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Exploring the *in vitro* activity of the combination of Manuka honey and *Cinnamomum verum* bark oil against carbapenem-resistant *Acinetobacter baumannii* clinical isolates

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ABSTRACT

Background: The spread of carbapenem-resistant *Acinetobacter baumannii* (CRAB) globally is a serious public health challenge due to its limited therapeutic options and high morbidity and mortality rates. This study was aimed to examine the *in vitro* activity of Manuka honey and cinnamon bark oil (CBO) against CRAB isolates obtained from various clinical samples. The broth microdilution technique was used to determine minimum inhibitory concentrations (MICs). The checkerboard test was additionally used to evaluate synergistic interactions between Manuka honey and CBO. **Results:** Both Manuka honey and CBO showed antibacterial activity against all CRAB isolates. The MICs for Manuka honey and CBO were determined as 103.88 mg/mL, and 1.9 µg/mL respectively, while all isolates tested had additive interactions with Manuka honey plus CBO. **Conclusion:** From this study, it can be inferred that there is *in vitro* efficacy of Manuka honey and CBO on CRAB isolates with potential for synergy interaction. However, further studies should investigate the possible clinical usefulness as well as mechanisms of action for these natural products against CRAB infections.

Introduction

On a global scale, many infectious diseases are being faced with various multi-drug resistant (MDR) pathogens emerging everywhere, making this become a critical public health issue [1]. According to the World Health Organization (WHO), the priority pathogens list categorizes drug-resistant bacteria into three levels according to the urgency of developing new antibiotics: critical, high, and medium. In particular, the list marks how Gram-negative bacteria are a challenge due to their multi-drug resistance. These pathogens possess the ability to develop new defenses and transfer genetic materials responsible for other bacterial infections becoming resistant to drugs [2]. The critical group includes carbapenem-resistant *Acinetobacter baumannii* (CRAB), carbapenem-resistant *Pseudomonas aeruginosa*, (*P. aeruginosa*) and various carbapenem-resistant and extended-

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spectrum beta lactamase (ESBL) *Enterobacteriaceae* (including *Klebsiella*, *Proteus*, *Serratia*, and *Escherichia coli* (*E. coli*)). They lead to severe and mostly fatal diseases like pneumonia as well as bloodstream infections. Consequently, this priority pathogens list provides a need for novel antibiotics or alternate therapy options, which can reduce death rates from drug-resistant infections worldwide [1, 2].

For many years, plant essential oils have been considered as potential and valuable sources of antimicrobial compounds that have long been utilized in traditional medicine. They are natural substances with a complex odoriferous volatile content, which has shown antibacterial and antifungal activities [3]. A variety of essential oils were tested for their antibacterial effect against MDR bacteria, of which cinnamon bark oil (CBO) extract demonstrated the strongest and highest antimicrobial activity [4]. Cinnamaldehyde and eugenol are the most active compounds of CBO, which showed powerful inhibitory effects. Both the CBO extract and cinnamaldehyde have been recognized as possible potentially active natural compounds that warrant further investigation as alternative treatment options for MDR bacterial infections including CRAB [5].

Much attention has been also directed to the future potential of natural pure honey as a promising alternative for treating MDR infections [6]. Honey is a natural source of a diverse range of beneficial probiotics and prebiotics, along with essential minerals such as calcium, potassium, and magnesium, as well as a variety of vitamins that contribute to overall health and well-being. Honey originates from different geographical and botanical origins and has shown antimicrobial efficacy due to its multi-mechanistic effect against even highly hypermutable and MDR bacterial strains [7].

Manuka honey was found to act effectively against antibiotic-resistant *A. baumannii*. Notably, it revealed the highest level of inhibition when treated with Manuka honey and appeared to be more sensitive to its effects compared to other bacterial species, despite its high levels of antibiotic resistance [8].

