



ORIGINAL ARTICLE

Enhancement of wound healing via topical application of natural products: *In vitro* and *in vivo* evaluations



Abdallah S. Abdelsattar^{a,b}, Salsabil Makky^a, Rana Nofal^a, Mariam Hebishy^a,
Mona M. Agwa^c, Rania G. Aly^d, Mohamed Y. Abo El-Naga^e, Yehia A. Heikal^e,
Mohamed S. Fayez^a, Nouran Rezk^a, Ayman El-Shibiny^{a,f,*}

^a Center for Microbiology and Phage Therapy, Zewail City of Science and Technology, October Gardens, 6th of October City, Giza 12578, Egypt

^b Center for X-Ray and Determination of Structure of Matter, Zewail City of Science and Technology, October Gardens, 6th of October, Giza 12578, Egypt

^c Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Giza 12622, Egypt

^d Department of Surgical Pathology, Faculty of Medicine, Alexandria University, Alexandria, Egypt

^e Food Science Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

^f Faculty of Environmental Agricultural Sciences, Arish University, Arish 45511, Egypt

Received 19 November 2021; accepted 24 March 2022

Available online 29 March 2022

KEYWORDS

Bee venom;
Antibacterial;
Antimicrobial;
Antibiotic-resistant bacteria;
Wound infection;
Propolis;
Cytotoxicity;

Abstract Intact skin is the first physical barrier against all microbial infections. Thus, in the cases of wounds, burns, and skin damage, bacteria can infect and invade the deeper layers of skin to the bloodstream and other organs leading to severe illnesses. Thus, our study aims to investigate the potential activity of natural products, propolis and honeybee venom, to control wound infections with multi-drug resistant *Staphylococcus aureus* (MDRSA) and safely accelerate the wound healing. First, this study characterized the clinically isolated *S. aureus* using biochemical, molecular, and antibiotic sensitivity tests. Then, the hydrogel was prepared via mixing chitosan with honey, propolis, and venom at different ratios, followed by physicochemical characterization and biological examination. The *in vivo* experiment results after topical application of optimum concentrations revealed that both venom and propolis have significant antibacterial activity at different

* Corresponding author at: Center for Microbiology and Phage Therapy, Zewail City of Science and Technology, October Gardens, 6th of October City, Giza 12578, Egypt.

E-mail addresses: p-abdallah.abdelsattar@zewailcity.edu.eg (A.S. Abdelsattar), syoussef@zewailcity.edu.eg (S. Makky), p-rnofal@zewailcity.edu.eg (R. Nofal), mhebishy@zewailcity.edu.eg (M. Hebishy), mohamedyousef@agr.asu.edu.eg (M.Y. Abo El-Naga), aelshibiny@zewailcity.edu.eg (A. El-Shibiny).

Peer review under responsibility of King Saud University.



Synergetic effect;
Honey;
Chitosan

temperatures. The IC₅₀ of both propolis antioxidant and cytotoxicity assays was found to be 40.07 ± 2.18 µg/mL and 18.3 µg/mL, respectively. The cocktail bacteria showed both minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 10 µg/mL and of 300 µg/mL with venom respectively & MIC and MBC of 100 µg/mL, 300 µg/mL with propolis respectively. The use of hydrogel was effective against wound infection and enhanced wound healing during 14 days. Before starting clinical trials, further studies can be done on large animal models.

© 2022 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Due to the misuse of antibiotics over the past six decades, almost all pathogenic bacteria express antibiotic resistance characteristics. Moreover, the annual death rate due to the infection with anti-microbial resistant bacteria exceeds the cancer death rate by 2050 (Zolfagharian et al., 2016). Thus, the world is looking for alternatives to antibiotics to control the increasing bacterial infections. Among the suggested alternatives are the active compounds of natural products, bacteriophage (bacterial viruses) therapy, a synthetic compound, and metal nanoparticles. Although all the suggestions are promising, natural products are more recommended due to the low toxicity level, high accessibility, and social acceptance by consumers. Natural products may include plant extracts, essential oils, herbs, and honey. Here, we focus on honey and other bee-related compounds (propolis and bee venom).

Bee venom is a transparent, odorless compound composed of various bioactive proteins. It has around 18 active elements with many pharmaceutical properties ranging from enzymes and peptides to biogenic amines (e.g., apamin, melittin, adolapin, degranulating peptides, and hyaluronidase enzymes), making it effective against various diseases (Al-Waili et al., 2015; Wehbe et al., 2019). In addition, the bee venom has a therapeutic efficiency in treating inflammatory diseases including arthritis, fibrosis, tendonitis, lupus, rheumatoid and multiple sclerosis when administered at a therapeutic dose. However, bee predators have reported toxicity when administrated at high doses (El-Seedi et al., 2020).

On the other hand, Propolis is a resinous compound with a dark color and is collected from nectars by honeybees from the flora and trees buds of the area surrounding the hives and used to maintain the hive structure (Hannan et al., 2015). Hence, its chemical composition varies from one place to another. Propolis is mainly composed of wax (30%), resin (50%), pollen (5%), essential oils (10%), and other substances (5%), including minerals, debris, and organic compounds (El-Guendouz et al., 2018). A broad spectrum of biological activities such as antioxidant, antifungal, anticancer, antibacterial, and anti-inflammatory responses has been linked to propolis active compounds (Hannan et al., 2015; Fernandes Júnior et al., 2005). In particular, the antimicrobial properties of propolis are due to the synergistic effect of phenolics and other compounds such as flavonoids, galangin, pinocembrin, and pinobanksin (Fernandes Júnior et al., 2005). Chitosan was used in several studies as an accelerator for wound healing in animal models (Ueno et al., 1999; Tang et al., 2016; Ueno et al., 2001), in addition to its antimicrobial activity that makes the possible risks at the lowest level in wound infections (Mi et al., 2002; Ong et al., 2008). Recently, the gelatin was used in the preparation of wound dressing due to its ability to absorb the water and stimulate the macrophages (Pawde and Deshmukh, 2008; Saarai et al., 2012).

Among the isolated bacteria from infected wounds, especially diabetic foot ulcers, *Staphylococcus aureus* (*S. aureus*) is considered one of the most pathogenic bacteria to treat due to the multiple virulence factors and its ability to resist various antibiotics (Serra et al., 2015; Halcón and Milkus, 2004). *S. aureus* is a cluster coccus bacterium, frequently associated with the human body parts, either the throat, nose, vagina, or skin, due to its capability of causing various infections and

its competence to adapt to diverse environmental conditions (El-Guendouz et al., 2018). Accordingly, these characteristics gave *S. aureus* the privilege of antibiotic resistance development, making it one of the most difficult to treat pathogens. The Multi-Drug Resistant (MDR) *S. aureus* exploits a variety of virulence factors, including coagulase, collagenase, protein A, hemolysins, lipase, hyaluronidase, adhesive proteins, multiple toxins, and also proteins involved in biofilm formation (Szweda and Kot, 2017).

