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Research Article

In-vitro antimicrobial and antibiofilm activities of honey from *Apis cerana* and *Apis dorsata* against selected microbial strains of wound pathogens

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Abstract

Introduction: Microbial biofilms play a main role in chronic wounds. Due to the broad-spectrum antimicrobial activity of honey, it has been revisited as an alternative treatment option for chronic wound infections. In this study, we aimed to evaluate the anti-bacterial, anti-candidal, and anti-biofilm effects of natural Sri Lankan *Apis cerana,* and *Apis dorsata* honey against selected bacterial, and *Candida* species. Methods: The physicochemical properties of both honey types were assessed. The antimicrobial efficacy was tested against reference strains of *Staphylococcus aureus* (including MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, and *Candida tropicalis*. The agar well diffusion method and Minimum Inhibitory Concentration (MIC) assays were utilized to evaluate antimicrobial activity. Anti-biofilm activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The ultrastructure of biofilms following treatment with *A. cerana* honey was visualized via Scanning Electron Microscopy (SEM). Results: The physicochemical properties of *A. cerana* and *A. dorsata* honey types were comparable. Both honey types demonstrated antibacterial activity, while anticandidal activity was observed only with *A. cerana* honey. Both honey types effectively inhibited established biofilms, with *A. cerana* honey specifically inhibiting initial biofilm adhesion and development. SEM images revealed cell shrinkage in mature sessile cells following treatment with *A. cerana* honey. Conclusion: Sri Lankan *A. cerana,* and *A. dorsata* honey types exhibit potent antimicrobial and anti-biofilm activities, highlighting their potential as wound-cleansing agents for chronic wound management.

Keywords: Honey, Antimicrobial, Biofilm, Physicochemical

Introduction

Natural honey is a viscous solution of a supersaturated complex mixture of various sugars including glucose, sucrose, and fructose [1]. Other than sugars, natural honey contains water (approximately 20%) as the major component [2]. It also contains proteins, amino acids, lipids, various enzymes (amylase, glucose oxidase, catalase, and phosphatases etc.), organic and

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phenolic acids (succinic acid, malic acid, citric acid, formic acid gluconic acid, acetic acid etc.), vitamins and minerals (ascorbic acid, pyridoxine, niacin, sodium, calcium and potassium etc.) as minor components [2]. The chemical composition of honey varies depending on the bee species, floral sources, environmental factors, and geographical sources [3]. In rural and urban Sri Lankan landscapes, there are four well-known hive-nesting honey bee species belonging to the family *Apidae* [4,5]. They are *Apis cerana* (the domesticated Asian honeybee), *Apis dorsata* (the feral giant honeybee), *Apis florea* (the feral dwarf honeybee), and *Trigona iridipennis* (the stingless bee) [4].

Bee honey can be considered as one of nature's excellent products that has a wide range of beneficial uses including food sweetening, and meat and fruit preservation [6]. Natural honey produced by bees possesses a number of medicinal properties and is used in traditional and modern medicine to treat various conditions including eye diseases, rheumatoid arthritis, hepatitis, obesity, diabetes mellitus, constipation, tuberculosis, diarrhea, and fatigue [7].

Chronic wounds and related complications are emerging global health problems, which adversely affect the length of hospitalization, treatment efficacy, and quality of life of patients and contribute significantly to economic burden [8]. Biofilms play a major role in these chronic wounds as they lead to delayed wound healing [9]. A biofilm is defined as an aggregate (usually polymicrobial) of microorganisms that are irreversibly attached to a surface or associated with interfaces, and it is often encased in an outer polymer layer [10]. Biofilm formation is a complex process that involves different developmental stages including initial attachment, irreversible secondary attachment, biofilm maturation, and dispersion of peripheral microbial cells to distal sites [10]. Microbial biofilms demonstrate significant resistance to antimicrobial therapeutics and external physical or chemical stresses which ultimately cause difficulty in treating or controlling biofilm infections in healthcare settings [11]. Further, antimicrobial resistance (AMR) has become a challenging situation and a critical public health problem [12]. Therefore, alternative or complementary antimicrobial strategies should be discovered in order to combat resistant microbial pathogens [13]. In this case, scientists have focused their studies on discovering natural antimicrobial agents that possess minimal toxicity to the human host, wide availability, cost efficacy, and high therapeutic efficacy, as well as antibiofilm activity, especially in the initial stages of biofilm development (adhesion and microcolony formation) rather than combating mature, matrix enclosed biofilms, since the early stages are more susceptible to therapeutic agents [14].