Consequently, the aim of the current study was to investigate safe and natural alternative to traditionally ineffective antibiotics through evaluating the antibacterial activities of Manuka honey, CBO and the combination of both against CRAB clinical isolates.

Material and methods

Bacterial isolates

Ten clinical isolates of CRAB were collected from different sites of infections. The isolates were collected from a microbiology laboratory unit in Alexandria, Egypt and verified using VITEK-2 compact automated system to check their identity to the species level and to test the antimicrobial susceptibility pattern using the minimum inhibitory concentration (MIC) method [9,10].

Honey sample acquisition and processing

A commercially available Manuka honey 20+ UMFTM (Hill Laboratories, Hamilton, New Zealand) was purchased. According to the manufacturer's product specifications, the main active ingredients included were leptosperin (760 mg/kg), dihydroxyacetone (DHA) (1182 mg/kg), methylglyoxal (MGO) (831 mg/kg), and hydroxymethylfurfural (HMF) (27.80 mg/kg). The honey samples were gently warmed in a water bath to reach approximately 50 ^{o}C to liquefy, so that they could be aliquoted for culture plates. The honey samples were kept in sealed containers and at ambient temperature [7].

CBO extraction and characterization

The CBO was purchased as a trademark product from the Egyptian market. The product label explicitly confirmed the botanical source and origin of the bark, identifying it as *Cinnamomum verum* (*C. verum*) at the species level. This ensured the authenticity of the material used in the current study's experiments.

i- Extraction of cinnamon oil

One hundred grams of cinnamon bark was accurately weighed and macerated for soxhlation with one liter of 96% ethanol. The solution was kept for 24 hours, then filtered. To obtain cinnamon oil extract, the filtrate was placed in rotary evaporator device (Scilogex RE100-Pr, USA) and heated to 70 ^{o}C at 50 rpm for 90 minutes. The obtained yield was subjected to further analysis [11].

ii- Characterization of CBO extract:

a- Gas chromatography-mass spectrometry (GC-MS)

GC-MS analysis was conducted at the central laboratory of a governmental institute in

Egypt using a Trace 1300 GC Ultra/Mass Spectrophotometer ISQ QD (Thermo Scientific) equipped with X-Calibur 2.2 software (Thermo X-Calibur). Helium served as the carrier gas, maintaining an average velocity of 39 cm/sec, with the column flow rate set at 1.00 mL/min and a total run time of 31 minutes. A TG-5MS Zebron capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness, Thermo) was used for separation. A 1 µL sample of cinnamon oil was injected in split mode with a 20:1 split ratio at an injection temperature of 300 °C. The column temperature was initially set at 80 °C for 0 minutes, then gradually increased to 200 °C at a rate of 4 °C/min under an electron ionization energy of 70 eV. Ethanol was used as the solvent, and the total elution time was 31 minutes. The relative percentage of each component was determined by comparing its average peak area to the total peak areas. System control and data acquisition were performed using the provided MS Solution software. Component identification in the cinnamon oil extract was based on retention indices, with mass spectrum interpretation conducted using the Replib database library [12].

b- High-performance liquid chromatography (HPLC)

Each of the standard cinnamaldehyde– a chemical reference substance – and cinnamon oil extract were dissolved in acetonitrile. One µL of each was injected into the HPLC and separated by C18 column (4.6 x 260 mm) with a UV detection wavelength of 280 nm. The highest peaks of the standard as well as the extract were obtained, and the concentration of cinnamaldehyde in the cinnamon oil extract was subsequently calculated [13].

