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An Experimental Evidence for Antibacterial Activity of Propolis on Bacteria from Lebanese Patients

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Abstract
Propolis, with its distinctive chemical composition, has been known for therapeutic potential in treating numerous diseases. This study investigated whether propolis has an antibacterial effect on bacteria in Lebanon. Twenty- eight samples of gram-negative and gram-positive bacteria were collected from Raii Hospital and Al-Taakhi Medical Center in Gazieh, South Lebanon. Each sample was streaked on three different Petri dishes with different percentages of propolis under aseptic conditions. The results were recorded visually and confirmed by Gram staining after incubation for 24 h at 37°C. The obtained results show that propolis inhibited the growth of 18% of the total number of collected bacteria. The majority of bacteria on which propolis had an inhibitory effect were gram- positive bacteria, which inhibited 50% of them, whereas it only inhibited the growth of 9% of the gram-negative bacteria. Propolis was also shown to have an antifungal effect.

Keywords: Propolis, Anti-Bacterial, Anti-Fungal, Lebanon

1. Introduction

Propolis is a natural mixture produced by bees. It is gathered from substances collected from parts of plants, buds and exudates (Burdock, 1998). Honey bees use a combination of bees' wax and saliva to produce propolis (Cornara et al., 2017). The propolis name is derived from Greek origin "Pro" which means in front of and "polis" means community or city (Aminimoghadamfarouj & Nematollahi, 2017) which indicates that propolis is the hive defensive substance (Al-Hariri, 2011; Araujo et al., 2012).). Propolis is one of the famous honeybee products used in folk medicine since ancient times for its various health effects (Ahangari et al., 2018).

2. Physical properties of Propolis

There are several types of propolis based on the area they originate from and their plant source (Ahmed et al., 2017). Propolis is a lipophilic substance that is also hard, brittle, and very sticky when heated (Hausen el al., 1987). Depending on its source and age, propolis could be a yellow green substance, or a red to dark brown

substance (Marcucci, 1995). The melting point of propolis at which it turns into its liquid state is 60ºC to 70ºC but for some samples, it may be 100ºC (Wagh, 2013).

Propolis is commercially extracted by a suitable solvent, of which ethanol, methanol, water, hexane, acetone, dichloromethane, and chloroform are most common used (Gómez-Caravaca et al., 2006 ; Kumar N. et al., 2008). These solvents should remove the inner materials and reserve the desired compounds (Marcucci, 1995).

3. Chemical Composition of Propolis

The Biological activity and quality of propolis are directly related to its chemical constituents (Zhang X. et al., 2014). More than 300 constituents were identified in different propolis samples (De Castro SL., 2001), depending on its place of origin and time of collection (Kujumgiev A. et al., 1999). The main groups of propolis constituents are Plant resins (50%) in addition to other components such as waxes, essential oil, pollens, and other organic substances in different percentages as shown in Figure 1 (Przybyłek, I., & Karpiński, T. M. 2019).

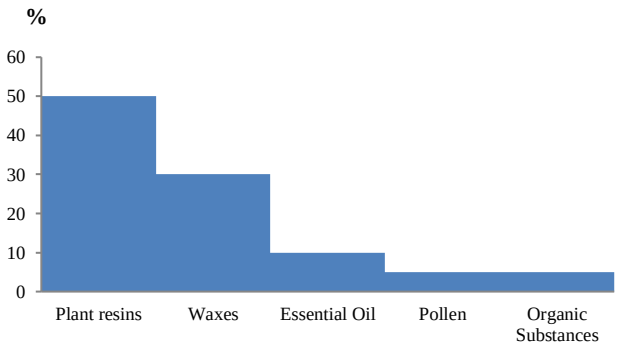

Figure 1: Raw Propolis chemical composition (%).