Biofilm has a vital role in bacterial infection as it acts as a physical barrier preventing antibiotics from penetrating the polysaccharide matrix and halting the antibiotics clearance mechanisms (Halcón and Milkus, 2004). Moreover, it is the most critical virulence factor playing an essential role in passing the virulent genes between different bacteria species, enhancing antibiotic resistance (Høiby et al., 2010; Stewart, 2002; Fadl, 2018). The rise in antimicrobial resistance opens the door for searching for new alternatives with varying modes of action. Moreover, various studies have been established to testify the ability of bee venom and propolis to control *S. aureus* and its biofilm formation (Szweda and Kot, 2017; Fadl, 2018; Han et al., 2016; Galdiero et al., 2019; Grecka et al., 2020). Many previous studies tried to evaluate the anti-biofilm activity of bee venom, melittin, and macropin peptides against *S. aureus* (Picoli et al., 2017; Alni et al., 2020; Ko et al., 2017). However, to date, this is the first trial to evaluate the ability of bee venom and propolis to control the biofilm formed by a cocktail of MDRSA bacteria isolated from patients.

The process of combining the potential compounds with natural products was used successfully to obtain the synergism against multidrug-resistant bacteria (Cheesman et al., 2017). In some cases, the combination between compounds generates synergism and tackles the efflux pump mechanism in *S. aureus* (Pereira et al., 2019). Therefore, the propolis extract became one of the antibacterial agents that was combined with antibiotics (Neto et al., 2017) or the plant extracts (da Silveira Regueira-Neto et al., 2019) against *S. aureus*. Furthermore, in several studies, propolis (Oryan et al., 2018), bee venom (Al-Waili et al., 2015), chitosan, glycerol (Zoghi et al., 2021), and honey (Molan, 1999) were used separately or in combinations to treat the wound infection and accelerate the healing process.

This study aims to provide an alternative to antibiotics to treat a cocktail of MDRSA isolated from clinical samples using hydrogels through both *in vitro* and *in vivo* treatment models. In addition, various concentrations of venom and propolis were tested to inhibit and disrupt MDRSA biofilm formation. Moreover, providing a chemical profile and analytical data that can be used in future studies.

2. Results and discussion

2.1. Bacterial characterization and antibiotic sensitivity

Using colony PCR, the isolated bacteria depicted amplicons of 279 bp for the nuc gene: catalase-positive and oxidase-negative. Moreover, the antibiotic sensitivity disk diffusion test showed that all isolates (n = 11) are resistant to Amoxicillin, whereas they are all sensitive to Vancomycin and Linezolid.

Table 1 Antibiotic sensitivity profile results in millimeters (mm) for the MDRSA clinical isolates.

Class	Clinical Isolates																			
	Antibiotics (µg)		ZCSA/02	ZCSA/03	ZCSA/04	ZCSA/05	ZCSA/08	ZCSA/06	ZCSA/14	ZCSA/15	ZCSA/16	ZCSA/17	ZCSA/24							
β-lactam	Amoxicillin(10)	R-10	R-10*	R-6	R-6	R-6	R-6	R-6	R-6	R-6	R-6	R-6	R-8							
Penicillins	Oxacillin(1)	S-14	S-13 ⁺⁺	R-0	R-8 ⁺⁺	R-9	R-9	R-9	R-9	R-9	R-9	R-9	R-9							
Aminoglycoside	Gentamicin (30)	R-12 ⁺	R-8*	R-9*	R-11	S-24	S-26	R-8	R-8	R-8*	S-21	S-26	S-24							
Glycopeptide	Vancomycin(30)	R-14	R-13	S-19	R-13	R-12	R-13	R-14	R-14	R-14	R-13	R-14	R-14							
Macrolide	Erythromycin(15)	S-24	I-22	S-25	S-24	R-12*	S-25	R-11	S-24	S-24	I-19	S-23	S-23							
Quinolone	Ciprofloxacin(5)	S-26	I-19	S-24	S-22	S-22	S-24	I-20	S-24	S-24	S-23	S-25	S-22							
	Levofloxacin(5)	S-25	S-21	I-16	S-24	S-24	S-26	S-23	S-28	S-28	S-21	S-26	S-24							
Tetracyclines	Tetracycline(10)	R-8*	R-9	R-8	R-7	R-8	R-6	R-8	R-8	R-8	S-18*	R-9	S-21 ⁺⁺							
	Doxycycline(30)	S-20*	I-14	R-9	R-11	R-12	R-11	R-8	R-11	R-11	S-23 ⁺⁺	S-19 ⁺⁺	S-25							
Phenicol	Chloramphenicol (30)	R-9	S-20	S-24 ⁺	S-20	R-10*	R-10*	R-10*	R-9	R-9	S-21	S-21	S-22							
Oxazolidinones	Linezolid(30)	S-30	S-25	S-28	S-25	S-32	S-36	S-26	S-28	S-28	S-31	S-30	S-26							
Lincosycin	Clindamycin(2)	S-21 ⁺	S-21 ⁺	S-23	I-20	R-6*	I-20	R-5	S-24	S-24	20 ⁺	I-19	I-20							
Minobenzesulfonamides	Sulfamethoxazole trimethoprim (25)	S-16	R-10	S-19	S-21	I-14	S-17	S-22	S-19	S-19	S-19	S-25	S-23							

R = Resistant I = Intermediate S = Susceptible.
⁺ Bacteria developed resistance in the inhibition zone after 24 h (large colonies); ⁺⁺ Bacteria developed resistance in the inhibition zone after 24 h (small colonies); *Bacteria are showing efflux bump signs on the plates.

Only two isolates were susceptible to Oxacillin. The rest of the antibiotic panel results are presented in (Table 1).

The clinical isolates were confirmed to be *S. aureus* using colony PCR, in which the *muc* gene was detected. Furthermore, the antibiotic sensitivity profile showed that many isolates, including ZCSA/04, 05, 08, 09, 15, 16, and 24 are resistant to Penicillin (64%) and β-lactam (100%) drug classes. Moreover, many of the isolates showed resistance to Tetracycline (73%), Gentamicin (55%), Doxycycline (55%), Chloramphenicol (36%), Clindamycin (18%), and Sulfamethoxazole trimethoprim antibiotic (9%). The most effective antibiotics were Linezolid, Ciprofloxacin, and Levofloxacin, all of which did not display (0%) resistance. Yet, the last two antibiotics, Ciprofloxacin and Levofloxacin, presented intermediate effects on some strains. On the plates, Linezolid produced the widest inhibition zone. Some bacterial isolates expressed efflux pump in the plate as they showed an inhibition zone followed by an outer concentric bacterial growth followed by a second outer concentric inhibition zone. Among the antibiotics that showed an inconsistent efflux bump were Oxacillin, Gentamicin, Erythromycin, Tetracycline, Doxycycline, Chloramphenicol, and Clindamycin.

