# Antibacterial activity of Algerian hydro-ethanolic propolis extract against *Escherichia coli* and *Staphylococcus aureus*-induced breastfeeding women mastitis

Kadda CHEBLI 1, Farouk BOUDOU 1,2\*, and Abdekrim BERROUKCHE1,3

1. Department of Biology, Faculty of Natural and Life Sciences, Dr MOULAY Taher University of Said, Algeria.
2. Department of Biology, Faculty of Natural and Life Sciences, DjillaliLiabes University of Sidi-Bel-Abbes, Sidi-Bel-Abbes, Algeria.
3. Research Laboratory of Water Resources and Environment, Biology Department, Faculty of Sciences, Tahar-Moulay University of Saida, Algeria.

***\* Corresponding author****. Email:* [farouk.boudou@yahoo.fr](mailto:xyz@gmail.com) *(F. BOUDOU)*

**Abstract:**

Mastitis, caused by *Escherichia* *coli* and *Staphylococcus aureus,* is a common infection in nursing mothers. Antibiotics (ATBs) are the mainstay of treatment for mastitis. However, given the potential for transfer of antibiotics from mother to infant, as well as the problem of bacterial resistance to synthetic ATBs, it is critical to develop new natural compounds with antibacterial properties. Thus, the aim of this work is to determine the phytochemical content, antioxidant capacity, and in vitro antibacterial activity of the hydroethanol extract of an Algerian propolis against the two above-mentioned bacterial strains, in comparison with four ATBs namely: Gentamicin (GEN), Cefazolin (CZ), Cefotaxime (CT), and Pipemidic acid (PI). Estimates of total polyphenol and total flavonoids revealed levels of 113.65±2.41 mg AGE/g DR, and 0.072±0.039 mg EC/g DR respectively. Evaluation of antioxidant activity revealed a significant ability to scavenge 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical (IC50=2.95±0.70 mg/mL). Bacterial inhibition tests performed by the agar well diffusion method showed at the concentration of 5000 µg/mL a strong antibacterial activity with inhibition zones of 22.5±1.67 mm and 25.5±0.50 mm against *S. aureu*s, and *E. coli* respectively, compared to GEN (31±1.00 mm and 35±0.50 mm), CZ (14.5±0.33 mm and 24.50±0.35 mm), CT (10.50±0.33 mm and 11±0.67 mm), and PI (06±0.00 mm and 35±0.50 mm) against *S. aureus*, and *E. coli* respectively. These results show that the efficacy of propolis extract exceeds that of the two ATBs belonging to the cephalosporin family (CZ and CT of the 1st and 2nd generation respectively) and the Quinolones family (PI of the 1st generation).