Propolis has many bioactive properties, such as antioxidant, antimicrobial, anti-inflammatory, wound healing and anti-hyperlipidemic (Ansorge S. et al., 2003; Pimenta, H. C. et al., 2015; Manso et al., 2021). The main chemical components that are responsible for this activity are phenolic compounds, including flavones and aromatic acids and their esters (Ansorge S. et al., 2003; Pimenta, H. C. et al., 2015 ; Manso et al., 2021). Several researches ensured that propolis from Asia, Europe and North America is mostly composed of phenolic compounds (Uzel et al., 2005). In a research done by Touzani et al. (2021) in determining the phenolic compounds in propolis samples collected from an African and an Asian region shows that phenolic content was the lowest in Palestinian sample of 74.71 ± 0.89 mg GAE/g while the highest in Moroccan sample of 148 ± 1.31 mg GAE/g. Similarly, flavone and flavonol content were higher in Moroccan sample of 118 ± 1.92 mg QE/g than Palestinian sample 26.97 ± 2.44 mg QE/g. The total antioxidant capacity is positively correlated with the content of phenolic compounds were it is 90.87 ± 2.91 mg AAE/g for Moroccan sample and 48.01 ± 0.51 mg AAE/g for Palestinian sample. Moreover Zeitouna et al. (2019) analyzed the chemical composition of Lebanese propolis and identified twenty-eight different compounds of which two phenolic acids and nine other flavonoids, also ferulic acid, caffeic acids, chrysin, galangin, quercetin, and pinocembrin were specified among the most representative compounds inside the Lebanese propolis.

4. Pharmacological Activity of Propolis

Several studies confirmed that Propolis has a therapeutic potential in pharmacology and medicines to treat various diseases due to its various chemical compositions.

4.1. Antibacterial Activity

Propolis is increasingly known for its antibacterial activity. The antibacterial activities depend on the concentration, treatment time, and bacterial mode of action (Clemente J.C. et al., 2012 ; Kim Y-H, 2011). It acts

either by directly interacting with the microbial cell or by stimulating the immune system of the host cells (Bouchelaghem S., 2022). One of the hypothesized mechanisms include the inhibition of bacterial adherence and division, decrease of bacterial mobility, disturbance of membrane potential, and increase in cell membrane permeability (Ristivojević et al., 2018). Phenolic compounds elevate the antibacterial activity of propolis by enhancing their interaction with the cell membrane (Sikkema et al., 1995). In addition to that Kumar N. et al. (2008) emphasized that this antibacterial activity is highly activated against Gram-positive bacteria and less activated against Gram-negative bacteria, unless high concentration of Propolis is used.

4.2. Antifungal Activity

Propolis shows anti-fungal activity against different fungi, such as Candida albicans, *C. parapsisolis, C. tropicalis, Saccharomyces cerevisiae and C. krusei* (Ożarowski et al., 2022). Ozcan, (1999) emphasized that different propolis chemical compositions such as, 3-acetylpinobanksin, pinobanksin-3-acetate, pinocembrin, p-coumaric acid and caffeic acid and more compounds enhances propolis antifungal activity. Previously, researches emphasized that propolis antifungal activity occurred by inhibiting the extracellular phospholipase, directing to alleviation of the fungal cell adhesion to epithelium (D'Auria et al., 2003). Recently, researchers spotted that propolis may affect the formation of the cell wall and inhibit the morphological transformation of fungi (Gucwa et al., 2018).

5. Methods

5.1. Sample Collection & Identification

A total of twenty-eight samples were collected from Raii Hospital and Al-Taakhi Medical Center- Gazieh. Both of these institutions are located in Ghazieh, a village in the vicinity of Said but receive patients from Saida, Ghazieh and several other villages and towns in South Lebanon. The collected samples that are available in the hospital or the medical center were samples taken from patients submitted to bacterial culturing and antibiogram process to specify the type of the bacteria and the effective drug to be prescribed. The samples were saved in incubators for short time to be taken and test propolis effect on these samples. The samples were including gram-negative and gram-positive bacteria that are randomly selected based on the available samples at the time of collection. The collection process took place over several days separated by two-week duration.

Figure 2 depicts the distribution of the bacterial samples such that the gram-negative bacteria exceed the gram-positive bacteria in number thus, in percentage as well. In total, there were 6 gram-positive bacteria, accounting for 21.4% of the total samples. On the other hand, 22 gram-negative bacteria were available accounting for 78.57% of the total number of samples.

