified by \((6)\). Propolis and some of its cinnamic acid derivatives and flavonoids were responsible for uncoupling the energy transuding cytoplasmic membrane inhibiting bacterial motility, which might contribute to the antibacterial action \((6)\). \((22)\) reported that the antimicrobial activity of phenolic compounds was concentration dependent, affecting enzymatic activity related to energy production at low concentrations and causing protein precipitation at high concentrations. Many plants contain non toxic glycosides which can get hydrolyzed to release phenolics which are toxic to microbial pathogens \((23)\). An important characteristic of essential oils and their components is their hydrophobicity, which enabled them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable \((24)\). \((25)\) reported that the antimicrobial activity of propolis is as a result of the high content of flavonoids. However, this activity varies according to geographic regions and pH of the culture medium \((26)\).

The presence of flavonoids and derivatives of caffeic acid is associated with the bactericidal activity \((27)\). The mechanism of antibacterial action of propolis has been the subject of only a few publications. \((28)\) showed through electron microscopy and micro-calorimetric assays that ethanolic extracts propolis (EEP) interferes with the division of Streptococcus through the formation of pseudomulticellular forms, cytoplasm disorganization, inhibition of protein synthesis leading to lysis of the bacteria. \((29)\) found that EEP and some 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 Bacillus subtilis. A synergistic effect with conventional anti-mycotic drugs was also observed \((30)\). \((28)\) 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, caused a partial bacteriolysis and inhibited 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 \((31)\).
These results were in agreement with ([32]) who pointed out that zones of inhibition of *S. aureus* and *E. coli* were 8 and 7 mm respectively.

The activities variation of ethanolic extract of propolis depend on types of microbes, and propolis concentration in the media. ([33]) reported that the zones of inhibition of *S. aureus* were 16 mm at 50% concentration of propolis collected from Al-Kufa, Iraq. ([34]) pointed out that the phenolic compound was causing protein denaturation of microbes through the pause of the enzymes action of metabolic reactions and dead the microorganism. Flavonoids were regarded as the largest component of the phenolic compound and it had pharmaceutical and antimicrobial activities. The concentration of flavonoids differ from sample to other sample of propolis, attributed to geographical area and concentration of propolis extracts ([35]). Tannins are toxic for bacteria, fungi and yeast due to their ability to combine with the microbial cell wall and cause growth inhibition ([36]). Antimicrobial activity of propolis contributed to the presence of alkaloids (metabolite products of proteins), nitrogen alkaline which has pharma-ceuticalproperties, it helps in the treatment of wounds and burn infection ([37]).

The results refer to the activity of volatile oil of proplis against fungal and bacterial isolates by inhibiting their growth completely because of the specialty of fatty acid in destroying microbial cells envelopes. ([34]) who pointed out that the phenolic compound of propolis was causing protein denaturation of microbes through the pause of the enzymes action of metabolic reactions and dies the microorganism. ([38]) showed that the differences in antimicrobial activities against microbes of types propolis extracts due to many factors such as type of extract, extracted methods, solvents nature, active component in propolis extract, concentration of these component, type of microbes that effected by this extracts, the nature and differences composition of cell wall of bacteria and yeast, subsequently slowly of permeability the extracts from outer membrane for antimicrobial agents of gram negative than gram positive bacteria.

Many studies ([39,40]) found that the ethanolic and aquatic extract were effect on nucleic acid DNA and RNA and some bioactivities of bacterial enzymes. Aquatic extract was inhibiting activity of dihydrofolate reducatase.

Propolis extract was considered as the best extract against yeast and bacteria. It has been used in this study which inhibited the microbial growth. ([41]) who found that the antimicrobial
activity of propolis may be due to inhibit of RNA-Polymerases during it loss of ability the combination with DNA.

**In vitro Antibacterial Activities of Active Components Extracts of Karbala Propolis**

Antimicrobial activities of active components of Karbala propolis at 25% concentration against Gram positive and negative bacterial isolates were studied (figure 1 and figure 2) respectively. In general, the results of this experiment revealed a variable action depend on both; type of the component and the type of organism, accordingly, the results of phenolic extracts of gram positive bacteria showed that this component was the most effective against *S. epidermidis*. The inhibitory zone ranged from 20-13 mm against the bacterial isolates.

Moreover, the results of phenolic extracts of gram negative bacteria showed that most effective against *P. fluorescence*, since the inhibitory zones ranged from 25-14 mm against the bacterial isolates. This result was in agreement with those results obtained by ([42]) who pointed out that the inhibition zones of phenolic extract of *S. aureus*, and *E. coli*, were 46, and 38 mm respectively at 48 mg/ml. Several authors ([4,11]) have reported that propolis antibacterial activity is attributed to a number of phenolic compounds, mainly flavonoids, phenolic acids and their esters and some prenylated -coumaric acids ([5]).

