

Propolis Extract: A Possible Antiseptic Oral Care against Multidrug-Resistant Non-Fermenting Bacteria Isolated from Non-Ventilator Hospital-Acquired Pneumonia

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Abstract

Non-ventilator Hospital-acquired Pneumonia (NV-HAP) is a significant burden in acute care hospitals and poses a risk to nonelderly, non-intensive care unit (ICU) patients, which have been increasing worldwide. In addition, poor oral hygiene has been associated to significant increases in the number of cases of NV-HAP. Unfortunately, preventive options are limited. Thus, there is a need for oral antiseptics, similar to those of natural products or plant sources. The aim of this study was to assess the antibacterial activity of various bee products (BPs); for example, honey, propolis, and bee venom against multidrug-resistant (MDR) non-fermenting bacteria (e.g., *Pseudomonas* and *Acinetobacter*), which were collected from NV-HAP patients to investigate its use as a possible antiseptic oral care. Bacterial susceptibility to different antibiotics were performed. The antimicrobial activity of BPs against non-fermenting bacteria, the minimum inhibitory concentration (MIC), and the minimum bactericidal concentration (MBC) were assessed. Eighteen *Pseudomonas aeruginosa* isolates and five *Acinetobacter baumannii* isolates were identified. *P. aeruginosa* isolates displayed high resistance to the antibiotics: meropenem and imipenem (55.6% and 77.8% respectively), whereas *A. baumannii* isolates were 100% resistant to meropenem and imipenem. All isolates remained sensitive to colistin. Propolis showed the best antibacterial activity ($p < 0.001$) in comparison to honey and bee venom against *P. aeruginosa* (13 - 36 mm, MIC = 1.4-22.5%, and MBC = 2.8-45%) and *A. baumannii* (7-20 mm, MIC = 5.6-22.5%, and MBC = 11.3 - 22.5%). While bee venom expressed the least antibacterial activity against all isolates with a zone diameter ranging from 0-12 mm, propolis, which is a non-toxic, natural, and inexpensive, had antibacterial activity towards the MDR bacteria: *P. aeruginosa* and *A. baumannii* collected from pneumonic patients. Additionally, we confirmed that propolis could be used as a potential antiseptic oral care product.

Keywords: Antimicrobial activity, Propolis extract, Pneumonia, *Pseudomonas aeruginosa*

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(Received: August 12, 2019; accepted: November 30, 2019)

Citation: Sylvana N. Gaber, Eman Elsayed Mahmoud Hemedat, Hebat -Allah Sayed Elsayeh, Wafaa Y. Abdel Wahed, Mahmoud A.F. Khalil and Enas G. Ibrahim, Propolis Extract: A Possible Antiseptic Oral Care against Multidrug-Resistant Non-Fermenting Bacteria Isolated from Non-Ventilator Hospital-Acquired Pneumonia, *J. Pure Appl. Microbiol.*, 2020; **14**(1):123-131. <https://doi.org/10.22207/JPAM.14.1.13>

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INTRODUCTION

Non-ventilator Hospital-acquired Pneumonia (NV-HAP) is one of the most common hospital-acquired infections (HAIs). Even though it conveys similar mortality rates as Ventilator-Associated Pneumonia (VAP), its incidence rates are higher; thus, the accompanying charges and deaths could be more considerable¹.

Three fundamental factors increase a patient's susceptibility to HAP: alterations in oral microorganisms (virulent gram-negative organisms), microaspiration (tiny droplets being aspirated), and a debilitated host. These factors generate a perfect environment for microorganisms to invade the respiratory area. In addition, neglected nursing care increased the likelihood of contracting NV-HAP triggered by an enlarged load of microbes². Fortunately, one's risk in contracting pneumonia can be reduced by simple care procedures; for example, evaluating the patient's aspiration hazard, regular mobilization, and uplifting the head off the bed. Additionally, the decontamination of the respiratory tract by topical antiseptic agents is an applicant protective interference³.