Since ancient times, bee honey has been used for therapeutic purposes, especially as a topical treatment of chronic infected wounds [15]. Recent studies have also focused on the antimicrobial effect of bee honey in order to use it in treatments [16]. Several countries have conducted studies to evaluate the antimicrobial properties of different types of honey produced by their endemic bees [17,18]. Even though these studies have identified the antimicrobial properties of bee honey against various microbial pathogens, the antimicrobial, and anti-biofilm activities of Sri Lankan *Apis cerana* (*A. cerana),* and *Apis dorsata (A. dorsata)* honey types have not been widely studied. Therefore, we aimed to fill this gap by investigating the antimicrobial and anti-biofilm activity of Sri Lankan bee honey (*A. cerana* honey, and *A. dorsata* honey) against selected bacterial and *Candida* species (*Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans,* and *Candida tropicalis*) at different biofilm developmental

stages using a series of standard *in-vitro* laboratory experiments.

Methods

Study design

The study was a laboratory based experimental study.

Study setting

The study was conducted at the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. The composition analysis was performed at the Department of Food Science and Technology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka. Scanning electron microscopy was performed at the Department of Material Science and Engineering, University of Moratuwa, Sri Lanka.

Collection of honey samples

Natural *A. cerana* (Asian Honey Bee), and *A. dorsata* (Giant Asian Honey Bee) honey were purchased from local vendors. The investigators directly observed the honey extraction process from the honeycombs to ensure its authenticity and quality. *A. cerana* honey was purchased from Muwankande area, Kurunagala District which belongs to the intermediate zone in Sri Lanka (7°24'13.3" N 80°25'30.2" E). *A. dorsata* honey was obtained from Mahiyanganaya, Badulla District which belongs to dry zone in Sri Lanka (7°19'51.1" N 80° 59'38.3" E). Honey samples were collected into sterile containers. Collected honey samples were aliquoted into sterile universal bottles. The aliquoted bottles were covered with aluminum foils separately and stored at 4 °C until use.

Chemical and physical analysis of honey

Density: The density of the honey samples was determined by measuring their mass and volume. The mass of the honey was recorded using a calibrated electronic analytical balance (RADWAG AS 220.R22 Plus, Poland). The density of each sample was calculated using the formula:

$$Density\ (mg/mL) = \frac{Mass\ (mg)}{Volume\ (mL)}$$

The procedure was performed in triplicate for both honey samples, and the mean density value was recorded for each sample.

Refractive index: The refractive index of *Apis dorsata* honey and *Apis cerana* honey was determined using a hand refractometer (OPTIKA HR-150N, Italy) based on the principle of total light refraction. The refractometer operates within a 0 to 80 Brix percentage scale, which measures the grams of sugar in an aqueous medium and assesses the sample's purity.

pH: This was measured by the benchtop pH meter (SPER Scientific LTD., Scottsdale, Arizona, USA) with a minimum deviation of 0.01.

Moisture content: The moisture content of the honey samples was determined using a moisture analyzer (Shimadzu MOC63u, Japan). The samples were heated to 105°C and maintained at this temperature for 25 minutes. The analyzer continuously monitored the weight loss until a constant weight was achieved, indicating no further moisture evaporation. The difference between the initial and final weights was recorded and was used to calculate the moisture content. All measurements were performed in triplicate to ensure accuracy [19].

Ash content: This was determined using the muffle furnace method as described by El Sohaimy, Masry, and Shehata (2015). A known amount of honey sample was accurately weighed (to 0.0001 g) using an analytical balance (RADWAG AS 220.R2 Plus, Poland) into a clean, dry, and pre-weighed porcelain dish. The sample was initially ignited slowly over a Bunsen flame until no visible fumes were emitted. The dish was then placed in a

muffle furnace and incinerated at 550°C for 5 hours until all black carbon particles were eliminated. After incineration, the dish was carefully removed, cooled in a desiccator, and reweighed. The process was repeated until a constant weight was achieved, ensuring complete combustion of organic matter. The ash content was calculated using the following formula: [20].

$$ASH\% = \frac{Weight\ of\ ash\ (g)}{Weight\ of\ sample\ (g)}\ X\ 100\%$$

Total phenolic content (TPC): The total phenolic content (TPC) of honey samples was determined using the Folin-Ciocalteu method, expressed relative to a gallic acid standard curve [21] Honey samples were diluted to 10% (w/v) with methanol (AR grade, India). An aliquot of 200 µL was mixed with 1 mL of 10% (v/v) 2N Folin-Ciocalteu reagent (Loba Chemie, India), covered with aluminum foil, and incubated for 3 minutes. Subsequently, 800 µL of 7.5% (w/v) sodium carbonate (Sigma-Aldrich, Germany) was added, stirred for 30 seconds, and kept in the dark for 120 minutes. Absorbance was measured at 760 nm using a UV-visible spectrophotometer (Shimadzu UV-1900i, Japan), with methanol as a blank. All measurements were performed in triplicate to ensure accuracy [21].