**Keywords:** Antibiotics; Antioxidant; *Escherichia* *coli*; Propolis; *Staphylococcus aureus.*

**INTRODUCTION**

Mastitis is a common condition in lactating women ([Wilson et al. 2020](#_ENREF_28)). It affects between 3% and 20% of all nursing women. Primiparity, breast-pump use, nipple-shield use, previous history of mastitis, and nipple trauma are all risk factors ([Mitchell et Johnson 2022](#_ENREF_15)). Mastitis is characterized by unilateral discomfort, redness, and swelling of the breast, and may be accompanied by flu-like symptoms (fever, chills and aches) ([Wambach 2003](#_ENREF_27)). Erythema, oedema, and unilateral tenderness of the afflicted breast are commonly seen on examination. In cases of breast abscess, a fluctuating, painful, hard breast mass and overlying erythema is seen. In order to distinguish infected mastitis from non-infectious mastitis, tests such as milk leukocyte count, bacterial colony count and culture may be useful ([Betzold 2007](#_ENREF_2); [Barlow 2011](#_ENREF_1)). *Staphylococcus aureus*, which includes methicillin-resistant *Staphylococcus aureus* (MRSA), *S. epidermidis*, streptococci, and gram-negative rods, are the most prevalent bacterium associated with mastitis ([Kateete et al. 2013](#_ENREF_9)). *Escherichia coli,* and[*Pseudomonas aeruginosa*](https://www.sciencedirect.com/topics/medicine-and-dentistry/pseudomonas-aeruginosa)have also been found to cause acute mastitis ([Underwood et al. 2015](#_ENREF_26)). Supportive treatment, efficient milk extraction, symptomatic treatment (pain medication, use of anti-inflammatory medications), probiotic treatment, and antibiotic therapy are all principles of mastitis treatment ([Jahanfar et al. 2009](#_ENREF_8)). For staphylococcal and streptococcal infections, penicillin, dicloxacillin and cephalosporins are recommended, while for gram-negative organisms, cephalexin or amoxicillin can be used ([Yang et al. 2018](#_ENREF_29)). A prospective study of a sample of 840 women in the United States found that 86% of women with mastitis received antibiotics, with the majority receiving cephalexin (46%). While, the remainder received amoxicillin, ampicillin and amdinocillin clavulanate ([Foxman et al. 2002](#_ENREF_5)). There is a high risk of transmission of antibiotics used by breastfeeding mothers to newborns through breast milk. While the majority of medicines used by breastfeeding women have no adverse effects on their newborns, they can sometimes have serious consequences ([Mathew 2004](#_ENREF_13)). Exposure to antibiotics in early childhood has been linked to an increased risk of non-communicable disorders, including allergies and obesity. Furthermore, the influence of antibiotics on the creation of the neonatal gut resistome, as well as the role of the microbiota as a reservoir of resistance genes, are linked to the increasing number of antibiotic-resistant pathogens ([Nogacka et al. 2018](#_ENREF_18)). However, faced with this dual constraint of transferring antibiotics from mother to infant, as well as the problem of bacterial resistance to synthetic antibiotics, it is essential to develop new natural compounds with bactericidal properties. Propolis, sometimes known as bee-glue, commonly referred as a “natural antibiotic” is a resinous compound produced and utilized by bees (*Apis mellifera L*.) to seal hive walls and strengthen the comb and hive entrance borders, which is already being used by humans since ancient times for its pharmaceutical properties, including antioxidant, anti-inflammatory, and antimicrobial activities ([Bruschi et al. 2017](#_ENREF_3)). It is relatively nontoxic and safe, and it's used in cosmetics and "natural products" for self-treatment because of its antiseptic, antimycotic, and bacteriostatic characteristics ([Desai 2016](#_ENREF_4)). The main compound categories present in propolis are phenolics and flavonoids. However, the composition of propolis varies greatly depending on geographical region and collection season ([Freires et al. 2018](#_ENREF_6)). All types of propolis possess significant antimicrobial activity against various types of microorganisms, including bacteria, fungi, and virus ([Mohammadzadeh et al. 2007](#_ENREF_16)). Anti-inflammatory and antimicrobial activity against *Streptococcos mutans*, *Staphylococcos aureus*, *Escherichia coli*, *Porphyromonas gingivalis*, *Pseudomonas aeruginosa* (among others) have been demonstrated ([Freires et al. 2018](#_ENREF_6)). Propolis is gaining popularity in the pharmaceutical, food, and cosmetic industries as a result of its medical characteristics, and it is now being used in products for human consumption, such as drinks and food additives ([Pascoal et al. 2014](#_ENREF_19)). Indeed, the objective of this work is to determine the phytochemical content, the antioxidant capacity and the in vitro bactericidal activity of the hydroethanolic extract of propolis against mastitis induced by *Escherichia coli* and *Staphylococcus aureus* in breastfeeding women in comparison with four antibiotics namely: Gentamicin (GEN), Cefazolin (CZ), Cefotaxime (CT), and Pipemidic acid (PI).

**MATERIALS AND METHODS**

**Preparation of propolis hydroethanolic extract**

The hydroalcoholic of a propolis from the region of Saida in Algeria was prepared according to the method described by Mohdaly et al. (2015) ([Mohdaly et al. 2015](#_ENREF_17)). 10 g of propolis powder samples were extracted overnight at room temperature with 100 ml of hydroethanol solution (70%). The extract was filtered through a Whatman filter paper (No. 1), and the solvent was evaporated using a rotary vacuum evaporator and the dry residue was stored at 4C° for further analysis.

The yield (R%) was calculated according to the following formula:

**Total Phenolic Content (TPC)**

The total phenolic content of the samples was determined using the Folin-Ciocalteu reagent, as reported by Serairi-Beji et al. (2018) ([Serairi‐Beji et al. 2018](#_ENREF_23)). 0.5 mL distilled water and 0.125 mL Folin-Ciocalteu reagent were mixed with an aliquot of diluted sample fraction. Before adding 1.25 mL Na2CO3, the mixture was mixed and incubated for 6 minutes (7 percent ). The solution was then diluted to a final volume of 3 mL with distilled water and properly mixed. The absorbance was measured at 760 nm against a prepared blank after incubation in the dark. Through the calibration curve with Gallic acid, TPC was represented as milligrams Gallic acid equivalents per gram dry weight (mg GAE/g d.w.). The range of the calibration curve was 0–100 g/mL. Three replications of each sample were performed.