2.2. Disc diffusion test for venom and propolis

The extracted bee venom and propolis showed a significant antibacterial activity starting from 41.2 µg/mL (0.4 µg/disc) on strains ZCSA/02, 06, and 15. However, the most effective concentration for inhibiting the bacteria was ≥ 123 µg/mL (1.23 µg/disc). In addition, the diameters of inhibition zones were between 13 and 10 mm for the 10 mg/mL (100 µg/disc) and from 8 to 10 mm in the concentration of 123 µg/mL. At the same time, propolis showed diameters of inhibition zones between 30 and 20 mm for the (100 µg/disc), displaying the most significant results as in Table 2.

Bee venom has similar inhibition zones to the snake *Echis carinatus* venom when tested on MDRSA isolates with 100 µg/mL (8.2 ± 0.5 mm). However, it produced larger inhibition zones (11.09 ± 2.3 mm) with *S. aureus*, showing sensitivity toward the methicillin antibiotics (Jami et al., 2010). In addition, using bee venom on MDRSA had fewer effects than other bee venoms tested on *S. aureus* (ATCC 25923) and presented an inhibition zone of 15.51 ± 1.0 mm at a concentration of 45 µg/disc (Zolfagharian et al., 2016). Furthermore, bee venom in this study showed similar inhibition zones to venom alkaloid solenopsin A (diameter = 11 mm) and slightly higher than that of solenopsin B and C (diameter = 9 mm and 8 mm, respectively) (Jouvenaz et al., 1972), and significantly greater than that of Heterometrus laoticus scorpion venom (inhibition zone = 2.4 mm around the disc) when tested on *S. aureus* (Uawonggul et al., 2007).

2.3. MIC and MBC of venom and propolis

The bacterial isolates presented an inhibition in the growth when treated with bee venom with 10 µg/mL and showed a complete bactericidal effect at the concentration of 30 µg/mL. For instance, the two isolates ZCSA/06 and ZCSA/16 showed a higher sensitivity towards bee venom since the growth of *S. aureus* was inhibited at a concentration of 3 µg/mL, whereas their MBC was 10 µg/mL. In the case of bacterial

Table 2 Shows the inhibition zones (mm) resulting from the venom and propolis disk diffusion test.

		100 µg/disc	33.3 µg/disc	11 µg/disc	3.7 µg/disc	1.23 µg/disc	0.4 µg/disc
ZCSA/02	venom	13	10	10	10	9	0
	propolis	24	15	14	12	8	0
ZCSA/03	venom	11	10	10	10	8	0
	propolis	25	16	15	12	8	0
ZCSA/04	venom	12	11	11	9	10	0
	propolis	20	11	10	8	0	0
ZCSA/05	venom	11	11	11	10	10	9
	propolis	28	15	15	12	7	0
ZCSA/06	venom	11	11	11	11	10	7
	propolis	23	15	16	12	7	0
ZCSA/08	venom	11	10	10	10	8	0
	propolis	22	13	14	11	7	0
ZCSA/14	venom	11	11	10	9	9	0
	propolis	24	14	13	11	8	0
ZCSA/15	venom	11	10	10	10	10	8
	Propolis	30	14	15	13	9	0
ZCSA/16	Venom	11	11	11	11	10	0
	Propolis	22	13	13	10	8	0
ZCSA/17	venom	11	9	11	10	8	0
	propolis	25	14	14	11	8	0
ZCSA/24	venom	10	10	10	10	8	0
	propolis	26	16	16	12	8	0

cocktails, venom MIC was 10 µg/mL, and the MBC was 300 µg/mL, which means that the MIC is one thirty-folds of MBC when used against different strains of bacteria in one cocktail. However, the propolis had a MIC of 100 µg/mL and MBC of 300 µg/mL in case of the cocktail. Whereas, with some strains (ZCSA/05, 06, 15, and 17), both propolis MIC and MBC were the same (100 µg/mL) (Table 3).

The MIC for bee venom against MDRSA was between 0.085 and 12.25 µg/mL, while the MBC was ≥ 12.25 µg/mL (Han et al., 2016; Shebeeb et al., 2020). The bee venom showed lower MIC and MBC than Snake venom (Boda et al., 2019) and lowered MBC than the spider *Lasiadora* sp. (Chen et al., 2018). Our results revealed a higher concentration of bee venom to kill the bacterial cocktail compared to the MBC of each isolate alone with the same MIC concentration for the bee venom in both cases. This finding suggested that the bacterial cocktail of the same species has a higher persistence ability. Thus, bee venom, in the case of the cocktail of cells, acts as a bacteriostatic agent instead of a bactericidal one because its MBC is more than four-folds of MIC (Salada et al., 2015). The results of the MIC and MBC of propolis were better as it was 100 µg/mL and 300 µg/mL for MIC and MBC respectively, compared to the previously reported results (El-Guendouz et al., 2018 (2018).) that were 360 µg/mL for MIC and 1220 µg/mL for MBC.

2.4. *In vitro* antioxidant activity of propolis

The preliminary screening of the propolis IC₅₀ turned out to be lower than 100 µg/mL. Consequently, the following concentrations of propolis 10, 20, 40, 50, 60, and 80 µg/mL were used to build a curve to estimate the IC₅₀ of the propolis. The IC₅₀ of the sample was found to be 40.07 ± 2.18 µg/mL compared to the Trolox standard (24.42 ± 0.87 µg/mL).

DPPH is a stable radical with maximum absorption at 517 nm and can be easily scavenged by antioxidants (Boly et al., 2016). The IC₅₀ was obtained by calculating the concentration of the propolis having the ability to reduce the initial concentration of DPPH by 50%. Consequently, higher antioxidant activity is implied by a lower IC₅₀ (Sowndhararajan and Kang, 2013). Several studies determined a correlation between the antioxidant activity of ethanolic extracts of propolis and their phenolic contents, such as flavonoids (Cunha et al., 2004; Kortenska et al., 2002; Kumazawa et al., 2004). As presented in (Fig. 1), the scavenging capabilities of propolis were expressed as IC₅₀ of 40.07 µg/mL, which would be sufficient for its antioxidant activity, which in turn scavenged half of the free radicals. The presence of flavonoids in the chemical composition of the propolis supports its antioxidant activity (Brito Pereira Bezerra Martins et al., 2018).