Propolis and some of its cinnamic acid derivatives and flavonoids were responsible for uncoupling the energy transuding cytoplasmic membrane inhibiting bacterial motility which might contribute to the antibacterial action ([6]). ([22]) reported that the antimicrobial activity of phenolic compounds was concentration dependent, affecting enzymatic activity related to energy production at low concentrations and causing protein precipitation at high concentrations. An important characteristic of essential oils and their components is their hydrophobicity, which enabled them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable ([24]). This result was closely compatible in agreement with the result obtained by ([43]) who had found that the inhibition zone of phenols of *S. aureus, S. pyogenes, E. coli* and *P. aeruginosa* were 11, 10, 10 and 8 mm respectively at 30%.

Phenols belonging to plant (especially some spice) which are rich in anthocyanins and hydrolysable tannins, gallic acid, anthocyanin fraction contained cyanidin, peonidin, pelargonidin, petunidin, and delphinidin glucosides and coumarates, hence the activity of phenols is closely related with these components ([44]). Traditional using of this spice may help
in protecting from several bacterial diseases spontaneously and may aid in control of bacterial growth in foods. Several studies mentioned that propolis extracts have been reported to potentiate some antibiotic effects attributing the antibacterial propolis activity mainly to flavonoids or to a synergism between some phenolic components.

On the other hand, the results of flavonoids extracts of Gram positive bacteria had good activity against bacteria, since the inhibitory zones ranged from 22-12 mm against the bacterial isolates, while the results of flavonoids extracts activity of Gram negative bacteria showed that most effective against *P. fluorescense*, since the inhibitory zones ranged from 22-13 mm against the bacterial isolates. This result agrees with that result obtained by who pointed out that the inhibition zones of flavonoids extract of *S. aureus, E. coli*, were 42, and 40, mm respectively at 48 mg/ml.

This result is closely compatible with the results obtained by who had found that the inhibition zone of *S. aureus* and *E. coli* at 30% concentration of flavonoids were 10, and 9 mm respectively. The presence of phenolic acids, derivatives of caffeic acid, coumaric acid, ferulic, flavonoids of guerctin, kaempheral, galangin were associated with the pharmacological, bactericidal and fungicidal activity. This can be attributed to denature of proteins and inhibition of enzymes activity of microbial metabolites, respiration enzymes such as cyclicphoptase, Lipoygenase, protein kinases, subsequently the organism could not continue and die.

Flavonoids were regarded as the largest components of phenolic compounds and they had pharmaceutical and antimicrobial activities due to quercetin which can format complex with bacterial cell wall and bacterial membrane rupture. Flavonoids form complex with bacterial soluble proteins and inhibition of DNA gyrase. The concentration of flavonoids differs from sample to other sample of propolis according to geographical area and concentration of propolis extracts. Many researcher showed that apigenins and flavonoids inhibited glucosyltransferases. This depends on enzyme structure and flavonoids structure when found double bond between C$_3$-C$_2$ of flavonoids which have inhibition effects.

This result was approximately compatible in agreement with the results obtained by who found that flavonoids have antibacterial activities against *S. aureus* (MRSA) and showed the importance of flavonoids as antistaphylococcal therapy.
The moderate activities of flavonoids were due to decrease of synergistic effect between active components of propolis. ([47]) asserted that the active components of extract that act together to antibacterial and antifungal activities. Pre-existing antifungal phenolics are simple phenols, phenolic acids, flavonols and dihydrochalcones. In addition, many flavones and flavanones have been shown to be active against fungal pathogens.

Table (1) Antibacterial activity of 25% concentrations of active components of propolis on Gram positive bacteria by agar well method.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Active component of propolis extracts (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td><em>S. aureus</em> marsa</td>
<td>18</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>20</td>
</tr>
<tr>
<td><em>S. mutanus</em></td>
<td>15</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>20</td>
</tr>
<tr>
<td><em>E. feacalis</em></td>
<td>13</td>
</tr>
</tbody>
</table>

Table (2) Antibacterial activity of 25% concentrations of active components of propolis on Gram negative bacteria by agar well method.

<table>
<thead>
<tr>
<th>Bacterial isolates</th>
<th>Active component of propolis extracts (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>25</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>15</td>
</tr>
<tr>
<td><em>P. merabilis</em></td>
<td>16</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>15</td>
</tr>
<tr>
<td><em>Acinetobacter</em></td>
<td>14</td>
</tr>
</tbody>
</table>

On the other hand, the results of agar diffusion methods of mucilage extracts showed that this component was highly effective against Gram negative and positive bacterial isolates.