Radical scavenging activity (RSA): This was determined using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. For extraction, 2 g of the sample was mixed with 10 mL of methanol. A 0.1 mM DPPH solution was prepared in methanol, protected from light using aluminum foil, and diluted until the absorbance ranged between 0.700–1.000 at 517 nm. An aliquot of 400 µL of the sample extract was mixed with 3.6 mL of the DPPH solution and incubated in the dark for 60 minutes. The absorbance was then measured at 517 nm, and the RSA was expressed as the percentage of DPPH discoloration [21,22].

Reducing sugars: Reducing sugar content was determined using the Lane and Eynon titration method [23]. Fehling's working solution was prepared by mixing equal volumes of Fehling's solutions A and B (Techno Pharmchem, India) and standardized with a glucose solution of anhydrous D-glucose. For sample analysis, 2 g/250 mL of the sample solution replaced the glucose standard, with 1% methylene blue as the indicator. Ten milliliters of Fehling's solution were boiled with 20 mL of distilled water. The sample was titrated dropwise until 90% of the blue color disappeared, after which 4 drops of methylene blue were added. Titration continued until the blue color disappeared, and a brick-red precipitate formed. The titration volume was recorded [23].

$$Reducing\ sugar\ \% = \frac{Dilutions\ x\ Fehling's\ factor\ \ x\ 100\%}{Weight\ of\ sample\ x\ Titre}$$

Bacterial and fungal strains

Pseudomonas aeruginosa (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), Methicillin Resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae* (ATCC 700603), *Escherichia coli* (ATCC 25922), *Candida albicans* (ATCC 10231), and *Candida tropicalis* (ATCC 13803) were obtained from the Department of Microbiology, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. Bacterial and fungal stock cultures were maintained at -70 °C. Bacteria and *Candida* were sub-cultured on freshly prepared Nutrient agar (NA, OXOID, England) and Sabouraud Dextrose Agar (SDA, OXOID, England) plates respectively, and incubated at 37 °C for 18-24 h aerobically.

Determination of the antimicrobial effect of honey on planktonic bacteria and Candida species

The antimicrobial activity of *A. dorsata,* and *A. cerana* honey was determined using agar well diffusion method [24]. Standard cell suspensions of test strains were prepared by dissolving a few isolated bacterial or fungal colonies in sterile

normal saline and the turbidity was adjusted according to the 0.5 McFarland turbidity standards. Lawn culture of the test organism was made on Mueller Hinton agar (MHA, Oxoid, England) plates using sterile cotton swabs separately. Four wells were made on the inoculated MHA plates with the base of a sterile 1000 µL pipette tip. Then, the bottom of four wells was sealed with molten agar. The wells were subsequently filled with 100 µL of honey samples, 100 µL of 0.2% chlorhexidine digluconate (CHG) serving as a positive control, and 100 µL of sterile distilled water as a negative control. Plates were incubated overnight at 37 °C aerobically. After incubation, zones of inhibition were observed. The test was performed in triplicates.

Determination of the Minimum Inhibitory Concentration (MIC) of honey

MICs of *A. cerana,* and *A. dorsata* honey towards the test organisms were determined by the agar well diffusion method [25] with few modifications. The starting concentrations of the tested substances were 1540 mg/mL and 1722.40 mg/mL, representing the neat (undiluted) concentrations. A series of two-fold dilutions (1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, and 1/2048 mg/ mL) were prepared by halving the concentration of antimicrobial agents (honey and CHG) stepwise in sterile Bijou bottles by using sterile distilled water. Culture plates were inoculated, and wells were prepared as explained previously. Dilutions of antimicrobial agents were filled into the wells, and plates were then incubated overnight at 37 °C aerobically. The highest dilution (lowest concentration) of honey that produced a visible zone of growth inhibition around the well was noted as MIC. The test was performed in triplicates.

Determination of the inhibitory effect of Asian bee honey on initial microbial adhesion

The ability of natural bee honey to inhibit the adhesion of test strains onto polystyrene surfaces was determined [14]. Standard cell suspensions of test strains were prepared by dissolving a few colonies of 24 h old fresh bacterial, and fungal cultures in sterile Brain Heart Infusion Broth (BHI, OXOID, England). The broth cultures were incubated at 37 °C for 18-24 h aerobically. After incubation, microbial cells were harvested by centrifugation at 3000 rpm for 5 min. Harvested cells were washed thrice with 5 mL of sterile phosphate-buffered saline (PBS) (Sigma, USA). Washed cells were re-suspended in BHI and the absorbance was adjusted to 0.08-0.10 at 570 nm using a microtiter plate reader (Multiskan Sky, India). Doubling dilution of antimicrobial agents (bee honey and CHG) was prepared in 96-well sterile flat-bottomed microplates (Corning Costar, USA) (50 µL/well), and 50 µl of prepared standard cell suspensions were added to corresponding wells. Microtiter plates were incubated at 37 °C for 2 h. After the incubation, plates were washed with 200 µL PBS (Sigma, USA) (14). Then, the adherent cell mass was quantified using Anti-biofilm activity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 100µL of 1 mg/ mL MTT (HiMedia, India) was added to each well. Plates were then covered with an aluminum paper to protect them from direct light and incubated at 37 °C for 4 h. After incubation, the remaining MTT solution was carefully aspirated and 100µL of Dimethyl sulfoxide (DMSO), (Merck-India) was added to each well. The absorbance of the content was measured at 570 nm using a microtiter plate reader (Multiskan Sky, India) [24]. The test was performed in duplicates.