2.5. Cytotoxicity assay

The sulforhodamine B (SRB) assay was conducted to examine the cytotoxic effect of bee venom using a noncancerous human skin fibroblast (HSF) cell line with different concentrations of bee venom (0.01, 0.1, 1, 10, 100 µg/mL). The results showed that the IC₅₀ of bee venom is 18.3 µg/mL by measuring the cell viability after adding the bee venom with the culture media.

The cytotoxicity assay results indicated that any concentration below 18.3 µg/mL is safe for treatment. However, concentration above or equal to this value will be lethal to the cell (Fig. 2), which clearly explains the significant reduction in cells at a concentration of 100 µg/mL. Previous results (Sobral et al., 2016) that tested the cytotoxicity effect of bee venom on non-cancerous liver cells gave a growth inhibitory 50 of 15.03 ± 0.28 , 13.86 ± 0.10 , 10.11 ± 0.91 , 12.94 ± 1.21 , 14.66 ± 0.66 for various bee venom samples. Another study

Table 3 Displays MIC and MBC values for bee venom and propolis against *S. aureus* bacterial isolates.

Concentration	MIC*			MBC**			
	3 µg/mL	10 µg/mL	100 µg/mL	10 µg/mL	30 µg/mL	100 µg/mL	300 µg/mL
Venom	ZCSA/06	ZCSA/02		ZCSA/06	ZCSA/02		Cocktail (11 strains)
	ZCSA/16	ZCSA/03		ZCSA/16	ZCSA/03		
		ZCSA/04			ZCSA/04		
		ZCSA/05			ZCSA/05		
		ZCSA/08			ZCSA/08		
		ZCSA/14			ZCSA/14		
		ZCSA/15			ZCSA/15		
		ZCSA/17			ZCSA/17		
		ZCSA/24Cocktail			ZCSA/24		
		(11 strains)					
	Propolis			ZCSA/02			
			ZCSA/03			ZCSA/06	ZCSA/03
			ZCSA/04			ZCSA/15	ZCSA/04
			ZCSA/05			ZCSA/17	ZCSA/08
			ZCSA/06				ZCSA/14
			ZCSA/08				ZCSA/16
			ZCSA/14				ZCSA/24Cocktail
			ZCSA/15				(11 strains)
			ZCSA/16				
			ZCSA/17				
			ZCSA/24Cocktail				
		(11 strains)					

*The MIC value means it has a bacteriostatic effect on equal or higher concentrations than MIC.

**The MBC value means it has a bactericidal effect on equal or higher concentrations than MBC.

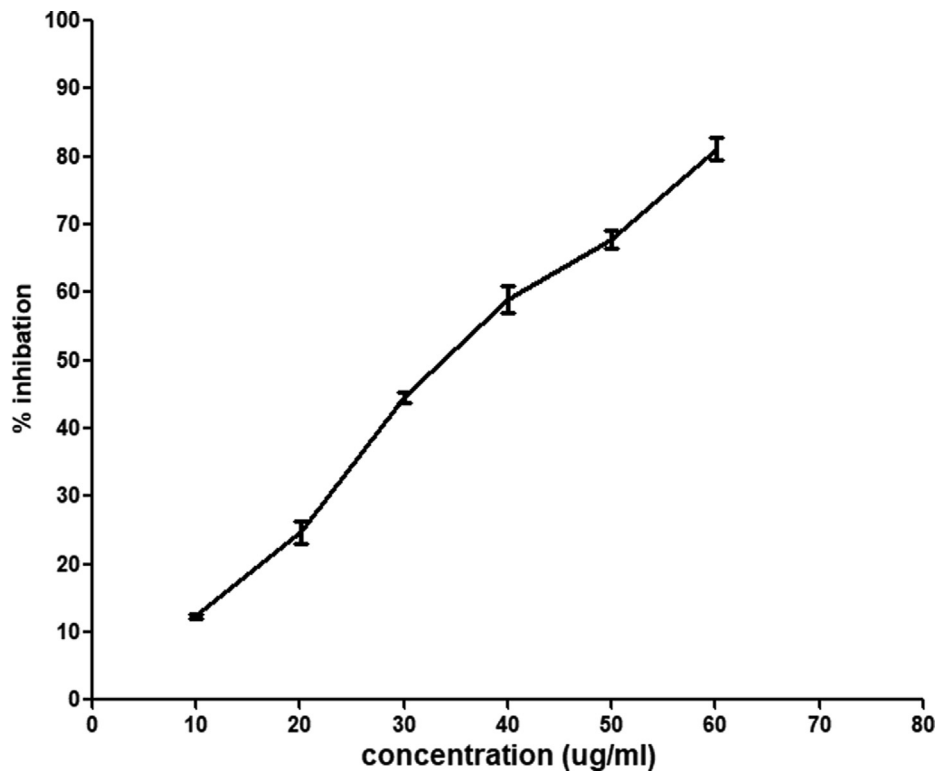


Fig. 1 Shows the relationship among the different concentrations of propolis and radical scavenging using DPPH assay. The results of antioxidants were carried out in triplicate (n = 3).