Non-fermenting Gram-negative (GN) species, particularly *P. aeruginosa* and *A. baumannii* have long been documented as common and recurrent pathogens that cause HAP. It has been observed that *P. aeruginosa* is the second leading cause of HAP, right behind *Staphylococcus aureus*, which may account for 15%-20% of patients infected with HAP⁴. Additionally, *A. baumannii* has developed a substantial presence for HAP in intensive care units⁵. *A. baumannii* has been associated with a high mortality rate, which is approximately 35% to 70% of cases.⁶

Currently, clinicians are faced with bacterial strains, such as *P. aeruginosa* and *Acinetobacter* species that are resistant to the majority of antibiotics, resulting in HAIs, with only a few available options⁷. Therefore, the development of new, naturally complementary, and alternative medicinal products against MDR GN pathogens is of the utmost importance in the field of HAIs⁸.

Bees are insects belonging to the genus *Apis*. Bees are nature's developers and accumulators of honey, which is their primary

product. However, bees also produce beeswax, royal jelly, bee venom, and propolis, which are derived from the excretion glands of the honeybee. It is worth mentioning that these products have been used for medicinal purposes for millennia⁹.

Honey comprises approximately 200 cellular molecules, such as, vitamins, amino acids, enzymes, and minerals. However, honey is primarily composed of water and sugar (i.e., glucose and fructose). The antibacterial agents in honey provide inhibitory properties to Gram positive (GP) and GN bacteria. Honey has low mean pH of 4.4, which is lethal to microorganisms. Even when honey is heavily diluted, the hydrogen peroxide activity of honey can still affect microbes by inhibiting their growth¹⁰.

Propolis is a resin-like material made by bees, produced through mixing beeswax and secretions of salivary glands with exudate collected from plants. Propolis is sometimes referred to as "bee penicillin"¹¹. A beehive is a very sterile environment found in nature. Propolis not only acts as glue but is also used to clean and disinfect the honeycombs. Ancient Egyptians used it for preserving bodies during mummification. Recently, Egyptian propolis has become a topic of interest and is receiving increasing attention from chemists and biologists¹².

Propolis is indicated in treating tuberculosis and upper respiratory tract infections, Human immunodeficiency Virus, H1N1 "swine" flu, and the common cold. Propolis contains phenolic substances, including flavonoids, with antimicrobial potential¹³.

Bee venom (BV) called apitoxin includes a combination of active enzymes, peptides, and amines. It is a colorless liquid with a pH of 5.0-5.5¹⁴. The antibacterial effect of BV has been recognized to be effective against *Salmonella* spp, *Escherichia coli*, *Citrobacter freundii*, *Enterobacter cloacae*, Coagulase-Negative *Staphylococcus*, and *S. aureus*¹⁰.

This study sought to assess the antibacterial effects of Egyptian Bee products (BPs): honey, propolis, and BV against MDR non-fermenting bacterial isolates collected from NV-HAP patients as a possible antiseptic oral care.

MATERIAL AND METHODS

Preparation of Bee products (BPs)

The study was carried out in El- Zawia village at EL-FAYOUM Governorate, a city near middle Egypt. Ten colonies were used for honey, propolis, and bee venom collection. Honeycombs were taken from these colonies where they were weighed, followed by honey-extraction. A stock honey solution of 50% (w/v) was prepared by liquefying 25 g of honey in 50 mL Muller-Hinton broth (Thermo Fisher Scientific Remel product, Lenexa, KS, USA) in a 50 mL flask. The solution was diluted for further analysis. Concentrations of honey were expressed as weight/volume percent (w/v %). Propolis was gathered by collecting the hive scrapings while washing the honey supers throughout the honey harvest. The scrapings were covered with 5.08 to 7.62 cm of water and placed in an oven at 93.3°C. The contents of the container were heated for approximately 2 h and were then moved. The liquefied wax is attached to pieces of wood, where it hangs to the surface of the water while the propolis attaches to the container bottom. After the container is detached from the oven and well-ventilated, the waxy layer on the surface of the water is detached, and the water is then cautiously poured off to reveal the colored propolis bulk underneath it. The cleaned propolis pieces are spread out on a piece of paper to dry before being stored. In total, 100 mg of the propolis powder sample was homogenized with 10 ml of 70% alcohol for 30 min. The alcoholic extract was separated by centrifugation at 6000-7000 rpm followed by evaporation. The electric shock method with the bee venom collector device was employed to gather the bee venom. The device was put vertically between the hive combs. Next, the collector device was used for 15 min/colony/week. The deposited venom between the two glass sheets was dried, then scraped off by a razor blade. Finally, the bee venom was collected and placed into dark bottles in a powder form¹⁴.

Next, the physicochemical properties for honey, bee venom, and propolis were determined.