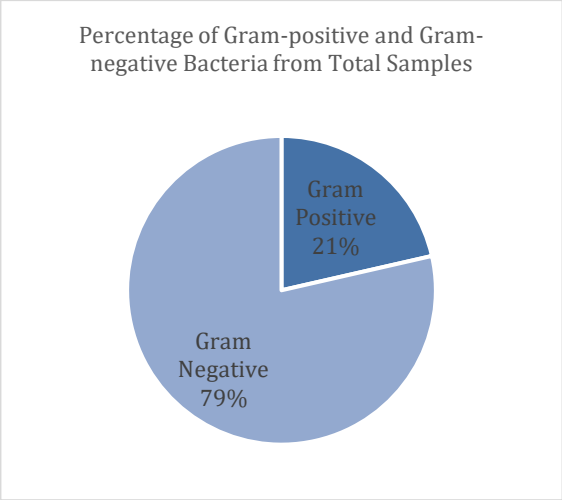

Figure 2: Distribution of Gram-positive and Gram-negative Bacteria Samples According to Percentage

The collected bacteria were diverse including Staphylococcus aureus, E. coli, Klebsiella, Proteus, Streptococcus and Klebsiella. Some of these samples are shown in the figure 3 below.

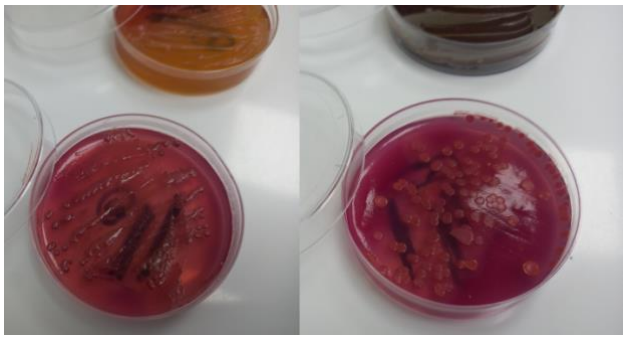

Figure 3: Examples of the Collected Bacteria Samples

5.2. Propolis Agar Preparation

In order to investigate the effect of propolis on the growth of several species of bacteria, a nutritional medium that permits the growth of these bacteria must be chosen so that there wouldn't be any need to change the medium type for each of the corresponding bacteria. For this reason, Mueller Hinton Agar (MHA) was selected. MHA is a non-selective and non-differential medium that allows the growth of a very wide range of bacteria and is often used for antibiotic susceptibility testing. Both of these characteristics make MHA a suitable medium for this study since the study includes different bacterial species and aims to determine the anti-bacterial effect of propolis. Therefore, MHA was used in the "control petri-dishes" for all the collected samples. On the other hand, propolis samples found in Lebanese market were in the form of a liquid state means that propolis dissolved in appropriate solvent. The liquid propolis found in the market were used in the "testing petri-dishes", varying volumes of Propolis were added and infused in the MHA agar medium to find out how different concentrations of Propolis might affect the bacterial growth. Rather than using agar diffusion method that is used by researchers such as (Balouiri et al., 2016; Stepanović et al., 2003; Alhassan Sa-eed et al., 2023) so that the bacteria diffused in the agar and the propolis tested by disk diffusion method. However, in this methods propolis diffused in the agar with different concentrations prepared as follows:

1) 0 mL of Propolis and 400 mL of MHA agar were added into petri-dishes as "Control"
2) 20 mL of Propolis were added to 380 mL of MHA agar, making a total of 400 mL, added to petri-dishes as "5% Propolis".
3) 40 mL of Propolis were added to 360 mL of MHA agar, making a total of 400 mL, added to petri-dishes as "10% Propolis".

5.3. Plating Samples on Petri-dish

It is critical in this study to maintain the same concentration of the bacteria sample between the three different petri-dishes. This is essential to ensure that the initial quantity of streaked bacteria doesn't affect the outcome in the 3 petri-dishes after 24 hours. The method used to plate the bacteria on different agar plates (one control, and the others contain propolis) is shown in the sketched diagram below:

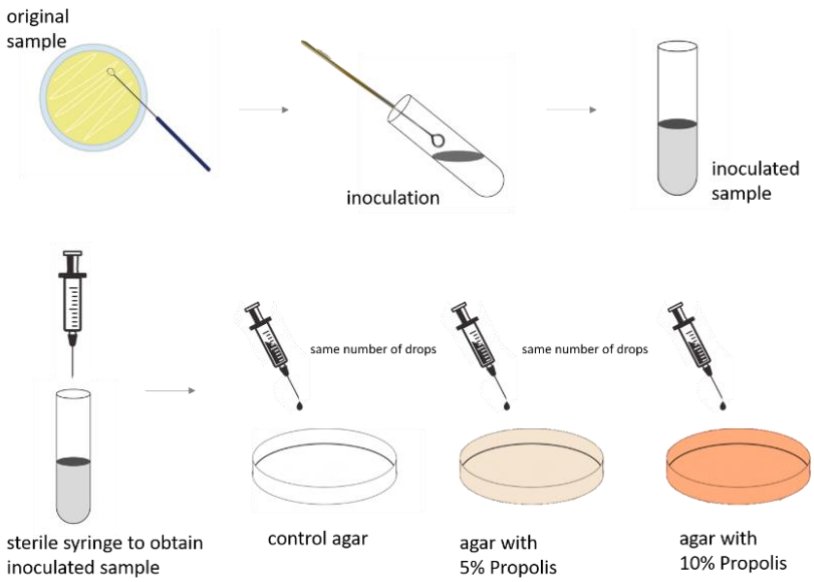

Figure 4: Procedure of Plating the Bacterial Samples in Petri-dishes

Figure 4 explains the detailed process by which the three different petri-dishes were streaked with bacteria. For each of the samples, an inoculum was transferred from the original sample into a sterile test tube with saline water to obtain a homogeneous bacterial suspension. Then, a sterile syringe was used to collect a volume of the prepared suspension and to transfer a limited number of drops into the control agar, the agar with 20 mL Propolis, and the agar with 40 mL Propolis, keeping in mind that the same number of drops is put into each petri-dish. After that, a sterile swab is used to uniformly distribute the drops of the bacterial suspension over the entirety of the petri-dish, and the process is repeated in each petri-dish separately to avoid contamination.
The process was performed for each of the twenty eight samples individually. Finally, the plated petri-dishes were incubated at 37°C for 24 hours.

5.4. Gram Staining

The results are viewed after 24 hours of incubation to determine the presence or absence of bacterial growth in each of the petri-dishes. Visual observation was done initially, followed by gram staining to confirm the observed results. Gram staining procedure took place according to the commonly accepted method starting with staining with Crystal Violet, followed by Iodine, Acetone Ethanol, and finally washing with Safranin. After staining, the samples were observed under the microscope.

6. Results

The main objective of this study was to investigate whether Propolis, a naturally produced substance, has an actual antimicrobial effect, and more specifically an antibacterial effect. For this reason, a control petri-dish, a 5% Propolis petri-dish and a 10% Propolis petri-dish were used for each of the tested bacterial samples. In order to identify the effect of Propolis on the different bacterial species, the bacterial growth was monitored visually and by gram staining for confirmation.

6.1. Growth Identification (visual and staining)

The following table illustrates the type of collected bacterial samples alongside their classification as gram-negative or gram-positive and their respective results in the three different mediums: control, 5% propolis and 10% propolis.

Table 1: The Collected Bacteria Species with Their Respective Results on the Control Agar And Propolis Containing Agar

Bacteria	*Gram + /Gram -*	*Control Agar*	*Agar with 5% Propolis*	*Agar with 10% Propolis*
Klebsiella	gram –ve	Growth	Growth	Growth
Pseudomona	gram –ve	Growth	Growth	Growth
Klebsiella	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
S. aureus	gram +ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	growth
Streptococcus	gram +ve	Growth	Growth	NO growth
S. aureus	gram +ve	Growth	Growth	NO growth
S. aureus	gram +ve	Growth	Growth	growth
S. aureus	gram +ve	Growth	NO growth	NO growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	NO growth
E. Coli	gram –ve	Growth	Growth	Growth
Klebsiella	gram –ve	Growth	Growth	Growth
Proteus	gram –ve	Growth	Growth	Growth
Proteus	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	NO growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
S. aureus	gram +ve	Growth	Growth	Growth
Klebsiella	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth
E. Coli	gram –ve	Growth	Growth	Growth

The table shows that all the samples presented growth in the control agar, as expected. However, only 1 bacteria sample presented no growth in the agar with 5% Propolis (S. aureus), and 5 samples presented no growth in the 10% Propolis agar. If the 10% Propolis results were taken into consideration, then 17.85% of the total bacterial samples were affected by Propolis to the extent of complete absence of growth.