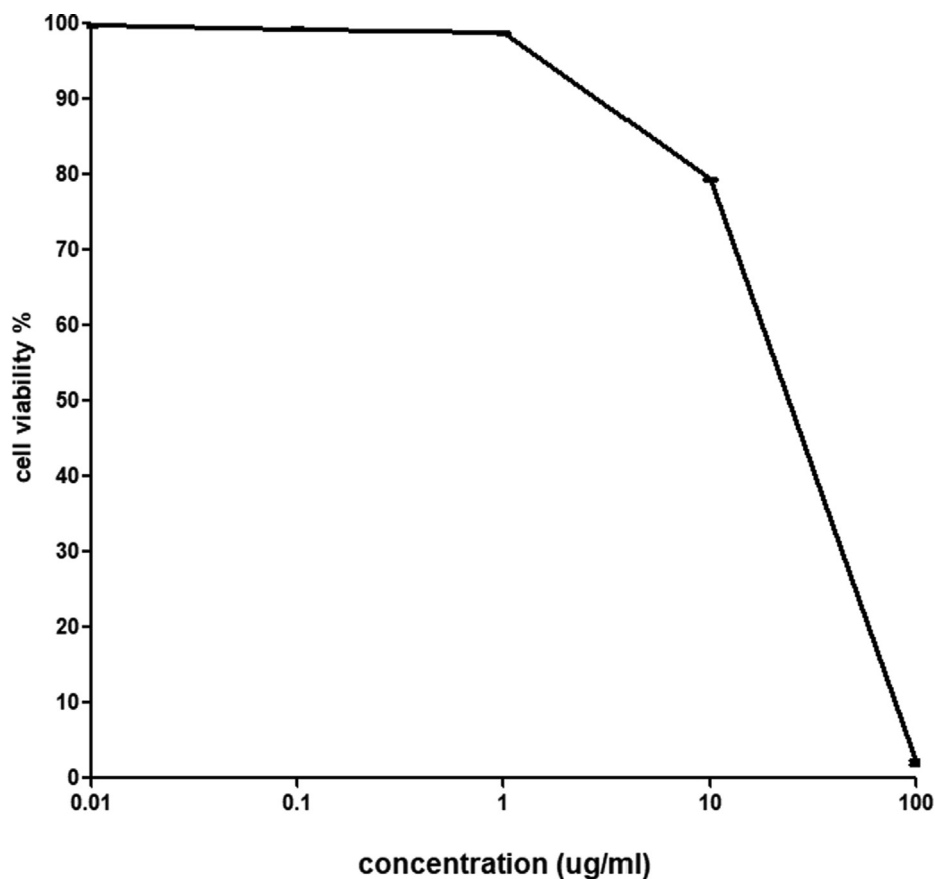


Fig. 2 Shows the percentage of cell viability after adding different concentrations of bee venom (IC_{50} is 18.3 $\mu\text{g/mL}$). The cell viability results were carried out in triplicate ($n = 3$).

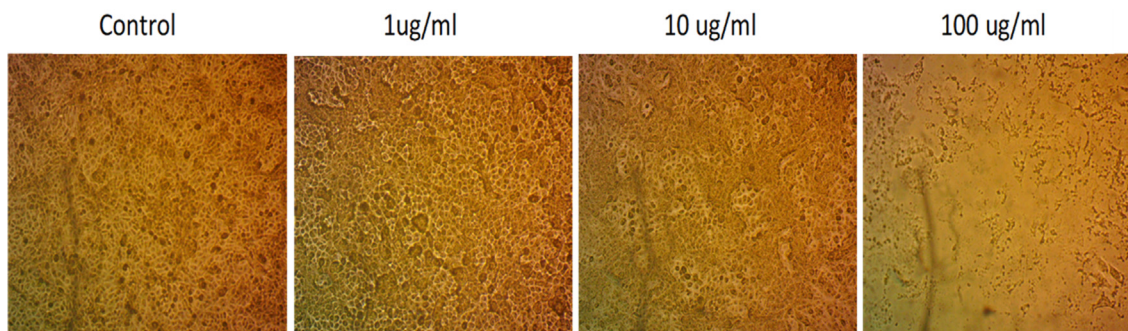


Fig. 3 Microscopic images of Human Skin Fibroblast (HSF). Cells were treated with bee venom at different concentrations without adding a stain.

by Ku et al. (Ku et al., 2020) demonstrated that bee venom has no cytotoxic effect on the cellular model of Alzheimer's Disease using a concentration below 10 $\mu\text{g/mL}$. These results indicate that the cytotoxic effect of our bee venom on non-cancerous cells fluctuates in the same range (see Fig. 3).

2.6. Gas chromatography/mass spectrometry (GC-MS)

Propolis samples were analyzed by gas chromatography and identified by the mass spectrum through an energy-rich ray of electrons ionized form of the separated molecules to study

active propolis compounds. The results highlighted 45 compounds separated from propolis samples over a run time of 45 min. Clear peaks were obtained, as seen in the chromatogram (Fig. 4). The separated components could be categorized as follows: Acids, Esters, Alcohols, phenols, aldehydes, ketones, amides, amines, flavonoids, hydrocarbons, and sugars. Table 4 gives the details of the separated components and their area percentage under the chromatogram.

As seen from Table 4, Egyptian propolis from the North-Sinai area consists of 20.66% acids (aliphatic and aromatic); 4.88% esters; 20.65% alcohols, phenols, aldehydes, and

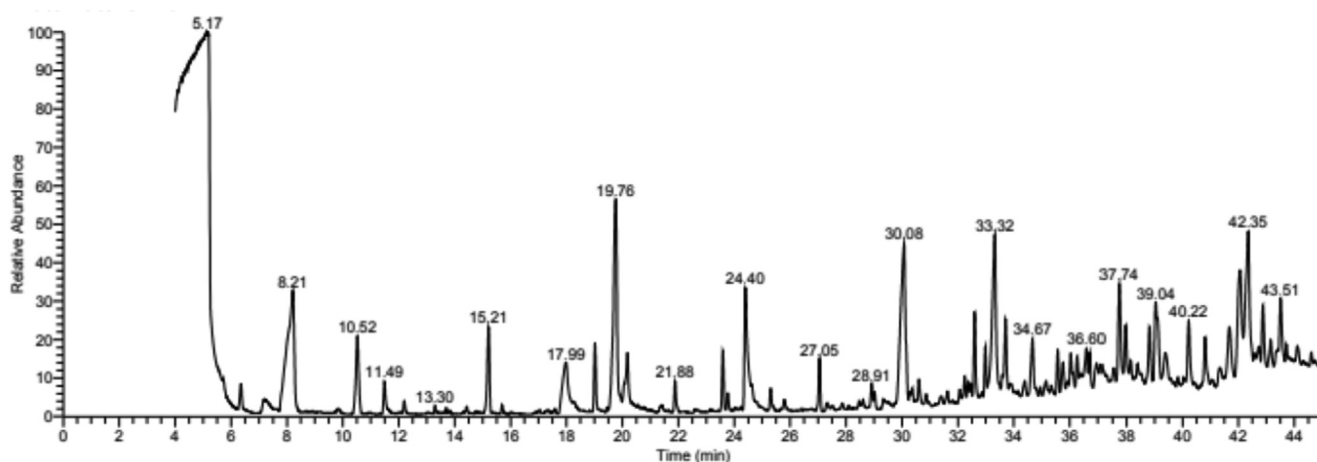


Fig. 4 Represents the GC–MS for ethanolic extract of propolis.

ketones; 27.84% amides and amines; 4.14% flavonoid; 18.63% hydrocarbons and terpenes as well as 3.21% sugar derivatives and silicon oil.