Collecting bacterial isolates

From November 2017 to May 2018, twenty-three bacterial isolates were collected from the sputum, pleural fluid, and bronchial alveolar lavage of non-ventilator HAP patients who were admitted to the Fayoum University

Hospitals in Fayoum, Egypt. The following criteria emphasized the definition and characteristics of pneumonia: (1) The presence of at least one of the following: hyperpyrexia, increased or decreased leukocyte levels, altered mental status for adults ≥ 70 years old. (2) Recent onset of purulent sputum, worsening cough, tachypnea or dyspnea, worsening gas exchange, or bronchial breath sounds. (3) Chest radiographs with one of the following: persistent infiltrate, recent or progressive consolidation, or cavitation. HAP was defined as pneumonia occurring within 48 h or more after hospitalization in non-ventilated cases¹⁵. The samples were transported within 2 h to the Fayoum University Hospitals laboratories and were cultured on Macconkey agar plates (Oxoid Ltd., Basingstoke, England). Isolated bacteria were identified according to the standard microbiological methods¹⁶. For bronchial alveolar lavage: potential pathogens present at $\geq 1 \times 10^4$ colony-forming unit/ml (CFU/ml) were considered significant. Any GN rods were additionally recognized by the Microbact™ GNB 12A kit (Oxoid Ltd., Basingstoke, England) and aerobically incubated at 37°C for 24 h. Testing of antibiotic susceptibility of the isolated colonies was performed according to the Clinical Laboratory Standards Institute (CLSI) guidelines¹⁷, by the disc diffusion method. The following antibiotics were tested: ampicillin (10 µg), gentamicin (30 µg), amoxicillin/clavulanic acid (30 µg), aztreonam (30 µg), piperacillin/tazobactam (100/10 µg), tetracycline (30 µg), meropenem (10 µg), imipenem (10 µg), cefoperazone (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), cefepime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (25 µg), tigecycline (15 µg), polymixin (300 U), and fosfomycin (50 µg). Additionally, MIC for colistin was performed. MDR-*P. aeruginosa* is defined as a strain resistant to one agent, in three or more antipseudomonal antimicrobial classes. *P. aeruginosa* ATCC 27853 was used as the control strain¹⁸.

Antimicrobial activity of bee products by disc diffusion test

The isolates were transported in a brain-heart infusion broth media to the research laboratory of Medical Microbiology and Immunology Department at the Fayoum University (Faculty of Medicine). For the detection

of antibacterial activities of the BPs, the agar disc diffusion method was performed. The suspension of the tested bacteria (10^6 CFU/ μ l) was spread on Mueller Hinton agar (Oxoid Ltd., Basingstoke, England). Then, bacterial strains were incubated for 20 min at 37°C. The agar was cut into discs of 5 mm in diameter, and wells were filled with 100 μ l containing 10%, 20%, 50%, and 90% concentrations of BPs. They were incubated at 37°C for 24 h. The inhibition zone diameters were detected in millimeters. The negative control was performed using distilled water. From triplicate measurements, the results were displayed as mean values³⁴.

Minimum inhibitory concentration determination (MIC) for Honey and Propolis

The Minimum Inhibitory Concentrations (MICs) of honey and propolis were assessed by a microdilution technique as both honey and propolis showing the highest antimicrobial activity through the agar disc diffusion method. The tests were performed in 96-well plates (Corning® Costar® Ltd., Corning, NY, USA) according to the CLSI guidelines¹⁹. Given that no diameter inhibitions were found at concentrations of 10%, 20%, and 50% of BPs, a volume of 100 μ l of 90% BPs concentration (w/v) was distributed in wells. Positive and negative controls (broth) were tested. Subsequently, wells were seeded with an aliquot of 10 μ l of approximately 10^6 CFU/mL of overnight bacterial culture and incubated for 24 h aerobically at 37 °C. The concentrations of BPs obtained in the well were between 45% and 0.01%.

To determine the minimum bactericidal concentration determination (MBC) of honey and propolis, 10 μ l of content from the MIC wells were spread out without visible turbidity onto Mueller Hinton agar and incubating (aerobically) as required for evaluation. Positive and negative control wells were included. MBCs were determined on three separate occasions³⁵.

Ethical considerations

This study was approved by the Faculty of Medicine Research Ethical Committee and Fayoum University Hospitals director. The study was conducted after explaining the study's aims.