Determination of the inhibitory effect of A. cerana honey on bacterial and candidal biofilm development

Flat-bottomed 96-well sterile microplates were seeded with standard microbial cell suspensions (100 µL/well). Plates were then incubated aerobically for 2 h at 37 °C. After incubation, plates were washed with 200 µL of sterile PBS

(Sigma, USA). Two-fold dilutions of bee honey ranging from 3 mg/ mL to 1540 mg/ mL were prepared in sterile BHI broth in separate sterile universal bottles. One hundred microliters of the prepared two-fold dilutions were added to the corresponding wells. Plates were then incubated at 37 °C for 24 h aerobically. The viability of the biofilms developed in the presence of antimicrobial treatments was quantified by MTT assay as explained previously. The test was performed in duplicates. BHI alone and CHG with different concentrations in BHI were used as negative and positive controls respectively [14].

Determination of Minimum Biofilm Inhibitory Concentration ($MBIC_{50}$)

Sterile flat-bottomed 96-well microplates were seeded with standard cell suspensions of test strains (100 µL/well). The plates were incubated for 24 h at 37 °C aerobically. Plates were then washed with 200 µL of sterile PBS. Two-fold dilutions of *A. cerana* honey in BHI ranging from 3 mg/ mL to 1540 mg/ mL were added to each well (100 µL/well). The plates were incubated for 24 h at 37 °C aerobically. Quantification of treated biofilm viability was done using MTT assay as mentioned previously. CHG and BHI alone were used as positive and negative controls respectively [11]. The test was performed in duplicates. The percentage of biofilm inhibition was calculated relative to the untreated control (i.e.; BHI alone). The $MBIC_{50}$ value represents the concentration of the tested honey that resulted in a 50% reduction in biofilm biomass compared to the control. This was determined by plotting the percentage inhibition against the concentration of the tested honey and identifying the concentration at which 50% inhibition occurred [26].

Scanning Electron Microscopy (SEM) of established biofilms

Ultra-structures of the established biofilms of *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), and *C. albicans* (ATCC 10231) after 24 h exposure to $MBIC_{50}$ of *A. cerena* honey were observed using SEM. A 24 h mature biofilms were established on sterile glass coverslips and treated with $MBIC_{50}$ of *A. cerena* honey in BHI broth for 24 h as explained previously and processed to visualize under SEM [26].

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 26 (2019) for Windows.

Results

Physiochemical Analysis

The physicochemical properties of *A. cerana* and *A. dorsata* honey were analyzed and compared, revealing distinct differences between the two (Table 1). According to the analysis results, *A. cerana* honey possessed a higher acidic pH, higher refraction index, and radical scavenging activity than *A. dorsata* honey. The average density, moisture content, total phenolic content, ash content, and reducing sugar content were perceived to be higher in *A. dorsata* honey compared to *A. cerana* honey.

Anti-bacterial and anti-candidal effects

The results for antibacterial and anti-candidal efficacy of natural *A. cerana* and *A. dorsata* honey against the selected bacterial and fungal ATCC strains (*S. aureus*, MRSA, *K. pneumoniae*, *P. aeruginosa*, *E. coli, C. albicans,* and *C. tropicalis*) are presented in Table 2. The presence of a zone of growth inhibition on the agar surface specifies the sensitivity of relevant microorganisms to the treatment [27]. All tested bacterial strains showed a zone of growth inhibition on the agar surface, which indicates the antibacterial effect of natural *A. cerana* and *A. dorsata* honey. Anti-candidal activity was exhibited only by *A. cerana* honey against the tested *Candida* spp. Overall, *A. cerana* honey appeared to have a higher antimicrobial activity against all the tested organisms compared to *A. dorsata* honey (Table 2).