Propolis is well known for its biological activities and can be used in wide applications in medicine, such as antibacterial, antifungal, antioxidant and anticancer, and food industries (Christov et al., 1998; Hegazi and Abd El Hady, 2001; Bakdash et al., 2018). The diversity of biological activities for the tested propolis sample could be due to different active components identified by GC analysis. For example, the antioxidant activity could be referred to as the presence of siloxans elements, 7,9-di-*tert*-butyl-1-oxaspirodeca-diene-dione, tetrahydro-hydroxy-methylethyl-methoxy,2-Naphthalenol, 3TMS derivative of methyl glycocholate, benz-indenophenanthrene, and Isochiapin A.

The predominant component in propolis is Trifluoro-N-methyl acetamide (14.89%), which is usually used to derive and prepare propolis extract for GC analysis. The relatively low content of esters and flavonoid compounds agrees with other researchers' results (Christov et al., 1998; Abd El Hady and Hegazi, 2002) in Egyptian propolis samples. In addition, palmitic and 9-octadecenoic acids were dominant acid components of the tested propolis sample (12.17%). On the other hand, ethanol, 2,2-dithiobis, tetrahydro-hydroxy-methyl ethyl-methoxy,2-Naphthalenol, and methyl-4-[(trimethylsilyl)oxy]-2-pentanone were the dominant alcohols and ketones in the North Sinai-propolis (12.61%). Amines compounds are present in a concentration of 8.87% (N,N-dimethyl-1-dodecanamine and N,N-dimethyl-1-tetradecanamine). Hydrocarbons and terpenes represented 18.63% of the area under the chromatographic curve, with siloxane components making more than 50% of this area. Sugar derivatives (Dimethyl-hexenyl-tetrahydro-hydroxy-dimethyl-naphthalenyl arabinopyranoside) are also present with a concentration of 2.82%.

The antimicrobial activity could be referred to as the presence of siloxans, methyl-4-[(trimethylsilyl)oxy]-2-pentanone, (dimethyl-7-oxo-7-hexahydro-2-naphthalenyl) propionic acid, N-[(fluorophenyl)methyl]-purin-amine, and phenoxazine phenol derivative components. In addition, two components with antiviral activity (N-methylbenzenesulfonamide and tri-*o*-acetyl-ingol) are present in the tested propolis sample. Also, methyl ester of eicosatriynoic acid, tetramethyl-tetrahydro-5

H-chromen-8-ol, dimethyl-hexenyl-tetrahydro-hydroxy-dimethyl-naphthalenyl arabinopyranoside (dimethyl-7-oxo-7-hexahydro-2-naphthalenyl), and propionic acid have anti-inflammatory effects. In addition, tetramethyl-tetrahydro-5H-chromen-8-ol could be considered as provitamin E, while dehydroergosterol tosylate is a precursor for vitamin D2.

The GC-analysis of North Sinai propolis proved the existence of compounds with antiulcer, anticancer, and immunity enhancers such as diethyl ester of 1,2-benzene dicarboxylic acid, tetrahydro-hydroxy-methylethyl-methoxy,2-Naphthalenol, spiro-tricyclodecane-oxirane, and hydroxy pregnanone. The tested propolis samples also contain skincare, emollient, and wound healing components such as dodecanol, octanal-2-phenylmethylene, naphthalene-dimethyl-methylethyl, acetoxypregnan-ol-one-oic acid lactone, (Carboxy-methyl-butenyl) decahydro-dimethyl-methylene-1-Naphthalenecarboxylic acid, palmitic acid ester, dotriacontane, fatty acids, and silicon oil. It also contains steroidal compounds such as; Androst-5,7-dien-3-ol-17-one, dehydroergosterol tosylate, tri-*o*-acetyl-ingol, hydroxy pregnanone, and Dihydroxy-oxoprostatrienoic acid, which works as male and female hormones. These compounds were not reported in propolis samples collected from upper Egypt (Hegazi and Abd El Hady, 2001) or the Nile delta (Said et al., 2006). Also, propolis samples collected in Saudi Arabia did not contain these components (Bakdash et al., 2018; Elnakady et al., 2017). These steroidal components in North Sinai propolis samples could be due to the remarkable flora of medicinal and ornamental plants in the Saini peninsula. Based on the results mentioned above, the tested propolis samples could be helpful in the treatments of wound infection.

The chemical composition of fresh bee venom that, was analysed using the high performance liquid chromatography (HPLC), is consist of 1–3.7% apamin, 9–12.9% phospholipase A2, and 63–75% melittin (Rybak-Chmielewska and Szczêsna, 2004), in addition to sugar content, including Fructose (3–7.9%), Glucose (0.1–5.6%), Sucrose (1.4–2%), Turanose (<0.7%), Maltose (<0.3%), isomaltose (<0.4%), Erllose (<0.65%), and Melezitose (<1.5%) (Samanci and Kekeçođlu, 2019). Moreover, the chemical composition of honey is water (13.2–26.5%), Fructose (37–42.6%), Glucose (18.2–32.1%), Sucrose (0.3–16.5%), other sugars around

Table 4 Chemical composition (%) for ethanolic extract of propolis obtained from the North Sinai area.