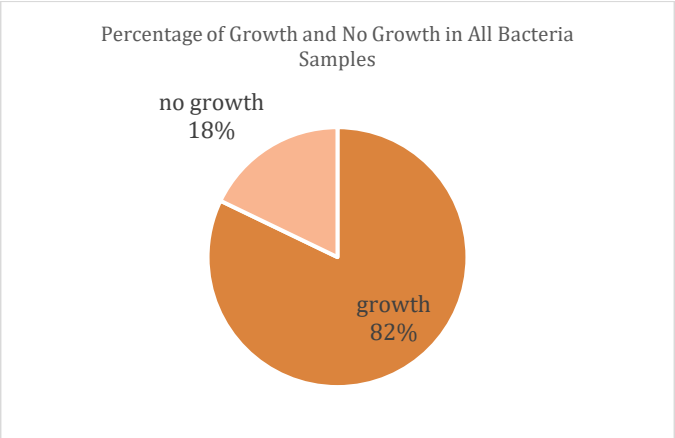

Figure 5: Percentage of Growth and NO Growth in All Bacterial Samples

Additionally, it can be seen from table 2 that three gram-positive bacteria (S. aureus) showed no growth in 10% Propolis, and two gram-negative bacteria (E. coli) showed no growth in 10% Propolis. In terms of percentages, this means that 50% of the gram-positive bacteria were negatively affected by Propolis, while only 9.09% of the gram-negative bacteria were affected by Propolis.

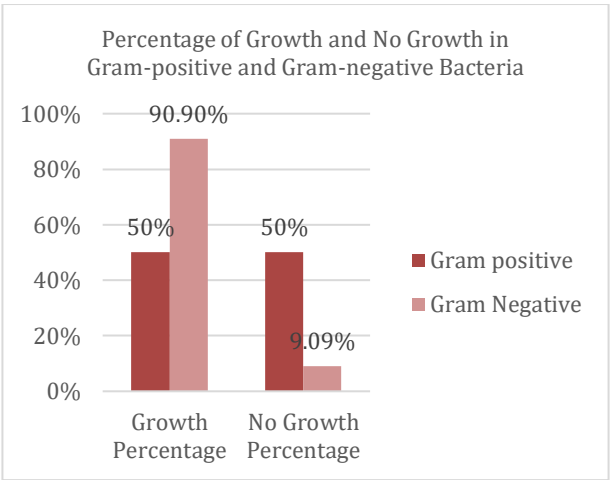

Figure 6: Percentage of Growth and No Growth in Gram-positive and Gram-negative Bacteria

Upon staining, gram-positive bacteria exhibit a purple color and a shape respective to the tested bacteria sample. For instance, grape like purple formation indicate S. aureus. On the other hand, a gram-negative bacteria display a pinkish color. For instance, E. coli appears as pink rod-shaped formations. Figure 7 shows some of the gram staining results at 5% Propolis through which two negative staining results and one positive staining result are shown.