Table 1: Physicochemical properties of natural Sri Lankan *A. cerana* honey and *A. dorsata* honey

Parameter	*A. cerana* honey	*A. dorsata* honey
Colour	Golden orange	Dark amber
Density	1540.00 mg/ mL	1722.40 mg/ mL
Refraction index (%±margin error)	75% (±0.50)	68% (±0.50)
pH (Mean±SD)	3.92 (±0.01) at 30 ^{0}C	4.17 (±0.01) at 30 ^{0}C
Moisture content	7.15%	10.70%
Total phenolic content	416.64 mg GAE/ kg	552.85 mg GAE/ kg
Ash content	0.56%	0.58%
Reducing sugar content	72.59%	76.48%
Radical scavenging activity	34.30%	33.52%

Minimum Inhibitory Concentration (MIC)

The MIC values corresponding to *A. cerana* honey, *A. dorsata* honey, and the positive control CHG are represented in Table 3. The MIC values of *A. cerana* honey ranged from 770.00 to 1540.00 mg/mL, while those for *A. dorsata* honey ranged from 430.60 to 1722.40 mg/mL. Notably, only *A. cerana* honey demonstrated MIC values (1540.00 mg/mL) against the tested *Candida* species (*C. albicans* and *C. tropicalis*), whereas *A. dorsata* honey showed no MIC even at the highest concentration tested (dilution factor 1). Overall, while both types of honey exhibited antimicrobial activity, their efficacy was generally lower than that of CHG, especially against Gram-negative bacteria and fungal strains.

Effect on initial biofilm adhesion

According to the data obtained from the MTT assay, Table 4 summarizes the concentrations of *A. cerana* honey, and CHG required to inhibit initial biofilm adhesion by 50%. *A. cerana* honey required significantly higher concentrations (12.00–192.50 mg/mL) to achieve 50% reduction across all tested organisms compared to CHG, which required much lower concentrations (0.39–6.25 mg/mL). Notably, *A. cerana* honey was most effective against *P. aeruginosa* (ATCC 27853), requiring 12.00 mg/mL, while its least effective concentration was against *S. aureus* (ATCC 25923), MRSA, and *Candida* species, all requiring 192.50 mg/mL.

Effect on biofilm development

According to the data obtained from the MTT assay, Table 5 shows the concentrations of *A. cerana* honey, and CHG required to inhibit biofilm development by 50%. *A. cerana* honey demonstrated varying effectiveness in inhibiting biofilm formation across different organisms. While it showed notable activity, especially against *E. coli* and *Candida* species, higher concentrations were required compared to CHG.

Anti-biofilm effect on established biofilms

Table 5 presents the effectiveness of different concentrations of *A. cerana* honey, *A. dorsata* honey, and CHG in reducing established biofilms across various strains. Both *A. cerana* honey and *A. dorsata* honey demonstrated efficacy in reducing established biofilms, when compared to the negative control. Notably, *A. dorsata* honey required substantially higher concentrations (ranging from 107.65 to 215.30 mg/mL) to achieve a 50% reduction in biofilm formation for all tested organisms. In contrast, *A. cerana* honey achieved similar levels of biofilm reduction at lower concentrations, ranging from 48.13 to 192.50 mg/mL. These findings indicate that while both types of honey are effective, *A. cerana* honey is comparatively more efficient, requiring lower

Table 2: Well diffusion results of *A. cerana* honey, *A. dorsata* honey, and 2% CHG against selected bacterial and *Candida* species (ATCC strains)

Organisms	*A. cerana* honey	*A. dorsata* honey	CHG
	ZDI (mm)	ZDI (mm)	ZDI (mm)
S. aureus (ATCC 25923)	20.33 ± 0.57	19.33 ± 0.57	23.66 ± 0.57
MRSA	20.00 ±1.00	17.00 ±1.00	20.33 ± 0.57
K. pneumonia (ATCC 700603)	15.33 ± 0.57	14.33 ± 0.57	18.33 ± 0.57
P. aeruginosa (ATCC 27853)	14.33 ± 0.57	14.33 ± 0.57	18.00 ±1.00
E. coli (ATCC 25922)	16.00 ±1.00	16.00 ±1.00	20.33 ± 0.57
C. albicans (ATCC 10231)	11.00	-	18.00 ±1.00
C. tropicalis (ATCC 13803)	11.00	-	14.33 ± 0.57

ZDI: zone diameters of inhibition (Mean ± SD), CHG: chlorhexidine digluconate

concentrations to achieve similar reductions in established biofilms.

SEM for determination of post-exposure ultrastructure of biofilms formed by S. aureus, P. aeruginosa, and C. albicans

Ultrastructure of 24 h established biofilms of *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), and *C. albicans* (ATCC 10231), after treating with $MBIC_{50}$ of *A. cerana* honey was quantitatively evaluated using SEM (Figure 1 (A, B, E, F), 2 (A, B). The untreated (negative control) biofilms were also examined throughout the experiment (Figure 1 (C, D, G, H), 2 (C, D). Untreated biofilms showed microcolonies, and a high number of microorganisms compared to the treated ones. Visualization of fewer microcolonies in the treated biofilm may be due to the inhibitory effect of bee honey against biofilm development.