**Total Flavonoid Content (TFC)**

The colorimetric method described by Kim et al. (2003) ([Kim et al. 2003](#_ENREF_11)) was used to determine the total flavonoid content of the extracts. 100 µL extract was combined with 0.4 mL distilled water, then 0.03 mL sodium nitrite solution (5 percent NaNO2) was added. After 5 minutes, 0.02 mL of a 10% AlCl3 solution was added. After 5 minutes, add 0.2 mL of 1 M Na2CO3 solution and 0.25 mL of distilled water. A vortex was used to agitate the mixture, and absorbance was measured at 510 nm. TFC was calculated as mg catechin equivalent per gram dry weight (mg CE/g d.w.) using the catechin 0–500 g/mL calibration curve (R2 = 0.99). Triplicate samples were tested.

**DPPH Radical-Scavenging Activity**

The method of Sanchez-Moreno et al. (1998) ([Sánchez‐Moreno et al. 1998](#_ENREF_21)) was used to measure DPPH radical-scavenging activity. Rapidly, 50 µL of each extract at various concentrations (from 0.078 to 5 mg/mL) were added to 1.95 mL of DPPH (0.025 g/L) methanolic solution. Simultaneously, a negative control is made by combining 50 liters of methanol with 1.95 mL of DPPH methanolic solution. The mixture was briskly shook before being allowed to stand at room temperature for 30 minutes in the dark. At 515 nm, the absorbance of the resultant solution was measured. The scavenging activity was represented as IC50 [mg/mL], which is the dose necessary to inhibit DPPH by 50%. Plant extracts with a lower IC50 value have stronger antioxidant activity. The percentage of radical-scavenging is calcula­ted according to the following equation:

where:

A0 is the absorbance of the control (DPPH solution without extract).

A is the absorbance in the presence of extract

**Antibacterial activity**

The inoculum was prepared by culturing the microorganisms in nutrient broth at 37°C for 12 hours and a concentration of approximately 1.5 x 108 colony forming units (CFU/ml) used for the antimicrobial analysis ([Hayouni et al. 2007](#_ENREF_7)). The Agar-well diffusion method was performed by spreading each bacterial suspension over the surface of Mueller-Hinton agar plates containing four wells of 6 mm diameter. Wells were filled with approximately 30 μL of each of the extract concentrations used (625, 1250, 2500, 5000 ug/mL). Ethanol was used as a control test. While the antibiogram were performed by the disc diffusion method using commercial antibiotic discs (Gentamicin, Cefazolin, Cefotaxime, and Pipemidic acid) of 6 mm diameter deposited in the previously prepared Petri dishes. The plates were incubated at 37°C for overnight. The results were expressed in terms of the diameter of the inhibition zone and sterile water used as control ([Kaushik et al. 2010](#_ENREF_10)).

**Statistical analysis**

The mean ± SD values were calculated for each group to determine the significance of intergroup differences. To find the difference between the groups, Student’s t-test was used. P values <0.05 were considered to be significant.

**RESULTS**

As shown in Table 1, the hydroalcoholic extraction of propolis using maceration method allows us to calculate the extraction yields (18.2%)expressed in percentages relative to the initial weight. The total phenol content was determined as Gallic acid equivalent in milligram per gram dry residue (113.65±2.41 mg GAE/mg DS.), while total flavonoid was calculated as catechin equivalent in milligram per gram dry residue (0.072±0.039 mg CE/g DS.). In addition, the antioxidant activity of the propolis extract against the DPPH radical was measured by tracking the reduction of this radical, which is accompanied by the passing of the purple color (DPPH-) to the yellow color (DPPH-H) at 515 nm. Thus, the hydroethanol extract of propolis has a significant capacity to trap the DPPH radical, as shown in Figure 1, with an inhibitory concentration of IC50=2.95 mg/mL.

