



Antibacterial Activity of Two Algerians Propolis

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ABSTRACT

Despite the availability of different approaches for the discovery of new therapeutically agents, natural products still remain as one of the best reservoirs of new structural types. Due to the notable medicinal value of bee glue, it was very interest to carry out a phytochemical investigation of this natural product. In our present study we report the result of our qualitative analysis of secondary metabolites extracted from algerian propolis. Ethanolic extracts of propolis were prepared for chemical analysis. For antibacterial assays, agar diffusion method, minimum inhibitory concentration (MIC) was determined against several species. We found that algerian propolis is very rich in flavonoids. Our propolis showed remarkable inhibitory effect on bacterial growth.

Keywords: Algerian propolis, Antibacterial activity, Flavonoids.

INTRODUCTION

Because of the appearance of bacterial resistance to antimicrobial agents, more effort is being made to find alternative antimicrobial components. It had been suggested that natural products are preferable to synthetic ones. The study of natural compounds has been considered as a fruitful approach in the search of new drugs. In this view, a series of antimicrobial studies reported therapeutic efficacy of propolis on different groups of microorganisms. Many authors demonstrated propolis antibacterial activity against *Enterococcus sp.*, *Escherichia coli*, *Pseudomonas aeruginosa* and especially *Staphylococcus aureus*.^{1,2} Reports have pointed out propolis efficient activity against Gram-positive bacteria and limited action against Gram-negative bacteria.²⁻⁵

Propolis is a sticky, gummy, resinous substance collected by honeybees from various plant sources. Bees collect propolis to seal holes in the hives, smooth out the internal walls and protect the entrance against intruders. This substance is also used as antibiotic and embalming substance to cover carcasses from hive invaders. The composition of propolis varies according to the plants that can be found in a specific region.^{6,7} The constituents of propolis vary widely due to climate, season, location and year.^{6,8} One of the most important pharmacologically active constituents in propolis are flavonoids (flavones, flavonols, flavanones), phenolics and aromatics. Flavonoids are thought to account for much of biologic activity in propolis.^{9,10}

Propolis has a long history of being used in folk medicine dating back to at least 300 BC¹⁶ and also has been reported to possess various biological activities, namely anticancer¹, antioxidant^{3,4}, anti-inflammatory, antibacterial⁵, antifungal² and antihepatotoxic.¹⁸

Although, numerous researchers have reported the antimicrobial activity and chemical composition of propolis collected worldwide, information about Algerian propolis are still insufficient. The aim of the present study is to carry out a comparative study of antibacterial activity of two algerian propolis harvested from north east algeria (Benibelaïd and El-malha). This diversity is kept in order to compare their chemical profile as well as their antibacterial effect for a possible use in therapeutic.

MATERIALS AND METHODS

Extraction

Propolis was collected from the north-east of Algeria from El-malha in Mila (Propolis 1) and Benibelaïd in Jijel (Propolis 2) by scraping the "bee glue" of walls, frames and entrance of the hive. Propolis of each region (10 g) was ground and extracted by 100 ml of 60%, 70%, 80% and 95% ethanol (% v/v) in absence of bright light. After two week extracts were filtered to remove debris such as wax and wood chips. The ethanolic extract of propolis then obtained was divided on two parts. One part was used to the realization of antibiogram and the other part was evaporated using a rotary evaporator and used to the realization of Minimal Inhibitory Concentration (MIC).

Determination of chemical profile

The flavonoids components were sought-after and separated by thin layer chromatography, performed according to the methods of Park and al.^{19,20} and Vanhaelen and al.²¹ The used solvent was ethanol 95°/distilled water (55/45 v/v). Ethanolic extract of propolis were also analyzed on silica gel (Alufolien Kieselgel Merck F254) with mobile phase petroleum ether/ethyl acetate 7:3. Spots were visualized by U.V light spraying with 60% sulfuric acid in ethanol and heating at

100 °C as described in the literature.¹⁸ Standard substances were used to the identification of some chemical components. Paper chromatography, was performed on Whatman paper n°3 in acetic acid 15%. The spectral absorption in UV-Visible of the two tested propolis were evaluated according to the methods used by Park and al.^{19, 20}

Antibacterial activity

Microorganisms tested

Earlier studies have determined the antibacterial activity of EEP (Ethanolic Extract of Propolis) against several strains of bacteria but few studies have tested its effect against resistant bacteria. A total of 11 Gram positive and Gram negative bacteria were used for antibacterial tests. In addition to determining the antibacterial effect of propolis on some species that have previously been tested like: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, β hemolytic streptococcus and *Escherichia coli*, this study also intends to examine resistant bacteria. The strains tested were as following:

- *Staphylococcus aureus* with homogenate resistance to β -lactamin associated to a resistance to aminosid.
- *Non hemolytic streptococci* with a resistance to erythromycin, spiramycin and to trimethoprime-sulfamethoxazole.
- *Klebsiella pneumonia* with a resistance to clavunilic acid associated to amoxicillin.
- *Proteus mirabilis* with a resistance to trimethoprime-sulfamethoxazole, nalidixic acid, ticarcillin, amoxicillin and pefloxacin.
- *Pseudomonas aeruginosa* with a resistance to pefloxacin.

Antibacterial activity

The antibacterial activity was determined using the agar diffusion method according to the National Committee of Clinical Laboratory Standard (NCLLS) guidelines.²² Sterile Mueller Hinton (MH) agar or Brain Heart Infusion, according to the requirement of microorganism, was poured into Petri dishes and left to set. Then, Petri plates were inoculated with the microorganism inoculum. The inoculum was prepared with an overnight culture of test microorganism and the size was adjusted to 0.5 McFarland standard turbidity. Sterile paper disk prepared according to Tichy and al²³ and Park and al^{19,20} method permeated with 100 μ l of ethanolic extract 60%, 70%, 80% and 95% ethanol of the two tested propolis (Benibelaïd and El-malha) were placed on to agar plates containing one of the mentioned bacteria. The plates were then left at room temperature for 30 minutes and incubated at 37°C for 24 hours. Antibacterial activity was determined by measuring the diameters of the inhibition zone of EEP. All tests were performed in triplicate in three different experiments.

Determination of Minimal Inhibitory Concentration (MIC) was performed by the agar dilution method following the National Committee of Clinical Laboratory standard guidelines. The inoculums suspensions were prepared with an overnight culture of the testing bacteria and the size was adjusted to 0.5 McFarland standard turbidity. Serial dilutions of propolis were made to obtain a concentration range from 10 μ g/ml to 1400 μ g/ml. 2 ml of each concentration were placed on Petri plates. Then 18 ml of Mueller Hinton agar were added to each of the Petri containing the dilution and swirled carefully until the agar began to set. The bacterial suspensions were inoculated using a steers replicator on Mueller Hinton agar surface and incubated at 37°C for 24 hours. Each antimicrobial test was also reproduced with plates containing the culture medium plus ethanol, in order to obtain a control of the solvent antibacterial effect. The MIC was considered as the lowest concentration of each extract that yields a negative culture. All tests were performed in triplicate in three different experiments.

RESULTS AND DISCUSSION

The phenolic compounds present in the two tested propolis samples have been investigated. Generally, our analysis showed that they contained similar patterns of phenolic compounds characterised by the presence of caffeic acid derivatives, and aglycones flavonoids.

The UV-visible spectra of the tested propolis (figure 1) present two peak of absorption corresponding to the streap I and II characteristic of the flavonoids. In the neutral methanol, the flavonoids compounds absorbed in two different regions within the ultra-violet spectral, between 300 and 385 nm (Strip I), and between 250 and 280 nm (Strip II). The strip I is related to the absorption of the system cinnamoyl that is associated with the conjugation of the CO grouping of the central heterocycl with the core B. The strip II is associated with the absorption of the benzoyl system; this strip permits to evaluate the level of substituting in core A.

The thin-layer chromatography analysis of the two tested propolis revealed the presence of flavonoids identified by co-chromatography as: pectolinargenin, pilosin, ladanein, chrysin, kumatakenin, 5, 6, 3', 4'-tetrahydroxy-7-methoxyflavone, 5, 4'-dihydroxy-7,3-methoxyflavone, apigenin and caffeic acid. In our previous investigation of Algerian propolis we isolated and identified five flavones: pectolinargenin, Pilosin, ladanein³, Chrysin and apigenin²⁴, caffeic acid and four caffeic acid derivatives: (+)-chicoric acid, (+)-chicoric acid monomethyl ester, caftaric acid, and caftaric acid 1-methyl ester²⁵ represented in figure 2.

Algerian propolis evaluated in this study showed antimicrobial activity against the tested bacteria. The diameters of the zone of inhibition of ethanolic extract of the two tested propolis (60%, 70%, 80% and 95% ethanol) illustrated in table 1 ranged from 8mm to 30mm.