Evaluation of antibacterial activity using broth microdilution assay

The antimicrobial activities of CBO extract and Manuka honey against ten CRAB clinical isolates were evaluated using the broth microdilution method in 96-well polystyrene plates. Bacterial suspensions were prepared from overnight cultures grown on Mueller-Hinton agar plates and adjusted to a 0.5 McFarland standard. Fifty µL of each of ten two-fold serially diluted concentrations of CBO extract and Manuka honey, based on MGO content (starting at final concentrations of 3.8 µg/mL and 207.75 mg/mL, respectively, using Cation-adjusted Muller Hinton Broth (CAMHB) as the diluent), were added to 50 µL of the bacterial suspension to achieve a final inoculum concentration of 5×10^5 CFU/mL. For each isolate, 50 µL of CAMHB was combined with 50 µL of the bacterial suspension in the growth control well, while 100 µL of CAMHB was used in the sterility control well. The microtiter plates were incubated at 37°C for 16–20 hours. The MIC was determined as the lowest concentration of the antimicrobial agent that completely inhibited microbial growth [14].

Checkboard microdilution assay

In microtiter plate, 25 µL of each dilution of Manuka honey were added to the wells in a vertical orientation, and 25 µL of each dilution of CBO extract were added to the wells in a horizontal orientation. Each well was inoculated with 50 µL of bacterial suspension (of final concentration of 5×105 CFU/ml). Microtiter plates were incubated at 37oC for 16-20 hours [15]. MIC values of Manuka honey were observed when combined with different CBO extract concentrations. Mean MIC values obtained for the combinations were used for calculating the FIC index. The FIC index was calculated using the following formula:

FIC index = (MIC of Manuka honey in combination / MIC of Manuka honey alone) + (MIC of cinnamon oil in combination / MIC of cinnamon oil alone)The average FIC was determined from eight individual FIC values to assess the combined antibacterial effect of Manuka honey and CBO extract. The interaction was classified as synergistic (FIC ≤ 0.5), additive (0.5 < FIC ≤ 1), indifferent (1 < FIC < 2), or antagonistic (FIC ≥ 2) [16, 17].

Results

The distribution of the ten studied *A. baumannii* clinical isolates according to sources and antimicrobial susceptibility testing (AST) showed recovery from different clinical samples (bronchoalveolar lavage, blood and wound). It was also noted that all the studied isolates were carpabenem resistant but sensitive to colistin. All isolates were resistant to ticarcillin, ticarcillin/clavulinic acid, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, and ciprofloxacn. Out of those isolates, 7 were sensitive to minocycline, 3 were sensitive sulfamethoxazole/trimethoprim and only one was sensitive to both gentamicin and tobramycin. (Table 1).

GC-MS analysis:

According to the Replib database, results illustrated that the most dominant compound of the

CBO extract showed the highest peak was cinnamaldehyde (molecular formula C_9H_8O and molecular weight 132) with a peak area of 68.89% at retention time 12.24 min (Figure 1).

High performance liquid chromatography (HPLC)

The cinnamaldehyde chemical reference substance exhibited its highest peak at a retention time of 3.477 (Figure 2). In comparison, the cinnamon oil extract displayed its highest peak at a retention time of 3.261, with the cinnamaldehyde concentration calculated to be 72.1 µg/g of the sample (Figure 3).

Antibacterial activity of Manuka honey, CBO and their combination

The MIC study for honey and oil tested against CRAB strains showed that all the tested isolates were sensitive to Manuka honey and CBO with MIC values of 103.88 mg/mL and 1.9 µg/mL respectively. In the Checkerboard technique, the interaction between the combination of Manuka honey and CBO against MDR *Acinetobacter* strains had an additive effect, although there were few variations. The ΣFIC results for the checkerboard method showed an additive effect, indicating the activity of both Manuka honey and CBO in combination being equal to the sum of their independent activity ΣFIC 0.54, 0.62, and 0.67 (Table 2)

Table 1. Sources and AST of the studied MDR *A. baumannii* isolates using MIC method according to CLSI 2023.