Table 1. Results extraction yield, total phenol, and total Flavonoid.

|  |  |  |  |
| --- | --- | --- | --- |
| Parametres | Exraction yield (%) | Polyphenols (mg GAE/g RS) | Flavonoids (mg CE/g RS) |
| Propolis hydroalcoholic extract | 18.2% | 113.65±2.41 | 0.072±0.039 |

Figure 1. Results of DPPH radical-scavenging activity

Figures 2, 3 and 4 reflect the results of the inhibition tests carried out by agar well diffusion and antibiotics disk diffusion methods and showed a strong antibacterial activity of propolis extract at the concentration of 5000 µg/mL with inhibition zones of 22.5±1.67 mm and 25.5±0.50 mm against *S. aureu*s, and *E. coli* respectively. At the two concentrations of 2500 and 1250 µg/mL the extract exhibit the same activity with inhibition zones of 20.5±0.33 mm and 14.5±0.33 mm against *S. aureus*, and *E. coli* respectively. While for the concentration of 625µg/mL the propolis extract had more effect on *S. aureus* with an inhibition zone of 12±0.00 mm, and an inhibition zone of 10.5±3.00 mm against *E. coli.* These results were compared to those of antibiotics which showed inhibition zones against *E. coli*, and *S. aureus* respectively expressed as follows: GEN (31±1.00 mm and 35±0.50 mm), CZ (14.5±0.33 mm and 24.50±0.35 mm), CT (10.50±0.33 mm and 11±0.67 mm), and PI (06±0.00 mm and 35±0.50 mm), where it has been found that the efficacy of propolis extract exceeds that of the two ATBs belonging to the cephalosporin family (CZ and CT of the 1st and 2nd generation respectively) and the Quinolones family (PI of the 1st generation).

Figure 2: Results of the antibacterial activity of propolis extract.

Figure 2: Results of the antibacterial activity of the tested antibiotics:

Gentamicin (GEN), Cefazolin (CZ), Cefotaxime (CT), and Pipemidic acid (PI).

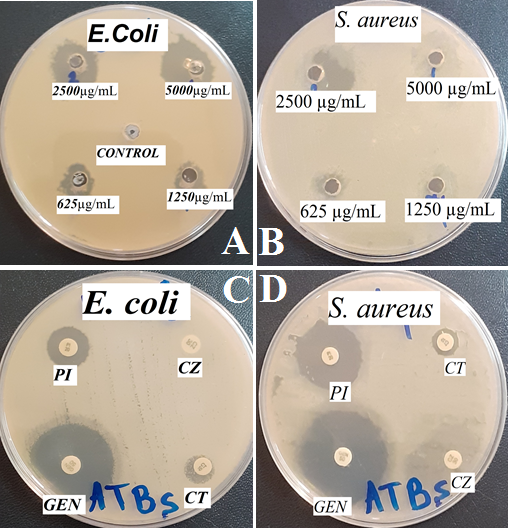


Figure 4. The antibacterial activity by Agar-well diffusion, and antibiotics disk methods.

A and B: Propolis antibacterial activity against *E. coli,* and *S. aureus* respectively. C and D: Antibiotics antibacterial activity against *E. coli,* and *S. aureus* respectively.