No.	Component	Formula	Area %
Acids			
1	n-Hexadecanoic acid (Palmitic acid)	C ₁₆ H ₃₂ O ₂	7.79
2	(Dimethyl-7-oxo-7-hexahydro-2-naphthalenyl) propionic acid	C ₁₅ H ₂₀ O ₃	1.56
3	Acetoxypregnan-ol-one-oic acid lactone	C ₂₃ H ₃₂ O ₅	1.58
4	Dihydroxy-oxo-prostatrienoic acid (Prostaglandin)	C ₂₀ H ₃₀ O ₅	1.61
5	9-octadecenoic acid	C ₁₈ H ₃₄ O ₂	4.38
6	Octadecanoic acid (stearic acid)	C ₁₈ H ₃₆ O ₂	1.92
7	(Carboxy-methyl-butenyl) decahydro-dimethyl-methylene-1-Naphthalenecarboxylic acid	C ₂₀ H ₃₀ O ₄	1.82
Total percentage area of acids		20.66	
Esters			
8	1,2-Benzene dicarboxylic acid, diethyl ester	C ₁₂ H ₁₄ O ₄	0.74
9	Hexadecanoic acid, 2,3-dihydroxy-propyl ester (glyceryl monopalmitate)	C ₁₉ H ₃₈ O ₄	0.96
10	Eicosatriynoic acid, methyl ester	C ₂₁ H ₃₀ O ₂	0.57
11	Dehydroergosterol tosylate	C ₃₅ H ₄₈ O ₃ S	0.95
12	3TMS derivative of methyl glycocholate	C ₃₆ H ₆₉ NO ₆ Si ₃	1.66
Total percentage area of esters		4.88	
Alcohols, Phenols, Aldehydes, and Ketones			
13	Ethanol, 2,2-dithiobis	C ₄ H ₁₀ O ₂ S ₂	2.27
14	Dodecanol	C ₁₂ H ₂₆ O	1.63
15	Tetramethyl-tetrahydro-5H-chromen-8-ol	C ₁₃ H ₂₀ O ₂	0.69
16	Tetrahydro-hydroxy-methyl ethyl-methoxy,2-Naphthalenol	C ₁₄ H ₂₀ O ₃	2.17
17	3H-Phenoxazine phenol derivative	C ₂₀ H ₁₆ N ₂ O ₂	1.69
18	Naphthalene-dimethyl-methylethyl	C ₁₅ H ₁₈	0.51
19	Octanal-2-phenylmethylene	C ₁₅ H ₂₀ O	0.60
20	2-Phenanthrenecarboxaldehyde,9-dodecahydro-hydroxy-tetramethyl	C ₁₉ H ₃₀ O ₂	0.76
21	Methyl-4-[(trimethylsilyl)oxy]-2-pentanone	C ₉ H ₂₀ O ₂ Si	8.17
22	Hydroxy pregnanone	C ₂₁ H ₃₄ O ₂	1.29
23	Androst-5,7-dien-3-ol-17-one	C ₁₉ H ₂₆ O ₂	0.87
Total percentage area of alcohols, phenols, aldehydes, and ketones		20.65	
Amides and Amines			
24	Trifluoro-N-methyl acetamide	C ₃ H ₄ F ₃ NO	14.89
25	N-Methylbenzenesulfonamide	C ₇ H ₉ NO ₂ S	0.77
26	N,N-dimethyl-1-dodecanamine	C ₁₄ H ₃₁ N	5.78
27	N,N-dimethyl-1-tetradecanamine	C ₁₆ H ₃₅ N	3.09
28	N-[(Fluorophenyl)methyl]- purin-amine	C ₁₂ H ₁₀ FN ₅	1.29
29	N-methyl-N-benzyl-dodecanamine	C ₂₀ H ₃₅ N	2.02
Total percentage area of amides and amine		27.84	
Flavonoids			
30	7,9-Di- <i>tert</i> -butyl-1-oxaspirodeca-diene-dione	C ₁₇ H ₂₄ O ₃	0.60
31	Spiro-tricyclodecane-oxirane	C ₁₅ H ₂₄ O ₃	3.54
Total percentage area of flavonoids		4.14	
Hydrocarbons and Terpenes			
32	Hexadecamethyl-cyclooctasiloxane	C ₁₆ H ₄₈ O ₈ Si ₈	1.40
33	Octamethyl-cyclotetrasiloxane,	C ₈ H ₂₄ O ₄ Si ₄	0.69
34	Octadecamethyl-cyclononasiloxane	C ₁₈ H ₅₄ O ₉ Si ₉	1.24
35	Docamethyl-cyclopentasiloxane	C ₁₀ H ₃₀ O ₅ Si ₅	2.32
36	Dodecamethyl-cyclohexasiloxane	C ₁₂ H ₃₆ O ₆ Si ₆	2.41
37	Tetramethyl-dioxa-dithia-disiladodecane	C ₁₀ H ₂₆ O ₂ S ₂ Si ₂	1.47
38	Dotriacontane	C ₃₂ H ₆₆	1.33
39	Benz-indeno-phenanthrene	C ₂₅ H ₁₆	4.10
40	Hexadecamethyl-octasiloxane (separated in two peaks at 32.98 and 35.87 min)	C ₁₆ H ₅₀ O ₇ Si ₈	2.08
41	Tri-o-acetyl-ingol	C ₂₆ H ₃₆ O ₉	0.78
42	Isochiapin A	C ₁₉ H ₂₆ O ₆	0.81
Total percentage area of hydrocarbons and terpenes		18.63	
Others			
43	Dimethyl-hexenyl-tetrahydro-hydroxy-dimethyl-naphthalenyl arabinopyranoside	C ₂₅ H ₃₈ O ₆	2.82
44	Silicon oil	N/A	0.39
Total percentage area of other components		3.21	

8.5%. In addition to enzymes includes esterases, Acid phosphatase, protease, catalase, glucose oxidase, amylase, and glucosidase (Santos-Buelga and González-Paramás, 2017).

2.7. Evaluation of antibacterial activity

The bactericidal activity of bee venom and propolis was measured after 24 h of incubation at two different temperatures (4 and 37 °C). The bacterial cell count at 4 °C decreased from 5.01×10^8 CFU/mL to lower than the detection limit, 10^3 CFU/mL. In addition, a significant ($p \leq 0.0001$) reduction in the bacterial count at 37 °C was observed when the titer was reduced from 1.28×10^9 CFU/mL to 5×10^4 CFU/mL (Fig. 5). The cocktail of bacteria treated with propolis at 37 °C was found to be decreased lower than the limit of detection (10^3), while at 4 °C, propolis was not effective. Moreover, treatment with honey alone was not effective. However, the combination of venom, propolis, and honey with 1x MBC reduced until the limit of detection at 4 °C as well as at 37 °C.

The results of antibacterial activity after 24 h showed the ability of bacteria to persist (not to resist) the effect of bee venom. A population of bacteria with small size was below the limit of detection at 4 °C. However, at 37 °C, this population started to grow slowly. According to the antibacterial activity of propolis at 37 °C and 4 °C, the results showed that propolis is better to be used at 37 °C, not at 4 °C and gave better effect when it is combined with both honey and venom.

2.8. Time-kill kinetics

The analysis of results of the time-kill curve proclaims a rapid bactericidal activity of bee venom at different concentrations, with more than 99.99% killing rate within two hours for the treated cocktail of 11 MDRSA isolates with 10x MIC concentrations and more than 99.999% killing rate after 4 h (Fig. 6). The bacterial killing was faster and more potent with 100x MIC (MBC concentration) as it reached the limit of detection in < 2 h and was stable for 6 h. However, the titer of bacteria began to increase after 6 h of incubation with 10x MIC and after 24 h of incubation with 100x MIC of venom. The result of the time-kill curve of bee venom and propolis at both 4 °C and 37 °C using a cocktail of the 11 bacteria showed that bacteria treated with bee venom at 37 °C was reduced to 10^3 CFU/mL after 70 min compared to 10^6 CFU/mL in control. In comparison, at 4 °C, it was reduced to 10^4 CFU/mL after 70 min. However, propolis at 37 °C reduced the bacterial titer to 10^3 CFU/mL, with no antibacterial effect at 4 °C.