Mature biofilms treated with *A. cerana* honey caused microbial cell shrinkage, deformities, cell wall damage, and decreased biofilm cell density when compared to the negative controls. When having a closer look at the cell walls of negative control, it was observed that their cell walls were smooth and regular.

Discussion

This study provides a comprehensive analysis of the physicochemical properties, and antimicrobial efficacy of *A. cerana*, and *A. dorsata* honey against both planktonic and sessile cells (biofilms) of selected reference bacterial and *Candida* species. Our findings reveal that *A. cerana* honey demonstrated superior antimicrobial activity compared to *A. dorsata* honey across all tested bacterial and fungal strains. Notably, *A. cerana* honey exhibited a higher acidic pH, refraction index, and radical scavenging activity, while *A. dorsata* honey was characterized by higher density, moisture, total phenolic content, ash content, and reduced sugar levels.

The physicochemical composition is one of the key facts that confirms the antimicrobial

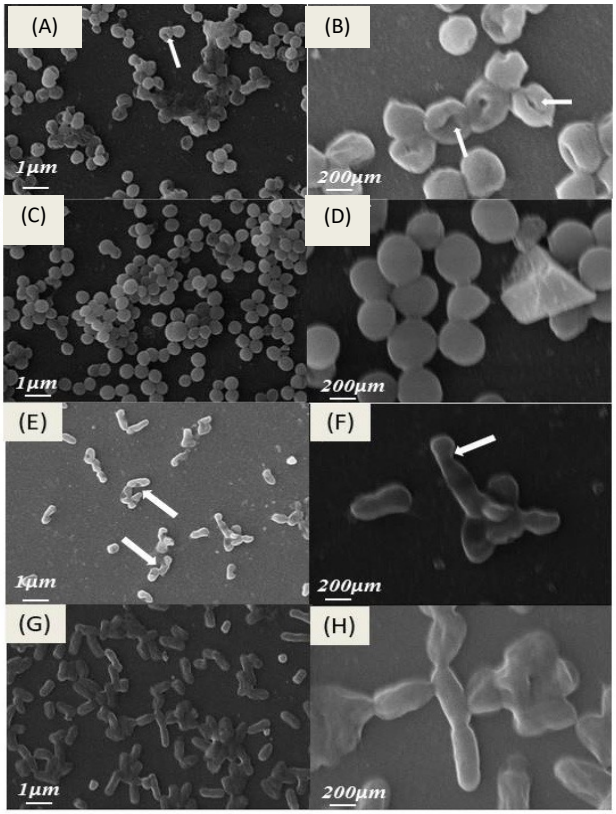

Figure 1. Ultrastructure of 24 h established biofilms of *S. aureus* and *P. aeruginosa* (A, B, E, F) after treating with $MBIC_{50}$ of *A. cerana* honey, and (C, D, G, H) the untreated - White solid arrows: Cellular changes

properties of honey [28]. According to the results of the current study (Table 1), all the analyzed parameters including density, refractive index, pH, moisture content, ash content, total phenolic content, reducing sugar content, and radical scavenging activity of both samples, complied with the European regulations on honey quality criteria [29]. Both honey samples exhibited an acidic pH ranging from 3.92 to 4.17, consistent with previous findings [30,31]. This low pH plays a critical role in inhibiting the growth and persistence of microorganisms [30]. Further acidic pH of honey is worthwhile since wound healing is promoted by acidification, which causes to release of oxygen from hemoglobin [32]. The results of the reducing sugar contents (72.59% - 76.48%), and moisture contents (7.15% - 10.70%) of both honey samples were in agreement with the

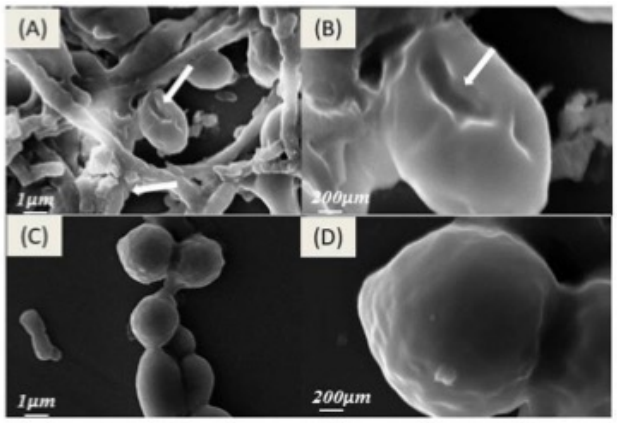

Figure 2: Ultrastructure of *A. cerana* honey-treated biofilms formed by *C. albicans* (A) and (B) and untreated biofilms formed by *C. albicans* (C) and (D) - White solid arrows: Cellular changes