This result agrees with result that obtained by ([42]) who pointed out that the zones of inhibition of mucilage extract of *S. aureus*, and *E. coli* were 40, and 16mm respectively at 48 mg/ml. ([50]) clarified that the mucilage was complex carbohydrates had antimicrobial activities. He treated many types of burns, pharynx and stomach infections, effectively without any side effect on human.
On the other hand, the results of alkaloids showed that this component was the most effective against Gram positive and Gram negative bacteria. However, slight activity of alkaloids against *E. faecalis*. The inhibitory zones ranged from 25-10 mm against the bacterial isolates. Antimicrobial activity of propolis contributed to the presence of alkaloids (metabolite products of proteins) is nitrogen alkaline which had pharmaceutical properties due to its ability to penetrate the microbial cell and interference with DNA synthesis \(^{(147)}\). It helps in treatment of wounds and burns infections \(^{(137)}\).

Furthermore, the results of terpenoids extracts of Gram positive and Gram negative bacteria were recorded that this component was the most effective against *P. fluorescense*. However, the inhibitory zones ranged from 22- 14mm against the bacterial isolates.

The most familiar phytotoxins are sesquiterpenoids or amino acid derivatives. Triterpenoids are toxic and many have pharmacological activity. Most of the antimicrobial constituents such monoterpenes contributed to the antimicrobial effect particularly against *L. monocytogenes* \(^{(51)}\). \(^{(2)}\) found that the antimicrobial activity of propolis may be due to the action of interference between sesquiterpenes and flavonoids hydroxyl acids resulting in inhibition of cell mitosis. \(^{(52,53)}\) stated that the antimicrobial activity of propolis may be due to the action of diterpenic which inhibits the growth of *S. aureus*.

Moreover, the results of saponin extracts showed that this component was highly effective against *Acinetobacter*. The inhibitory zones ranged from 12-28 mm against the bacterial isolates.

On the other hand, the results of agar diffusion methods of tannins extracts revealed that tannin had highest activity against most Gram negative and positive bacteria. The inhibitory zones ranged from 12-30 mm against the bacterial isolates. Tannins are toxic for bacteria, fungi and yeast due to combine with the microbial cell wall which inhibits the bacterial growth. The use of tannins showed a high activity against isolated fungi and bacteria by inhibiting their growth by stopping one or more of physiological processes of microorganisms \(^{(36)}\). \(^{(54)}\) pointed out that that the ability of tannins in disturbing the growth or changes the morphology of certain microorganisms. It was suggested that the main action of this extract would be primarily on the cell envelope of fungal cell.
The activity of tannins as antimicrobial drug lies in that they may act according to one of these mechanisms; tannins may interact with one of the biological reproduction of fungal or bacterial cells and prevent germination process. Tannins might interact with the physiological process of MO as a respiratory chain and cause failure in respiration by inhibition of NADP which has a main role in this process ([54]).

Last but not least, the results of agar diffusion methods of coumarins extracts revealed that this component was the most effective against *Acinetobacter* and *S. aureus*. The inhibitory zones ranged from 12-30 mm against the bacterial isolates.

The activity of coumarins showed little activity compared with other extracts. However, the coumarin activity represented by reducing the rate of fungal and bacterial growth by inhibiting reproduction process ([47]).

The effect of propolis extracted compounds against the growth of *C. albicans* may stop exponential growth phase, and kill yeast by rapid and sensitive leakage of intracellular K+, permeation to propidium iodide, lysis of spheroplast and severe membrane ultrastructural alteration as explained by ([55]).

It has been reported that pathogenic isolates have a relatively large potential for developing antibiotic resistance ([56,57]). The increase of bacterial resistance to antibiotic is largely due to the widespread use of antibiotics in medicine, in animal care and in agriculture. The problem is compounded by the lack of new antibiotics to attack bacteria in different ways to circumvent the resistant genes. Therefore, finding antimicrobial agents which are effective or might enhance the antibiotic efficacy against resistant bacteria would be of great advantages. *S. aureus* and *E.coli* are the major causes of hospital-acquired infections ([41,56]). These organisms occur naturally in human body. However, certain strains can lead to infections and are becoming resistant to antibiotics.

Preliminary results of this study indicate that the compounds in these active components are flavonoids. This result is in agreement with the findings in the literature, which indicate that the active compounds present in propolis include mainly flavonoids, phenolic acids and esters ([58,59]).
CONCLUSION
Thus, it was concluded that the EEP was the most active against all bacterial isolates. Showing the maximum zone of inhibition of 30 mm at the 25% concentration *S. pneumoniae* was more sensitive to propolis extract than other. Mucilage was butter active component of propolis, Mucilage which had highest antibacterial activity against bacterial isolates. Further studies can be done for the identification of the chemical compounds responsible for the antimicrobial activity and its isolation along with its characterization.

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REFERERANCES


47. Cowan MM. (Plant productsz as antimicrobial agents). Clinical Microbiology reviews, 1999;12(4) : 564 – 582 .