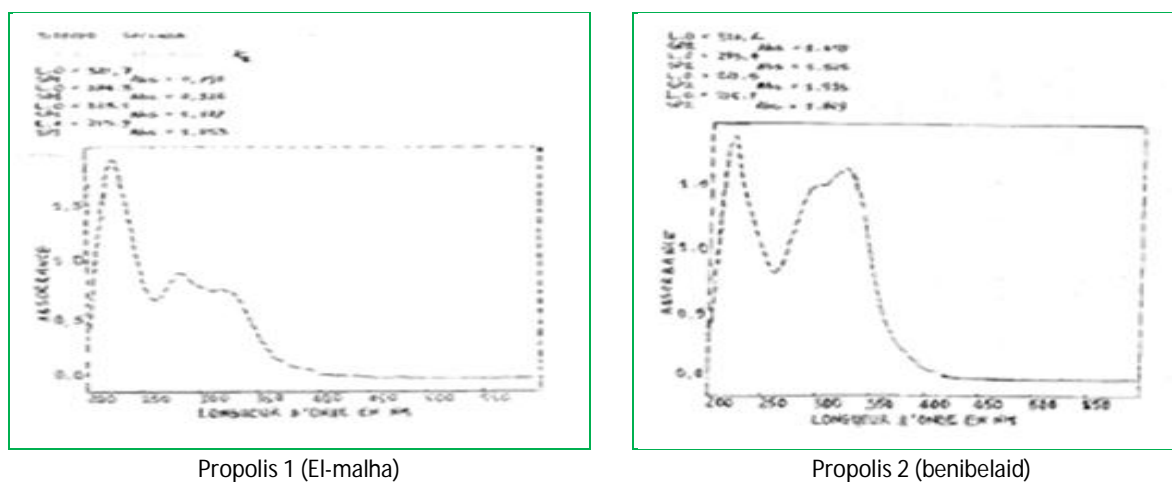


Figure 1: UV-visible spectra

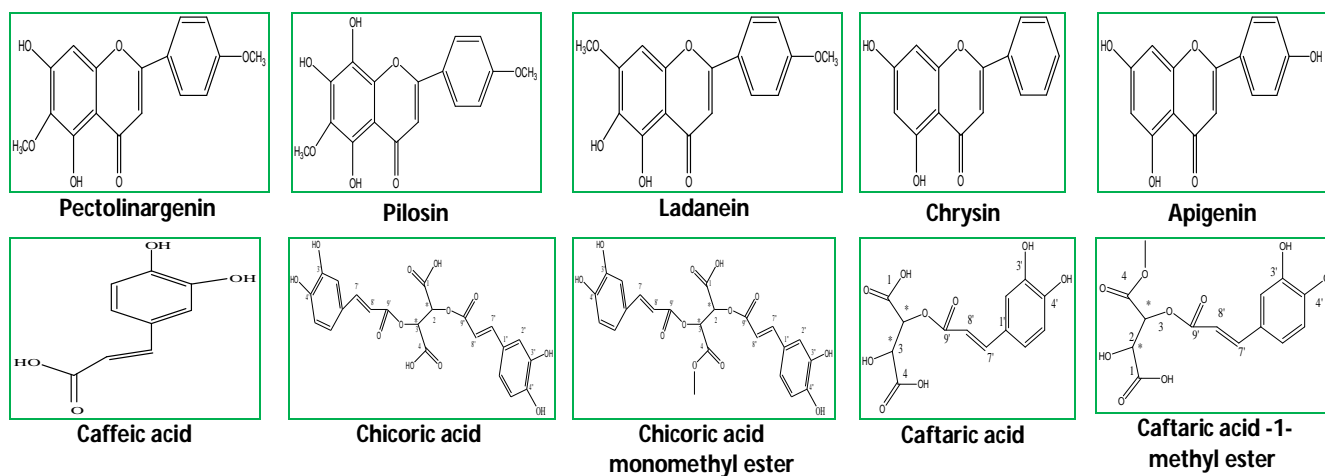


Figure 2: Chemical structures of identified compounds from Algerian propolis

Table 1: Susceptibility of microbial strains to EEP prepared with different percentage of ethanol