Table 3: MIC assay results of *A. cerana* honey, *A. dorsata* honey, and 2% CHG

Organism	MIC (mg/ mL)		
	***A. cerana* honey**	***A. dorsata* honey**	**CHG**
S. aureus (ATCC 25923)	770.00	861.20	0.78
MRSA	770.00	430.60	1.56
K. pneumoniae (ATCC 700603)	770.00	861.20	25.00
P. aeruginosa (ATCC 27853)	770.00	1722.40	6.25
E. coli (ATCC 25922)	1540.00	1722.40	1.56
C. albicans (ATCC 10231)	1540.00	No MIC point	6.25
C. tropicalis (ATCC 13803)	1540.00	No MIC point	3.12

MIC- Minimum inhibitory concentration CHG: chlorhexidine digluconate

findings of previous studies [33-35]. Since the antibacterial property of honey is also derived from the osmotic effect, the presence of both high sugar content and low moisture content is crucial (36). Several studies have proved that phenolic compounds have the ability to induce the growth of a broad spectrum of Gram-positive and Gram-negative bacteria [37-39].

In this study, we assessed the *in vitro* antimicrobial activity of *A. cerana,* and *A. dorsata* honey against selected reference bacterial and *Candida* species (Table 2). Both honey samples exhibited significant antibacterial activity against both Gram-positive and Gram-negative bacterial strains. According to the data obtained from the agar well diffusion method, both honey samples demonstrated good capability to inhibit the growth of all the tested bacterial strains. As reported by Grego et al in 2016, *S. aureus* and MRSA strains demonstrated more sensitivity than Gram-negative bacteria [40]. The reason for this difference may be the cell membrane structures where Gram-positive bacteria consist of a single plasma membrane whereas Gram-negative bacteria are bound by two membranous structures (i.e.; due to the presence of inner and outer membrane) [41]. Notably, *A. cerana* honey also exhibited anti-candidal activity against *C. albicans* and *C. tropicalis*, while *A. dorsata* honey did not demonstrate anti-candidal efficacy, which may be attributed to differences in honey composition and phenolic content [38,42,43]. These data are important in order to implement new therapeutic strategies for the treatment of infected wounds since they are the major pathogens associated with wound infections [44].

The antimicrobial activity of both honey samples is attributed to the presence of active compounds such as the flavanone pinocembrin, caffeic acid phenethyl ester, flavonol galangin, and quercetin [45,46]. Flavonol galangin, flavonone pinocembrin, and caffeic acid phenethyl ester are responsible for the inhibition of bacterial RNA polymerase activity while quercetin increases the membrane permeability, and prevents bacterial ATP synthesis. Galangin degrades the cytoplasm membrane of the bacteria, which leads to a loss of potassium ions [45]. Further physico-chemical

Table 4: Concentrations of *A. cerana* honey, and CHG for 50% reduction of initial biofilm adhesion

Organism	Concentration for 50% reduction (mg/ mL)	
	***A. cerana* honey**	**CHG**
S. aureus (ATCC 25923)	192.50	3.12
MRSA	192.50	0.39
E. coli (ATCC 25922)	24.10	3.12
P. aeruginosa (ATCC 27853)	12.00	1.56
K. pneumoniae (ATCC 700603)	24.10	3.12
C. albicans (ATCC 10231)	192.50	6.25
C. tropicalis (ATCC 13803)	192.50	3.12

CHG: chlorhexidine digluconate

properties, entomological origin, botanical origin, and symbioses with beneficial microbes are also influenced for the antimicrobial activity of honey [32].

Wound pathogens that exist as microbial biofilms contribute to delayed wound healing resulting in chronic wounds [47]. Microorganisms get adhered to the wound bed, and form microcolonies which are embedded in extracellular matrix (EPS) [48]. Microorganisms in biofilms are more resistant to antibiotics [49]. Due to these microbial biofilms, most of the time chronic wounds exhibit poor wound healing [9]. Diffusion of antimicrobial agents into the biofilm is inhibited by the extracellular biofilm matrix, which acts as a chemical or physical barrier [11].

Biofilm formation begins with adhesion to either biotic or abiotic surfaces [50]. In this study, *P. aeruginosa* was particularly vulnerable to the inhibition of initial microbial adhesion when treated with *A. cerana* honey. This effect may be attributed to the honey's ability to inhibit PA-IIL, a virulence factor of *P. aeruginosa* that plays a critical role in biofilm formation and adherence [51]. According to the data obtained, *E. coli* and *C. albicans* were more susceptible to biofilm development whereas MRSA was more susceptible in established biofilms with the treatment of A. *cerana* honey. These findings are in line with the published studies from different parts of the world using medical-grade honey types (i.e.; Manuka honey) [38,52].