	Source	TC	TIM	PRL	TZP	CAZ	FEB	IMP	MEM	CN	TOB	CIP	MH	CT	SXT
Ac1	Blood	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	>=16 R	<=0.5 S	<=20 S
Ac2	BAL	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	<=1 S	1 S	>=320 R
Ac3	BAL	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	4 S	<=0.5 S	>=320 R
Ac4	Blood	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	<=1 S	<=0.5 S	>=320 R
Ac5	BAL	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	>=16 R	<=0.5 S	<=20 S
Ac6	Wound	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	4 S	<=0.5 S	<=0.5 S
Ac7	BAL	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	<=1 S	<=0.5 S	80 R
Ac8	Wound	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	8 I	<=0.5 S	>=320 R
Ac9	Blood	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	>=16 R	>=16 R	>=4 R	2 S	<=0.5 S	>=320 R
Ac10	BAL	>=128 R	>=128 R	>=128 R	>=128 R	>=64 R	>=64 R	>=16 R	>=16 R	<=1 S	2 S	>=4 R	<=1 S	<=0.5 S	>=320 R

AC: *Acinetobacter* - S: Sensitive - I: Intermediate sensitivity - R: Resistant - TC: Ticarcillin - TIM: Ticarcillin/Clavulinic acid - PRL: Piperacillin - TZP: Piperacillin/Tazobactam - CAZ: Ceftazidime - FEB: Cefepime - IMP: Imipenem - MEM: Meropenem - CN: Gentamicin - TOB: Tobramycin - CIP: Ciprofloxacin - MH: Minocycline - CT: Colistin - SXT: Trimethoprim/Sulfamethoxazole

Table 2. MIC of Manuka honey (mg/mL), *C. verum* bark oil (µg/mL), and their combination on different CRAB strains.

		MICo	MICc	FICc	FICI	R%
AC 1	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 2	Manuka	103.88	51.4	0.49	0.54	51
	Cinnamon	1.9	0.098	0.05		95
AC 3	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 4	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 5	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 6	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 7	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 8	Manuka	103.88	51.4	0.49	0.67	51
	Cinnamon	1.9	0.35	0.18		82
AC 9	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87
AC 10	Manuka	103.88	51.4	0.49	0.62	51
	Cinnamon	1.9	0.24	0.13		87

MICo: MIC of a single component tested without any other components; MICc: MIC of each component in the combination at the concentration that produces the greatest inhibition of growth.; FIC: Fractional inhibitory concentrations is determined by the ratio MICc/MICo; FICI: Fractional inhibitory concentration index = FIC of Manuka honey + FIC of CBO; R% constitutes the percent decrease in the quantity of each associated component relative to each single component. FIC and FICI are reported as means of three replicates.

Figure 1. Chemical characterization of *C. verum* bark oil extract using GC-MS.

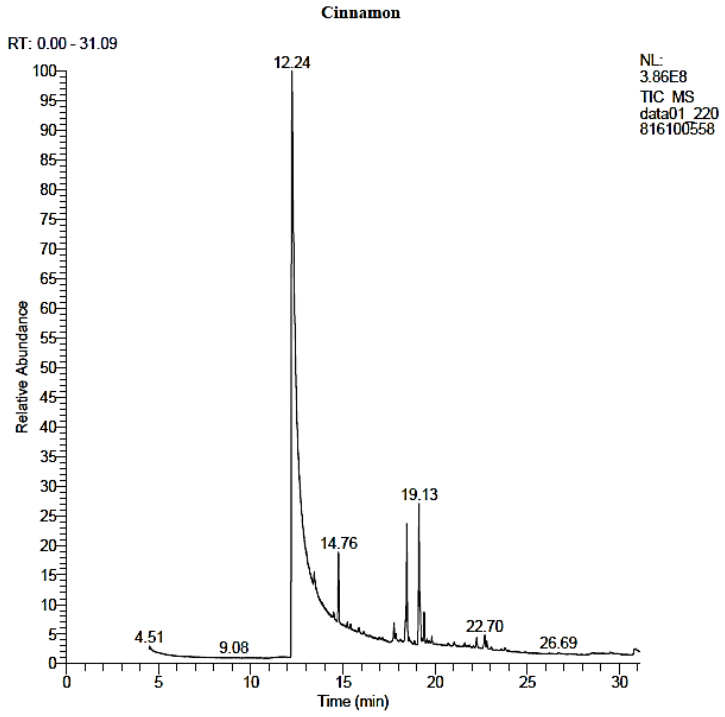


Figure 2. Chemical composition of standard cinnamaldehyde chemical using HPLC.