**DISCUSSION**

The results of this study revealed that the hydroethanol extraction of propolis obtained by maceration during a period of 24 hours resulted in an important extraction yield evaluated to 18% and that the dry residue obtained contained a significant amount of total phenols estimated at 113.65±2.41 mg GAE/g DR, with a negligible flavonoid content of 0.072±0.039 mg CE/g DR. Indeed, our results are in agreement with those of Socha et al, (2015) ([Socha et al. 2015](#_ENREF_24)) who showed in their study conducted to estimate the phenolic composition and the antioxidant activity of propolis from various regions of Poland that the total phenolic content of propolis samples ranged from 150.05 to 197.14 mgGAE/g, while the total flavonoid content was 35.64-62.04 mgQE/g. They add that the dominant phenolic acid was p-coumaric acid, and ferulic acid. While, among the flavonoids, chrysin, naringin and galangin were dominant. On the other hand, the study of Segueni et al (2021) ([Segueni et al. 2021](#_ENREF_22)) shows after the analysis of two samples of propolis of Algerian and Turkish origin that the levels of phenolic compounds are lower than ours findings, varying from 19.51 ± 0.86 to 219.66 ± 1.23 mg GAE/g for total phenols and from 5.27 ± 0.07 to 74.57 ± 1.03 QE/g for flavonoids. Similarly, a study carried out on the effects of different ethanol concentrations (20, 50, 80%) on the total phenolic content and total flavonoid content in Malaysian propolis also shows low levels compared to our study where the highest TPC and TFC were recorded at 80% ethanol, i.e. 8.898 mg GAE/ml and 0.034 mg QE/ml respectively ([Yusof et al. 2020](#_ENREF_30)). Freires et al, (2018) ([Freires et al. 2018](#_ENREF_6)) explain that the main compound categories present in propolis are phenolics and flavonoids. However, propolis composition varies significantly depending on geographical region and collecting season. Furthermore, the antioxidant activity of propolis extract against the DPPH radical which is measured by following the reduction of this radical, which is accompanied by the change from purple colour (DPPH-) to yellow colour (DPPH-H) measured by a spectrophotometer at 515 nm shows that the hydroethanol extract of propolis has a significant capacity to scavenge the DPPH radical, with an inhibitory concentration of IC50 = 2.95 mg/mL. These findings are in agreement with those of Mihai et Mărghitaş, (2010) ([Mihai et Mărghitaş, 2010](#_ENREF_14)) which indicate an antioxidant capacity of Transylvanian propolis that varies between 1.67 mg/ml and 3.28 mg/ml. However, our results remain low compared to a study conducted on the DPPH radical scavenging activity of Cameroonian propolis which showed that the radical scavenging activity varied from the Hexane extract of Foumban propolis (IC50 = 5. 6 mg/mL) to the Methanol extract of Foumban propolis (IC50 = 1.07 mg/mL) for the extracts and from 3β-hydroxylanostan-9,24-dien-21-oic acid (IC50 = 1. 22 mg/mL) compared to those of the standard antioxidants gallic acid (IC50 = 0.30 mg/mL) and vitamin C (IC50 = 0.80 mg/mL) ([Talla et al. 2017](#_ENREF_25)). Whereas, the results of the inhibition tests carried out by agar well diffusion showed a strong antibacterial activity of propolis extract at the concentration of 5000 µg/mL with inhibition zones of 22.5±1.67 mm and 25.5±0.50 mm against *S. aureu*s, and *E. coli* respectively. At the two concentrations of 2500 and 1250 µg/mL the extract exhibit the same activity with inhibition zones of 20.5±0.33 mm and 14.5±0.33 mm against *S. aureus*, and *E. coli* respectively. While for the concentration of 625µg/mL the propolis extract had more effect on *S. aureus* with an inhibition zone of 12±0.00 mm, and an inhibition zone of 10.5±3.00 mm against *E. coli.* Indeed, the antibacterial activity obtained in our results exceeds that found in the study carried out by Motior et al. (2010) ([Rahman et al. 2010](#_ENREF_20)) on the antibacterial activity of propolis against *Staphylococcus aureus* and *Escherichia coli* with moderate zone inhibition of 13.0 ± 0.09 to 15.0 ± 0.11 mm against these two bacteria respectively using a concentration (5.48 mg ml-1) close to the one used in our study. They also suggest that *S. aureus* is more sensitive to the effect of propolis than its Gram-negative counterpart *E. coli*. Similarly, Arum et al (2020) ([Lestari et Permana, 2020](#_ENREF_12)) in their study to determine the effect of propolis on the growth of *Staphylococcus aureus* and *Escherichia coli* bacteria at different concentrations (30%, 50%, 70% and 90%) confirms that propolis has an inhibitory effect on the growth of *S. aureus* colonies with the best concentration value of 90%, while it has no effect on *E. coli* colonies. These findings are in disagreement with our results, as the extract used in our study had a good effect on both bacterial strains (gram negative and positive).In addition, these results were compared to those of antibiotics which showed inhibition zones against *E. coli*, and *S. aureus* respectively expressed as follows: GEN (31±1.00 mm and 35±0.50 mm), CZ (14.5±0.33 mm and 24.50±0.35 mm), CT (10.50±0.33 mm and 11±0.67 mm), and PI (06±0.00 mm and 35±0.50 mm), where it has been found that the efficacy of propolis extract exceeds that of the two ATBs belonging to the cephalosporin family (CZ and CT of the 1st and 2nd generation respectively) and the Quinolones family (PI of the 1st generation).

**CONCLUSION**

The increase in bacterial resistance to synthetic antibiotics, as well as the potential risk of drug transfer via breast milk, has prompted the search for alternative antibacterial drugs. This research has shown that Algerian propolis may be an appropriate substrate of these synthetic drugs due to its high phenolic acid content, which gives it a powerful antioxidant and especially antibacterial effect against gram positive and negative bacteria.

**CONFLICTS OF INTEREST**

Authors have declared that they have no competing interests.

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