The decision to focus on *A. cerana* honey for the initial biofilm adhesion was guided by our preliminary results from well diffusion and minimum inhibitory concentration (MIC) tests. These results demonstrated that *A. cerana* honey exhibited greater antimicrobial efficacy compared to *A. dorsata* honey. Given this higher potency, we prioritized *A. cerana* honey for further investigation in the early stages of biofilm formation, as such assays often focus on planktonic bacteria. Both *A. cerana* and *A. dorsata* honeys were tested on mature biofilms to evaluate their broader anti-biofilm activity. Testing both honeys at this stage provided comparative insights into their efficacy against established biofilms.

The antimicrobial and anti-biofilm efficacy of honey is concentration-dependent [53]. At lower concentrations, glucose oxidase catalyzes the conversion of glucose into gluconic acid and hydrogen peroxide. An increased production of hydrogen peroxide significantly impairs biofilm formation [54]. The current study demonstrated that, when applied at optimal concentrations, both honey types effectively serve as potent antimicrobial and anti-biofilm agents against the tested microbial strains.

SEM imaging was utilized to gain a more detailed

view of the morphological changes in established biofilms exposed to the chemical stress of *A. cerana* honey. In this study, biofilms treated with $MBIC_{50}$ of *A. cerana* honey showed notable alterations in microbial morphology, including reduced cell density, cell wall deformities, and significant damage to cell walls. In this study SEM imaging was performed only for *A. cerana* honey, as it exhibited the most significant anti-biofilm activity. According to the findings of Campeau and Patel in 2014, the Manuka honey too caused cellular alterations when compared to the negative control revealing that both Manuka honey and Sri Lankan *A. cerana* honey have the same effect on *S. aureus,* and *P. aeruginosa* biofilms [55]. These morphological changes may be attributed to the presence of active constituents in honey, such as pinocembrin, caffeic acid phenethyl ester, galangin, and quercetin [14,45]. Previous studies have shown that quercetin enhances membrane permeability, while galangin degrades the cytoplasmic membrane, leading to potassium ion leakage and, ultimately, cell death [14,45]. The reduction in EPS observed in this study further indicates that *A. cerana* honey disrupts biofilm nutrient supply and impairs microbial attachment.

In this study, we used the agar well diffusion assay to assess the MICs of honey. The decision to utilize the agar well diffusion method was influenced by the physical properties of honey, particularly its thick and viscous nature, which posed significant challenges for performing accurate serial dilutions necessary for standard broth dilution assays. This limitation inherently restricts our ability to determine precise MIC values, a fact that we recognize as a limitation of our study. Despite this, the results obtained from the agar well diffusion method provided valuable insights into the antimicrobial properties of honey, demonstrating its potential efficacy against the tested reference bacterial and *Candida* species.

We plan to extend this work in future studies to include testing against multiple clinical isolates from each species to confirm and expand upon our

Table 5: $MBIC_{50}$ of *A. cerana* honey, *A. dorsata* honey, and CHG in developing and established biofilms

Organism	**$MBIC_{50}$ (mg/ mL)**				
	Developing biofilms		**Established biofilms**		
	***A. cerana* honey**	**CHG**	***A. cerana* honey**	***A. dorsata* honey**	**CHG**
S. aureus (ATCC 25923)	48.13	0.39	96.25	107.65	1.56
MRSA	96.25	0.78	48.13	107.65	0.39
E. coli (ATCC 25922)	24.10	0.19	192.50	107.65	1.56
P. aeruginosa (ATCC 27853)	96.25	12.50	192.50	107.65	3.12
K. pneumoniae (ATCC 700603)	48.13	6.25	192.50	215.30	6.25
C. albicans (ATCC 10231)	24.10	3.12	96.25	107.65	6.25
C. tropicalis (ATCC 13803)	48.13	6.25	192.50	215.30	3.12

MBIC: Minimum Biofilm Inhibitory Concentration; CHG: chlorhexidine digluconate

initial findings. This will help to determine the broader applicability and consistency of *A. cerana* honey's anti-biofilm effects across different strains.

Conclusion

Both Sri Lankan *A. cerana* and *A. dorsata* honey demonstrated antibacterial effects against planktonic growth of selected bacterial strains of wound pathogens: *S. aureus*, MRSA, *P. aeruginosa*, *K. pneumoniae*, and *E. coli*. Additionally, *A. cerana* honey exhibited anti-candidal activity against reference strains of *C. albicans,* and *C. tropicalis*, whereas *A. dorsata* honey showed limited anticandidal effects. Both types of honey also displayed anti-biofilm activity against all tested microorganisms. Comparatively, *A. cerana* honey was more effective than *A. dorsata* honey. The physicochemical properties of the honey play a substantial role in determining its antibacterial activity.

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