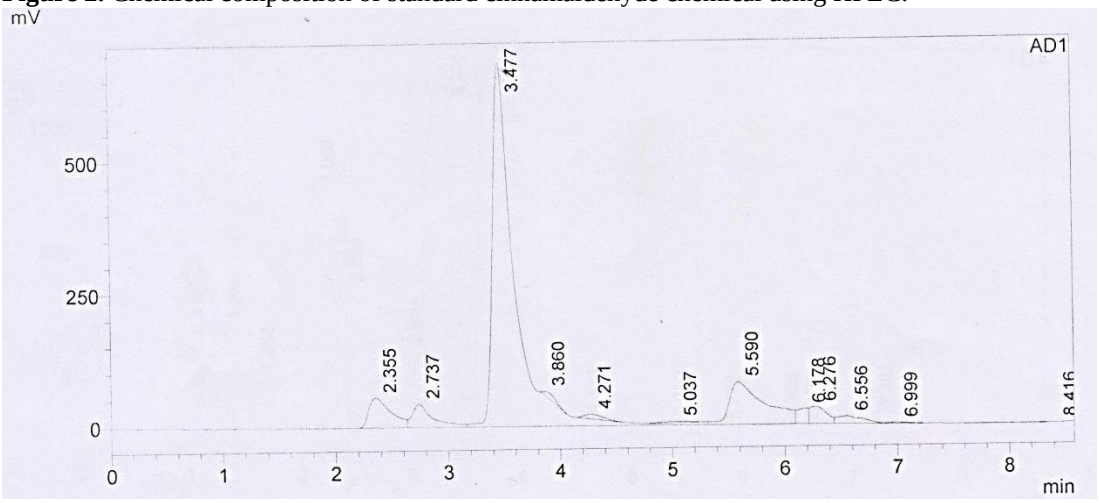
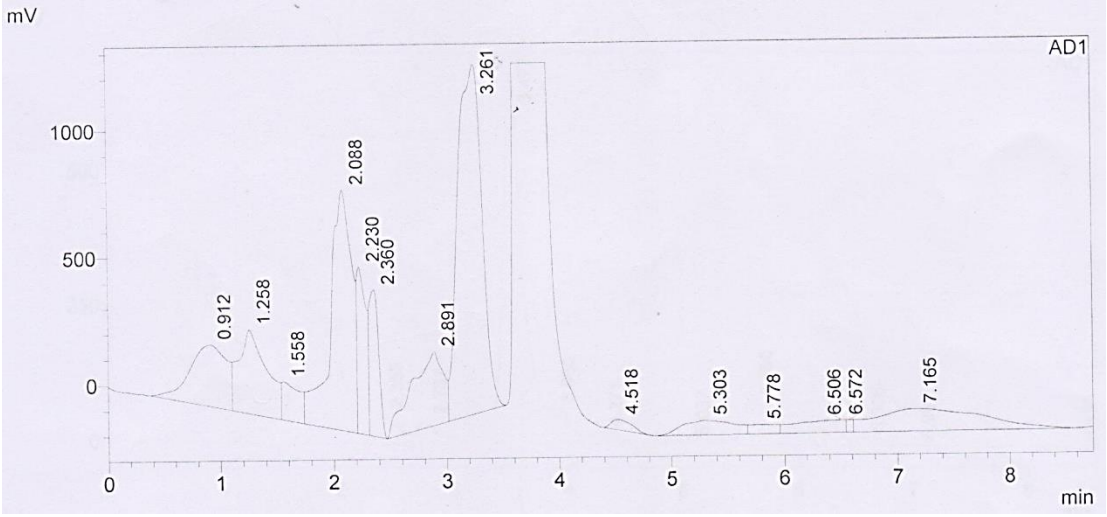


Figure 3. Chemical composition of *C. verum* bark oil using HPLC.