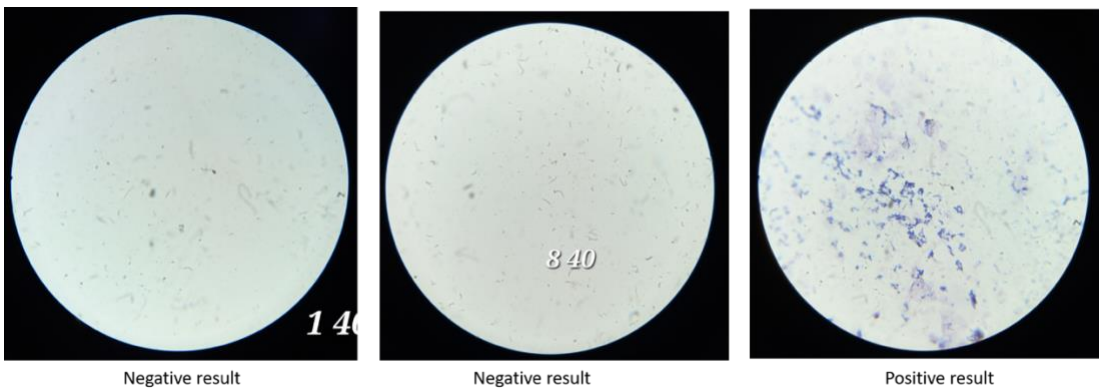

Figure 7: Gram Staining Results of some Bacteria Samples

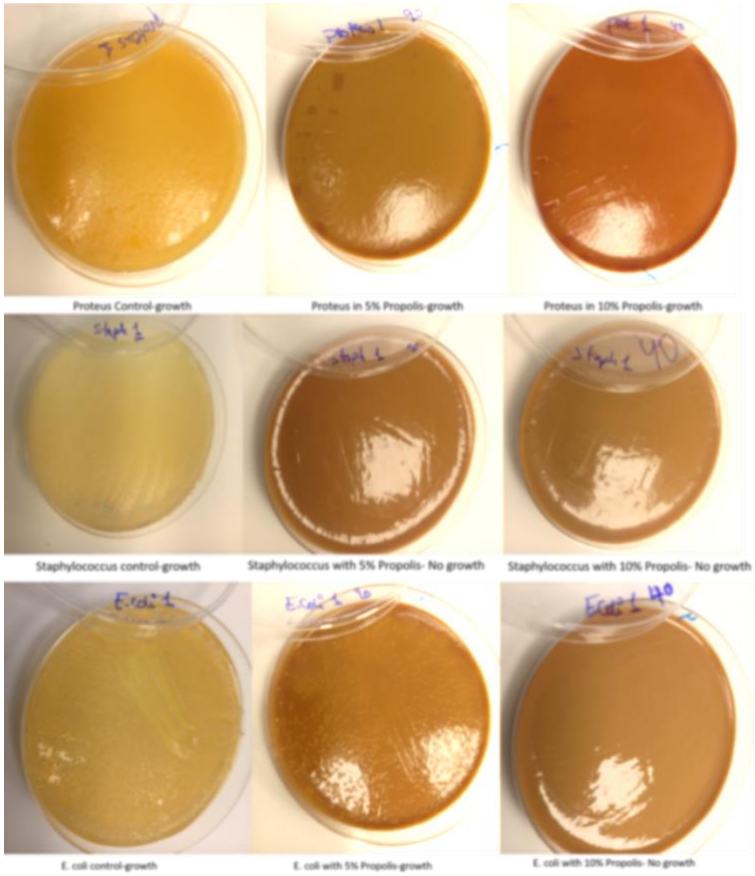

Figure 8: Examples of Growth Results for 3 Different Bacteria Species

From Figure 8, three different bacterial samples are presented where each sample illustrates the results in the control agar, the agar with 5% Propolis, and the agar with 10% Propolis. More specifically, the Proteus sample shows growth in all of the three cases: control agar, agar with 5% Propolis, and the agar with 10% Propolis. On the other hand, the staphylococcus sample showed growth only in the control agar and no growth in neither of the other cases. As for the E. coli sample, it showed growth in both the control agar and the agar with 5% Propolis whereas it showed no growth in the agar with 10% Propolis. These visual results were of course validated through the gram staining procedure.

6.2. Anti-fungal effect of Propolis

After leaving the plates for several days, fungi appear on the controlled plates while no appearance for any type on the plates containing Propolis as shown in Figure 9.