| Bacteria | EEP (1) | | | | EEP (2) | | | |
|---------------------------------|---------|------|------|------|---------|------|------|------|
| | 60 % | 70 % | 80 % | 95 % | 60 % | 70 % | 80 % | 95 % |
| <i>S. aureus</i> ATCC 25923 | 20 | 24 | 30 | 28 | 18 | 20 | 24 | 22 |
| <i>E. coli</i> ATCC 25922 | < 6 | 10 | 24 | 22 | < 6 | 8 | 14 | 12 |
| <i>P. aeruginosa</i> ATCC 27853 | < 6 | 12 | 12 | 12 | < 6 | 10 | 10 | 10 |
| <i>S. aureus</i> | 15 | 20 | 24 | 22 | 12 | 16 | 20 | 18 |
| β hemolytic streptococci | 12 | 18 | 20 | 20 | 16 | 18 | 18 | 18 |
| α hemolytic streptococci | 8 | 8 | 12 | 12 | 8 | 8 | 8 | 8 |
| non hemolytic streptococci | 18 | 20 | 22 | 22 | 16 | 18 | 20 | 20 |
| <i>E. coli</i> | < 6 | 8 | 10 | 10 | < 6 | 8 | 10 | 10 |
| <i>P. aeruginosa</i> | < 6 | 8 | 10 | 10 | < 6 | 8 | 10 | 10 |
| <i>P. mirabilis</i> | 10 | 10 | 12 | 12 | 10 | 10 | 12 | 12 |
| <i>K. pneumoniae</i> | 8 | 10 | 10 | 10 | 7 | 10 | 10 | 10 |

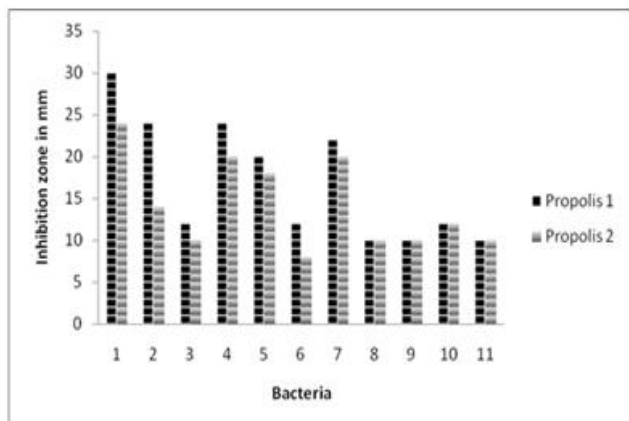
EEP (1): ethanolic extract of propolis of El-malha; EEP (2): ethanolic extract of propolis of Benibelaid

Propolis of El-malha is most active against *S. aureus* ATCC 25923, *S. aureus* and non hemolytic streptococci. The tested propolis has a less activity against *E. coli*, *P. aeruginosa*, *K. pneumonia* and *P. mirabilis* (Figure 2).

A critical step in the process of testing biological activity of propolis is its extraction. The solvents used for

extraction are usually alcohols: methanol and ethanol. The most often utilized solvent is ethanol containing different percent of water. Water has also been used on many occasions; however, it is important to note that in general, water dissolves a small part of propolis constituents whereas ethanol may dissolve better propolis samples depending on the wax amount. The

propolis tested showed the best diameter when 80% ethanol was used suggesting that most of the active components are dissolved and extracted using these percentage of ethanol (Figure 4).



1: *S aureus* ATCC 25923 ; 2: *E coli* ATCC 25922 ; 3: *P aeruginosa* ATCC 27853 ; 4: *S aureus* ; 5: β hemolytic streptococci ; 6: α hemolytic streptococci ; 7: non hemolytic streptococci ; 8: *E coli* ; 9: *P aeruginosa* ; 10: *P mirabilis* ; 11: *K pneumonia*.

Figure 3: Inhibition zone of 80% EEP according to the tested strain.

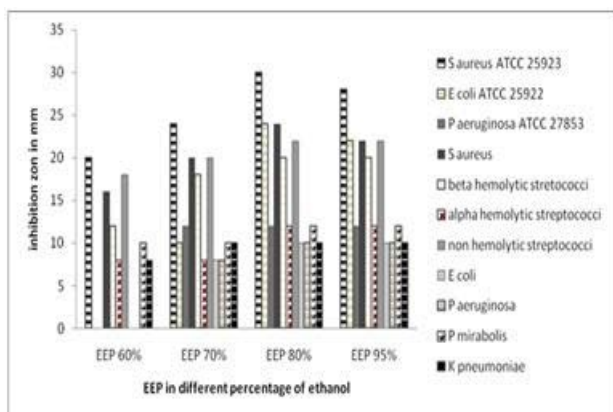


Figure 4: Inhibition zone depending on percentage of ethanol of propolis of El-malha