Statistical analysis

Using Statistical Package for Social Science (SPSS version 16, Chicago IL, USA), data were collected, coded, and analyzed. Data were

quantified by calculating the means, median, standard deviation (SD), and interquartile range (IQR) for numerical data. Frequencies and percent were completed for qualitative data. A comparison of quantitative variables was made using the Mann-Whitney U test between two groups and Kruskal-Wallis H tests (i.e., one-way ANOVA on ranks) for more than two groups. $P \leq 0.05$ was considered statistically significant.

RESULTS

A total of twenty-three isolates were collected from NV-HAP patients. Eighteen isolates were *P. aeruginosa*, and five isolates were *A. baumannii*.

P. aeruginosa isolates revealed a high resistance rate to meropenem and imipenem (55.6% and 77.8%, respectively). Additionally, all *P. aeruginosa* isolates were susceptible to polymyxin and colistin (Table 1). *A. baumannii* isolates were found to be resistant to all tested antibiotics except for colistin and polymyxin.

Table 1. Antibiogram of the isolated *P. aeruginosa*

Antibiotics	Resistance (%)
AMP	100
AMC	100
CTX	100
CRO	100
CAZ	100
EFP	72.2
TZP	94.4
ATM	77.8
IPM	77.8
MPE	55.6
CIP	77.8
GN	72.2
TE	100
TGC	94.4
SXT	100
C	100
FOX	88.9
PB	0
FOS	11.1

AMP: ampicillin, AMC: amoxicillin / clavulanic acid, CTX: cefotaxime, CRO: cefaprazone, CAZ: ceftazidime FEP: cefepime, TZP: piperacillin-tazobactam, ATM: aztreonam, IPM: imipenem, MEP: meropenem, CIP: ciprofloxacin, GN: gentamicin, TE: tetracycline, FOX: ceftoxitin, C: Chloramphenicol, TGC: tigecycline, SXT: sulfamethoxazole/trimethoprim, FOS: fosfomycin PB: polymyxin

Physicochemical properties of bee products

The physicochemical properties of propolis are comprised of the following flavonoids: quercetin, kaempferol, acacetin, isovanillin, pinostrobin, ramnocitrin, 5- hydroxy-7,4-dimethoxy flavone, 5,7-hydroxy -3,4- dimethoxy flavone, 3,5- dihydroxy- 4,7-dimethoxy flavone, and 5-hydroxy- 7,4-dimethoxy flavone. The physicochemical properties of honey are comprised of the following: 31% g glucose, 38.2% fructose, 7.2% maltose, 4.2% higher carbohydrates, 1.5% sucrose, 17.1% water, minerals, vitamins, and enzymes. The physicochemical properties of bee venom are summarized in Table 2.

Table 2. Composition of Bee Venom and its percentage

Class	Compound	percentage %	
1. Enzymes	Phospholipase A2	10-12%1%	
	phospholipase B		
	Hyaluronidase	1-2%	
	Phosphatase	1.0%	
	a – Glucosidase	0.6%	
	2. Proteins and peptides	Melittin	40-50%
		Apamine	2-3%
		Mast Cell	2-3%
		Degranulating peptide	0.5-2%
		Secapine	1-32%
Pamine		0.5-1%	
Minimine		1-2	
Adolapine	%0.1-0.8% 1-2%		
3. Amines	Histamine	0.5-2%	
	Dopamine	0.13-1%	
	Norepinephrine	0.1-0.7%	
4. Carbohydrates	Glucose and fructose	2%	
5. Amino acids	Amino butyric acid & Alpha-amino acids	1%	
6. Volatile ingredients	Complex Ether	4-8%	
7. Minerals	P, Ca, Mg	3-4%	

Antimicrobial activity of Bee Products

At the concentration of 90% (w/v), the inhibitory effect of BPs on bacterial growth was apparent. Significant differences in inhibition zone values, MIC, and MBC values for propolis in comparison to honey and bee venom ($p < 0.001$) were found (Table 3, 4). The best antibacterial

Table 3. Result of the Disc Diffusion method in millimeter (mm) of Bee Products against the studied isolates

	Bee Venom	Propolis	Honey	P-value
Mean	4.7mm	21.9mm	14.6mm	<0.001
SD	6.643	6.478	6.700	