Discussion

The dramatically increasing rates of antimicrobial resistance have emerged as a major global health challenge that threatens healthcare settings. The irrational overuse and misuse of antibiotics, mainly in the developing countries, have led to the rise of MDR pathogens, rendering many conventional antibiotics ineffective. This necessitates the exploration and development of alternative approaches to combat MDR microorganisms [18,19].

Natural products, derived from diverse sources, have been known for their medicinal properties and consequently used as remedies for a wide range of ailments. They may offer distinct advantages as potential antibiotics through possessing a broad range of diverse bioactive compounds with a wide spectrum of antimicrobial activities. In addition, the complex bioactive profiles and their ability to target various microbial targets simultaneously, result in a great challenge for pathogens to develop resistance. This offers potential innovative strategies to challenge MDR pathogens [20-22].

Both cinnamon, which is extracted from the bark of *Cinnamomum* trees, and Manuka honey, which is produced by bees from the nectar of the Manuka tree (*Leptospermum scoparium*, or *L. scoparium*), are two natural products that have been recognized for their antimicrobial activities due to their main bioactive components, cinnamaldehyde and MGO respectively. Both cinnamon and Manuka honey have broad-spectrum antimicrobial action against different types of bacteria, including either Gram-positive bacteria such as *Staphylococcus aureus*, Gram-negative bacteria including *E. coli*, *Helicobacter pylori*, or

even fungal pathogens such as *Candida* species [23-28].

Cinnamon and Manuka honey exhibit their antibacterial actions through different mechanism. Cinnamaldehyde may cause disruption of the microbial cytoplasmic membranes, leading to the leakage of essential intracellular components and consequently cell lysis. Furthermore, it may inhibit the activity of different microbial enzymes. Besides MGO, the high osmolarity, low pH, and production of hydrogen peroxide contribute to the antibacterial effect of manuka honey. Moreover, cinnamon and Manuka honey have shown antibiofilm activities against different microorganisms [24,29-31].

MDR *A. baumannii* is considered one of the threatening healthcare-associated pathogens that has gained attention due to its ability to develop resistance to different classes of antibiotics. It is the causative microbial agent for different infections, including skin and soft tissue infections, pneumonia, as well as bloodstream infections. The rise in MDR *A. baumannii* strains has become a significant challenge in healthcare settings, as limited treatment options are available [32]. The current study demonstrated the antibacterial activities of cinnamon, Manuka honey as well as the combination of both against CRAB clinical isolates.

The results of the GC-MS analysis of the current study showed that cinnamaldehyde is the major constituent of cinnamon oil extract which is in agreement with the documented literature [33-35]. Ooi et al. demonstrated that the antimicrobial activity of cinnamon oil is mainly due to cinnamaldehyde [36]. Therefore, the antibacterial activity of cinnamon oil in the present study can be attributed to the high percentage of cinnamaldehyde present in the extracted cinnamon oil.

Several studies have investigated the antibacterial activity of cinnamon essential oil extracts against *A. baumannii*. Ganić et al. reported that cinnamon essential oil and its emulsion have strong antibacterial/antibiofilm effect against *A. baumannii* [37]. Moreover, Kaskatepe et al. reported that cinnamon oil demonstrated antibacterial activity against healthcare-associated CRAB isolates using disc diffusion method [38]. In a study conducted in Egypt, it was observed that cinnamon essential oils showed strong inhibitory effects of against *A. baumannii* strains that were isolated as contaminants from raw milk and some milk products [39]. Furthermore, Saber and Sadek evaluated the activity of cinnamon oil nano emulsion against *A. baumannii*, suggesting that the nano formulation may increase the activity of the cinnamon essential oil. They also reported the synergistic inhibitory effects of cinnamon oil nano emulations with different antibiotics against *A. baumannii* [40].