After the evidence of in vitro antibacterial activity against all tested strains in the screening diffusion test, the minimum inhibitory concentration (MIC) was established using the agar dilution method (Table 2). The MIC values ranged from 40 µg/ ml to 1400 µg/ ml. The MIC values of the most effective propolis (El-malha) were 40 µg/ ml for *S aureus* ATCC 25923, 60 and 80 µg/ ml for *S aureus* and non hemolytic streptococci resistant strains, 100 µg/ ml for *E coli* ATCC25922, *P aeruginosa* ATCC 27853 and their clinical isolates, *P mirabilis*, *K pneumonia* and 200 µg/ ml for α and non hemolytic streptococci. Propolis of benibelaid has showed a less activity against all tested bacteria. The MIC values were 60 µg/ml for *S aureus* ATCC 25923, 200 and 600 µg/ml for *S aureus* and non hemolytic streptococci. All Gram-negative bacteria were inhibited by a concentration ranged from 1100 to 1200 µg/ ml.

Table 2: Minimal Inhibitory Concentration (µg/ml)

| Bacteria | Propolis (1) µg/ ml | Propolis (2) µg/ ml. |
|---------------------------------|---------------------|----------------------|
| <i>S. aureus</i> ATCC 25923 | 40 | 60 |
| <i>E. coli</i> ATCC 25922 | 100 | 1000 |
| <i>P. aeruginosa</i> ATCC 27853 | 100 | 1100 |
| <i>S. aureus</i> | 80 | 200 |
| β hemolytic streptococci | 60 | 600 |
| α hemolytic streptococci | 200 | >1400 |
| non hemolytic streptococci | 200 | 600 |
| <i>E. coli</i> | 100 | 1000 |
| <i>P. aeruginosa</i> | 100 | 1100 |
| <i>P. mirabilis</i> | 100 | 1200 |
| <i>K. pneumoniae</i> | 100 | 1200 |

Propolis (1): Propolis of El-malha; Propolis (2): Propolis of Benibelaid.

The comparison between the MIC values and the resistance of the tested bacteria against antibiotics showed a difference between Gram-positive and Gram-negative bacteria. The MIC of propolis 1 for β hemolytic streptococci sensitive to all tested antibiotics is 60 µg/ml, whereas for α and non hemolytic streptococci resistant to several antibiotics (erythromycin, spiramycin and to trimethoprim-sulfamethoxazol), the MIC are 3 times bigger (200 µg/ml). For propolis 2, the MIC of β and non hemolytic streptococci is 600 µg/ml, while α hemolytic streptococci persists to 1400 µg/ml. We noticed that there is a relation between sensibility of bacteria against propolis ethanolic extract and resistance to antibiotics. Concerning Gram positive bacteria, propolis 1 acts in the same way on all tested bacteria (sensitive and resistant). Whereas, propolis 2 acts according to the bacterial species.

CONCLUSION

Flavonoids have been found to be present as major components in propolis from temperate regions.²⁶ Our study confirm the presence of those compounds in algerian propolis. All propolis samples showed similar contents of flavonoids. Therefore a single species or a group of related species were the main source of propolis in the two algerian areas where propolis was collected.

In the present study Algerian propolis showed in vitro antibacterial activity. The standard strains were chosen according to the screening protocol including Gram positive and Gram negative bacteria. In the first step of an antibacterial screening, the product should be tested against these standard strains, which represent micro-organisms associated with important infections. It has been suggested that propolis has more antibacterial effects on Gram positive bacteria than Gram negative bacteria.^{1,4,9} In more recent studies propolis was found to have a weak activity against Gram negative bacteria.³ We found that Algerian propolis had a more pronounced

activity against *S aureus* and β hemolytic streptococci and a less evident activity against *E coli*, *P aeruginosa*, *P mirabilis*, *K pneumonia*, non and α hemolytic streptococci. Variations in the susceptibility to propolis among several microorganisms have been reported but not their specific mechanisms of action. Further studies are needed to explain whether the cell structure and permeability to such compounds or even specific targets in the cell enzymatic systems are involved in microbial susceptibility.

The lack of qualitative information hinders the wide use of propolis in medicine. It is clear that propolis possesses an important biological activity, but it would be difficult to determine quality control of propolis. This difficulty arises from the variation of the chemical composition of propolis depending on the geographic zone and the specificity of the local flora. It is essential to determine the biological active compounds of propolis and to characterize the abundance of them in a sample. Further in vitro and in vivo studies with raw extract and isolated compounds would be important for a better understanding of the biological activity and the variability of propolis.

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