Kruskal- Wallis test was done

P value ≤ 0.05 indicated a statistically significant

Table 4. Result of the Minimum inhibitory concentration % (MIC) and Minimum bactericidal concentration % (MBC) of Honey and Propolis against the studied isolates

	MIC of Propolis %	MIC of honey %	P- value
Mean	7.1	20.6	<0.001
SD	5.9	23.8	
Median (IQR)	5.6(2.8-11.3%)	11.3(5.6-22.5%)	
	MBC of Propolis %	MBC of honey %	<0.001
Mean	17.01	28.4	
SD	9.38	11.67	
Median (IQR)	22.5(11.3-22.5)	22.5(22.5->45.0)	

Mann-Whitney Utes was done

activity was observed for propolis against *P.aeruginosa* (13- 36 mm, MIC =1.4-22.5% with a mean 6.3% and MBC=2.8-45% with a mean 17%) and against *A. baumannii* (7-20 mm, MIC=5.6-22.5% with a mean 10%, and MBC=11.3 -22.5% with a mean 18%). This was followed by honey, that led to generation of a diameter zone of inhibition for *P. aeruginosa* (9–28 mm with MIC=5.6-45% with a mean 15%, and MBC=11.3->45% with a mean 26%); for *A. baumannii* the resultant diameter zone of inhibition was (0–16 mm, MIC=11.3-45% with a mean 18%, and MBC= 22.5- >45% with a mean 36%). Bee venom showed the least amount of activity against all isolates with a diameter zone of inhibition (0-12 mm with mean: 4.7 mm). MBC 50 and MBC 90 for honey and propolis are shown in Table 5.

Table 5. Result of the MIC50 MIC 90, MBC 50, and MBC 90 of Propolis and honey for the studied isolates

Bee	MIC	MIC	MBC	MBC
Products	50	90	50	90
Propolis	5.6	22.5	22.5	22.5
Honey	11.3	45	22.5	>45

Minimum inhibitory concentration (MIC)

Minimum bactericidal concentration (MBC)

DISCUSSION

Several hypotheses have explained the possible role of oral bacteria in the pathogenesis of respiratory diseases like pneumonia²⁰. *P. aeruginosa* and *A. baumannii* are important etiological factors of HAP, having a prominent ability to resist multiple categories of antibiotics²¹. Thus, research and investigation into potential antibacterials are currently of great interest. In recent times, public awareness in the therapeutic use of bee products has dramatically increased, especially since they have no reported record of adverse side effects on tissues²².

In the current work, among 18 isolates of *P. aeruginosa*, high resistance rates to meropenem and imipenem (55.6% and 77.8% respectively) were found. Additionally, five *A. baumannii* isolates were found to be resistant to all the antibiotics used in this study. These results were in agreement with the results reported by Liu *et al.* who reported the rates of resistance of *P. aeruginosa* to meropenem and imipenem (48.8% and 70.7%, respectively), and high resistance rates of *A. baumannii* to carbapenems (more than 70%) were found²³. Moreover, Chung *et al.* found *P. aeruginosa* with a resistance rate to imipenem (56.9%), and *Acinetobacter* spp. presented a high rate of resistance to imipenem (67.3%)²⁴. All isolates in our study persisted in being sensitive to colistin. However, in this report: the resistance rate to colistin was observed to be 0.8 %.

Several scientific studies showed propolis having antibacterial activity (with active constituents including flavonoids like galanin and hydroxyl-cinnamic acids)²⁵. The synergistic activity between various active constituents has been attributed as the chief reason for getting the complex antibacterial effect of propolis²⁶.

Campos *et al.* in Brazil aimed to study the biological properties and chemical composition of propolis. They reported legitimate antimicrobial activity of propolis, MIC for *P. aeruginosa* was 5.83 mg/mL, and MBC was 14.42 mg/ml²⁷. While Seidel *et al.* documented that propolis samples of European, North American, and South American origin had a MIC extending from 0.125 to >0.5 mg/mL whereas, African and Asian origin had a MIC extending from 0.08 to >0.5 mg/ml²⁸. Furthermore, Aissat *et al.* in Algeria reported propolis activity for *P. aeruginosa* was 9–19 mm, MIC in range of 17%–57%²⁹. These antimicrobial activity results have differed from our findings in which Egyptian propolis showed antibacterial activity against *P. aeruginosa* at 13- 36 mm, MIC =1.4-22.5% with a mean value of 6.3%, and MBC=2.8-45% with a mean value of 17%. Additionally, our study reported activity of propolis against *A. baumannii* to be the following: 7-20 mm, MIC=5.6-22.5% with a mean value of 10%, and MBC=11.3 -22.5% with a mean value of 18%. Hannan *et al.* in Pakistan aimed to determine the antibacterial activity of propolis for *A. baumannii* from two sites and observed that propolis from Sargodha and Lahore displayed inhibition zone diameters of 21.8 mm and 15.6 mm, respectively. MIC values of propolis from Sargodha and Lahore were from 1.5–2.0 mg/ml and 4.0–4.5 mg/ml, respectively³⁰.