The activity of Manuka honey was first reported in the 1980s by Peter Molan. Although the inhibitory effect of Manuka honey was suggested due to the high sugar content, low pH and production of hydrogen peroxide, the effect continues when these factors are diluted to negligible levels or neutralized [41].

In 2008, MGO was identified in Manuka honey as a result of spontaneous dehydration of the precursor dihydroxyacetone (DHA) which naturally occurred in the nectar of *L. scoparium* flower and other related species in New Zealand and Australia. Due to an unknown mechanism, MGO can selectively interact with bacterial macromolecules including DNA, RNA and proteins non-specifically without damaging the host cells [42-44]**.** Nevertheless, the level of leptosin, which is a glycoside that is exclusively found in *Leptospermum* honey, and the levels of various phenolics strongly correlate with the antimicrobial activity as well as the potency of manuka honey [45]**.** Manuka honey has been evaluated for its antibacterial activity *in vitro* on a broad range of pathogenic bacteria. Manuka honey has demonstrated effective and broad-spectrum inhibition of many problematic bacterial pathogens, particularly MDR clinical isolates [46-49]**.** According to the authors' best knowledge, no reports of resistance to Manuka honey have been acquired even experimentally in the laboratories [50,51].

In addition, Manuka honey is well known to disrupt cellular aggregates and can prevent biofilm formation by various variety of pathogens including *A. baumannii*, *Enterobacter cloacae*, *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *P. aeruginosa*, *Staphylococcus*, and *Streptococcus* species [52-57].

In the present study, Manuka honey was evaluated for its antibacterial activity against MDR *A. baumannii* clinical isolates recovered from different sites of infections. Results showed that Manuka honey demonstrated antibacterial activity with the MIC value of 103.88 mg/mL. (7% v/v). In 2007, George and Cutting reported that concentrations of 8% were required to inhibit 82% of tested *A. baumannii* clinical isolates including the

pan-resistant strains [47]. Similarly, Carnwath et al have showed that Manuka honey with different grades exhibited antibacterial activity against *A. baumannii* from equine source [58]. Another study highlighted the strong response of the CRAB isolates from wound infections to Manuka honey [7]. Moreover, in a study from Malaysia, it was stated that Manuka honey has an antibacterial effect against clinically isolated *A. baumannii* with MIC of 12.5% [48]. Other studies have documented the effective antibacterial activity of Manuka honey against resistant *Acinetobacter* species [50,59].

Due to their significant antimicrobial activities, great attention was paid towards the exploration of the effect of their combination. The current study aimed to evaluate the postulated beneficial effects of cinnamon oil extract and Manuka honey as well as their combination against the MDR *A. baumannii* clinical isolates. The results showed additive effect indicating that the activity of the combination is equal to the sum of their independent activities. According to the authors' best knowledge, this is the first study that demonstrates the effect of such a combination.

In conclusion, Manuka honey and cinnamon oil extract are believed to be promising in combating MDR *A. baumannii*. However, further rigorous studies are still required to determine their best applications and clinical benefits potential. Hence, it is highly crucial to identify, characterize and standardize their most effective constituents and concentrations to ensure their consistent and effective use. Well-designed clinical trials are also of great importance to investigate their efficacy and safety profiles when used as alternative options, which are critical to safeguarding public health, or if combined with other conventional antibiotics for treating various MDR *A. baumannii* infections

Conflicts of interest

None to be declared.

Financial disclosure

None.

Data availability

All data generated or analyzed during this study are included in this puplished article.

Authors' contribution

All authors made significant contributions to the work presented, including study design, data collection, analysis, and interpretation. They also contributed to the article's writing, revising, or critical evaluation, gave final approval for the version to be published.

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