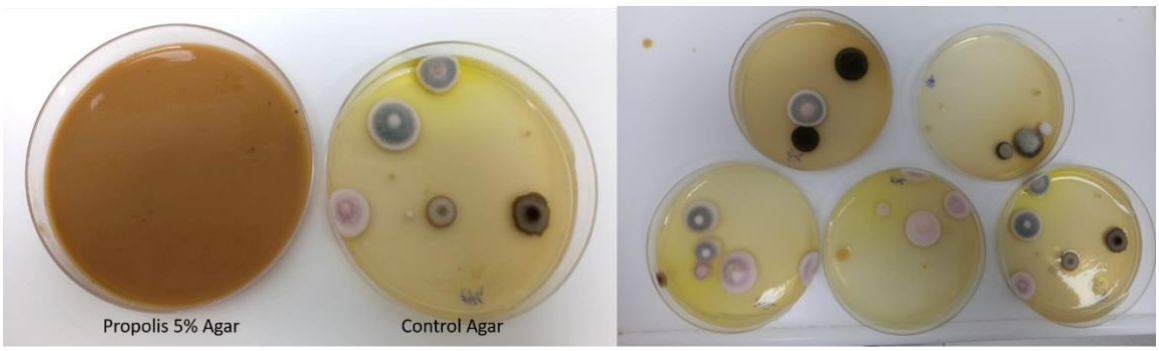

Figure 9: Anti-fungal effect of Propolis

7. Discussion of Results

The results obtained from the collected data in this study show that first of all, once randomly collected from a hospital or a medical center, the percentage of gram-negative bacteria (79%) exceeds the percentage of gram-positive bacteria (21%) highlighting the predominance of gram-negative bacteria in Lebanese patients. Furthermore, 53% (15 samples) of the total collected bacterial species in this study were E.coli. These results are in agreement with several previous studies that illustrated the predominance of gram-negative bacteria in Lebanon. For instance, in their published research, Moustapha Khodor et al., (2022) demonstrated that when monitoring the bacterial co-infection with covid-19 virus in the gram-negative bacteria accounted for 61.7% of the co-infections, whereas gram-positive bacteria accounted for 23.4% only. Among the gram-negative bacteria, E. coli was predominant (25.5%). Their study was conducted in Tripoli, Lebanon. Similarly, Kamal Chamoun et al. (2016) performed a nationwide investigation and concluded that from 16 hospitals, the percentage of collected gram-positive bacteria was 27.1% compared to 72.8% of gram-negative bacteria among which 54.7% was E. coli. In another study, the percentage of E. coli from urinary tract infection patients was 72% compared to only 2% UTI caused by staphylococcus aureus (Hamzé, M. et al., 2017).

As for the experimental results related to the antibacterial effects of Propolis, it was evident that Propolis had a greater effect on the gram-positive species, where it inhibited the growth of 50% of the total gram-positive bacteria. On the other hand, Propolis was able to inhibit the growth of gram-negative bacteria by 9.09%. These results were achieved by combining MHA agar with 10% Propolis preparation. Thus, the results confirm that Propolis is more efficient in inhibiting the growth of gram-positive bacteria than gram-negative bacteria. Studies such as (Przybyłek, I., & Karpiński, T. M. 2019) which tested the Propolis extract on a total of 600 aerobic and anaerobic bacteria showed with minimum inhibition concentration evidence that Propolis has a greater inhibitory effect on gram-positive bacteria. Similarly, Bratko et al. (2020) and Almuhayawi (2020) and Silva-Carvalho et al. (2015) confirmed the gram-positive bacteria susceptibility to Prolis, also confirmed the great inhibitory effect of Propolis on fungi, since 5% Propolis was able to prevent the natural growth of fungal contaminants in comparison to the control agar. These results confirmed the general knowledge of the anti-fungal effect of Propolis (Mutlu Sariguzel, F., 2016).

8. Conclusion

Propolis is a natural substance produced from honeybees after being collected from plant sources such as tree buds, sap flows, and botanical exudates. Even though bees use it for practical reasons such as sealing the gaps in their hives, human have used it as a healthy product for centuries after discovering its potential in preventing diseases. Numerous studies have been conducted to investigate the effect of Propolis against different strains and types of bacteria, and recently against antibiotic resistant bacteria as well. These studies concluded that Propolis has an antibacterial effect as well as an anti-fungal and antiviral effect.

In this study, the effect of Propolis was investigated on bacterial samples collected from south Lebanon. The samples included a number of gram-positive bacteria (21%) and gram-negative bacteria (79%). Each of the collected samples was incubated in a control Mueller Hinton Agar, an MHA agar with 5% Propolis preparation, and MHA with 10% Propolis. After a 24-hour incubation, the bacterial growth was investigated visually and confirmed by gram staining. As a result, the 10% Propolis preparation was able to inhibit the growth of 50% of the gram-positive bacteria and 9% of the gram-negative bacteria.

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Conflict of Interest: The authors declare no conflict of interest.

Informed Consent Statement/Ethics Approval: Not applicable.

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