Many researchers have shown that *Acinetobacter* spp exhibited either low sensitivity or total absence of sensitivity against propolis.^{12,31,32} These variations in the antibacterial activity of propolis among different studies could be due to the differences in origin.

In the present study, the antimicrobial activity of honey against MDR non-fermenter bacteria were less than propolis, which the diameter zone of inhibition of honey for *P. aeruginosa* was 9–28 mm with MIC=5.6-45% and MBC=11.3->45%, and for *A. baumannii* was 0–16 mm with MIC=11.3-45%, and MBC= 22.5->45%. Those results were higher than the results reported by Henriques *et al.* who found the values of MIC and MBC of manuka honey against *P. aeruginosa* (9.5% and 12%, respectively)³³. However, they were lower than the results revealed by Moussa *et al.* who observed the diameter zone of inhibition of honey for *P.*

aeruginosa to be 0–30 mm while the MIC ranged around 90–91%³⁴. Additionally, Al-Nahari *et al.* found that all tested types of honey employed a complete inhibition of bacterial growth at 50%³⁵. Furthermore, Wilkinson & Cavanagh concluded that 13 types of studied honey had an inhibitory effect on *P. aeruginosa*³⁶. Tirado *et al.* reported that the MIC and MBC of the Medihoney was 8.5%, and 0.5%, respectively, for *A. baumannii*³⁷. Adam also found *A. baumannii* susceptible to honey at a minimum concentration of 50% and 100 %³⁸. Contrarily, Basson & Grobler found no antimicrobial activity for honey native to South Africa³⁹. Kuś *et al.* tested 30 Polish monofloral honey-types and observed the type that had the highest antimicrobial activity was still dramatically less active against *P. aeruginosa* with values of MIC extended from 6.25 to 25%⁴⁰. It has been revealed that honey might have antibacterial action from less than 3% to 50% or higher concentrations³⁶. These results from different studies approved that honey from diverse regions and countries could have vast differences in their antibacterial activity.

Our results demonstrated little activity of bee venom against all isolates with zone diameter from 0-12 mm with mean: 4.7. Zolfagharian *et al.* agreed with our results, who evaluated the antimicrobial effect of bee venom against six GP and GN bacteria and found bee venom had no noticeable effect on *P. aeruginosa*¹⁴. Also, Fennell *et al.* reported that the bee venom is more active against GP than GN bacteria⁴¹.

The findings of our study showed that propolis had good antimicrobial activity against MDR non-fermenting bacteria, which helps to be used as a possible oral antiseptic in pneumonic patients.

CONCLUSION

Given the promising antimicrobial activity of propolis, low cost, and safety, we suggest its potential application as an oral antiseptic to reduce the NV-HAP incidence. Further investigation is warranted to confirm these promising findings, and additional studies on varied kinds of GP and GN bacteria are needed, and *in vivo* activity of propolis for the pneumonic patients would be essential for the assessment of its efficacy in pneumonia prevention.

ACKNOWLEDGMENTS

All authors acknowledge help from all paramedical personals to accomplish and finalize our research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING

The research was funded personally and by Microbiology and Immunology Department, Faculty of Medicine, Fayoum University.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

AUTHORS' CONTRIBUTION

S.N.G, Invited the idea, Share in writing, editing of the manuscript, Share in actual laboratory work. E.G.I, Share in writing, final editing of the manuscript, searched database. E.E.M, Collection of the bacterial samples, searched database. H.S.Elsayeh, Collection of Bee product samples, searched database. W.Y.A.W, Statistical analysis, Share in final English language editing. M.A.F.K, Share in actual laboratory work, Share in final English language editing.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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