Although the MIC experiment is the gold standard for measuring the activities of antibacterial agents, it does not reveal important data about the bactericidal effect concerning time. The time-kill kinetics can overcome this limitation. In previous studies, Mastoparan, isolated from wasp venom, against *A. baumannii* (ATCC 19606), was used with 4x MIC significantly reduced the bacterial titer after 4 h, then the bacterial titer increased again (Daikh et al., 2020). Another study using

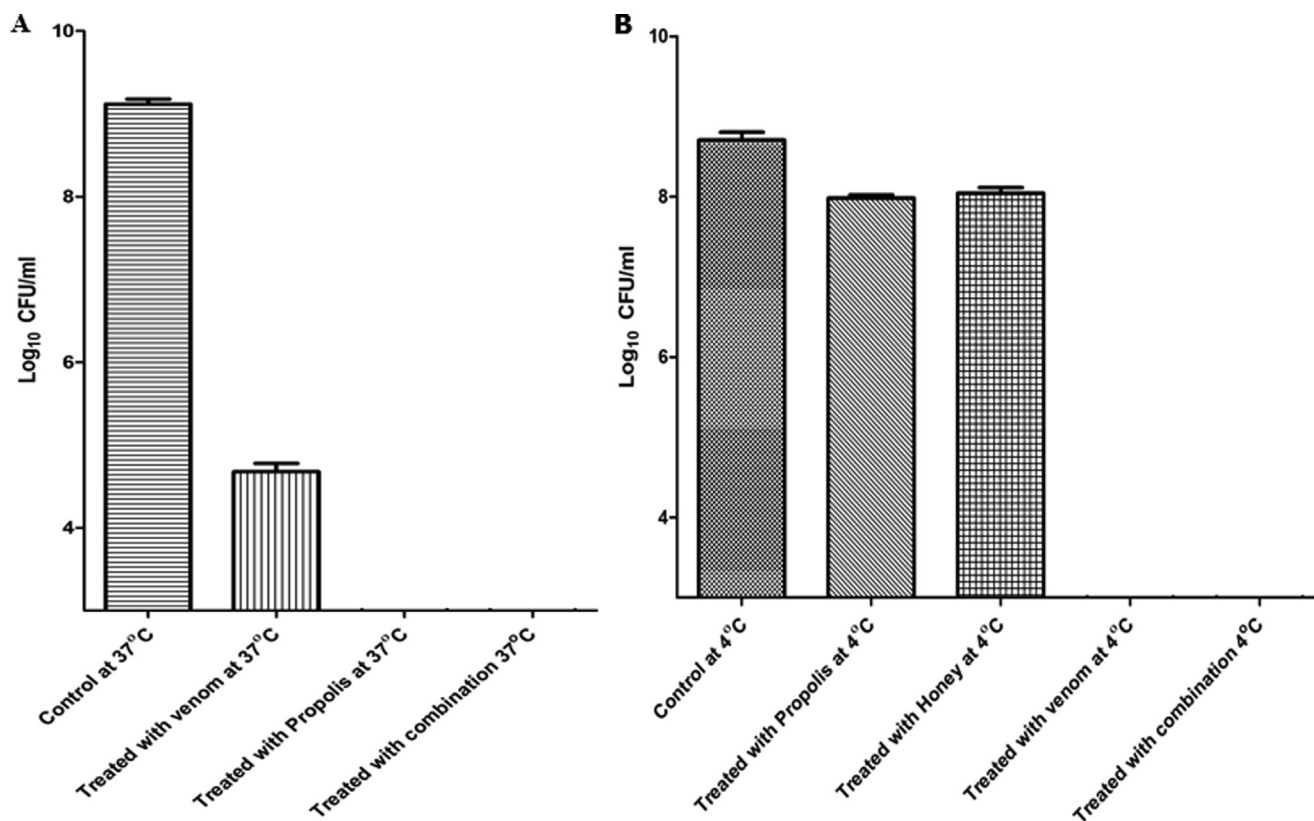


Fig. 5 Shows the ability of bee venom and propolis to kill the bacteria after 24 h of incubation at 37 °C A) and 4 °C B) with a concentration of 1x MBC. The results of antibacterial activity at 4 °C and 37 °C were carried out in triplicate (n = 3).

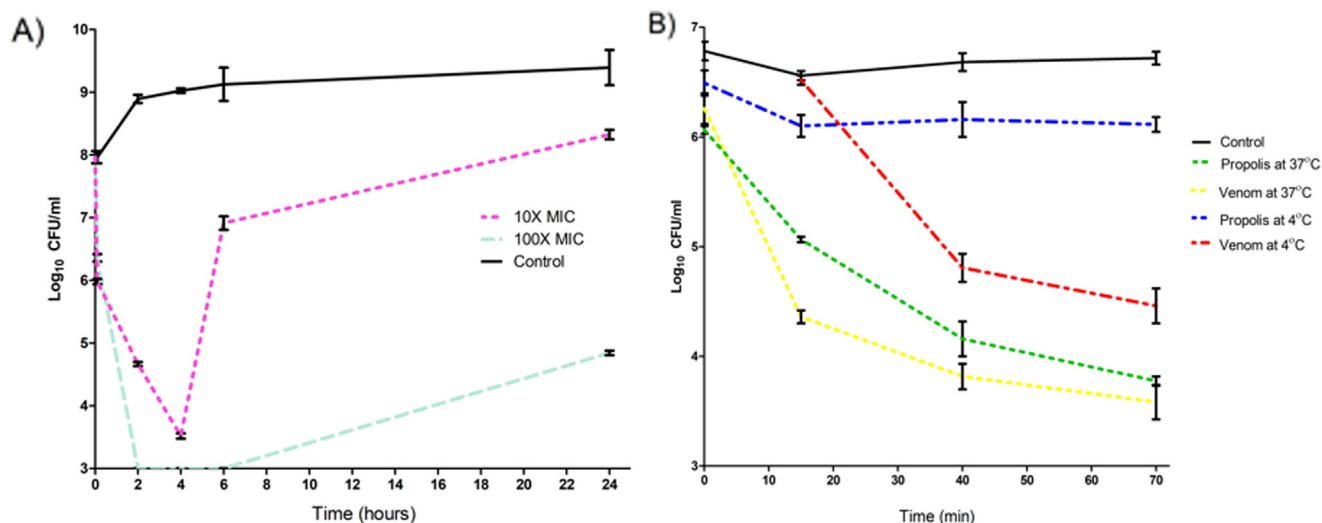


Fig. 6 Displays the time-kill curve for the cocktail of MDRSA isolates with A) two different concentrations of the bee venom at 37 °C for 24 h. B) the kill curve for 70 min at different incubation temperatures for 100X MIC of venom and propolis. The results of the killing curve were carried out in triplicate (n = 3).

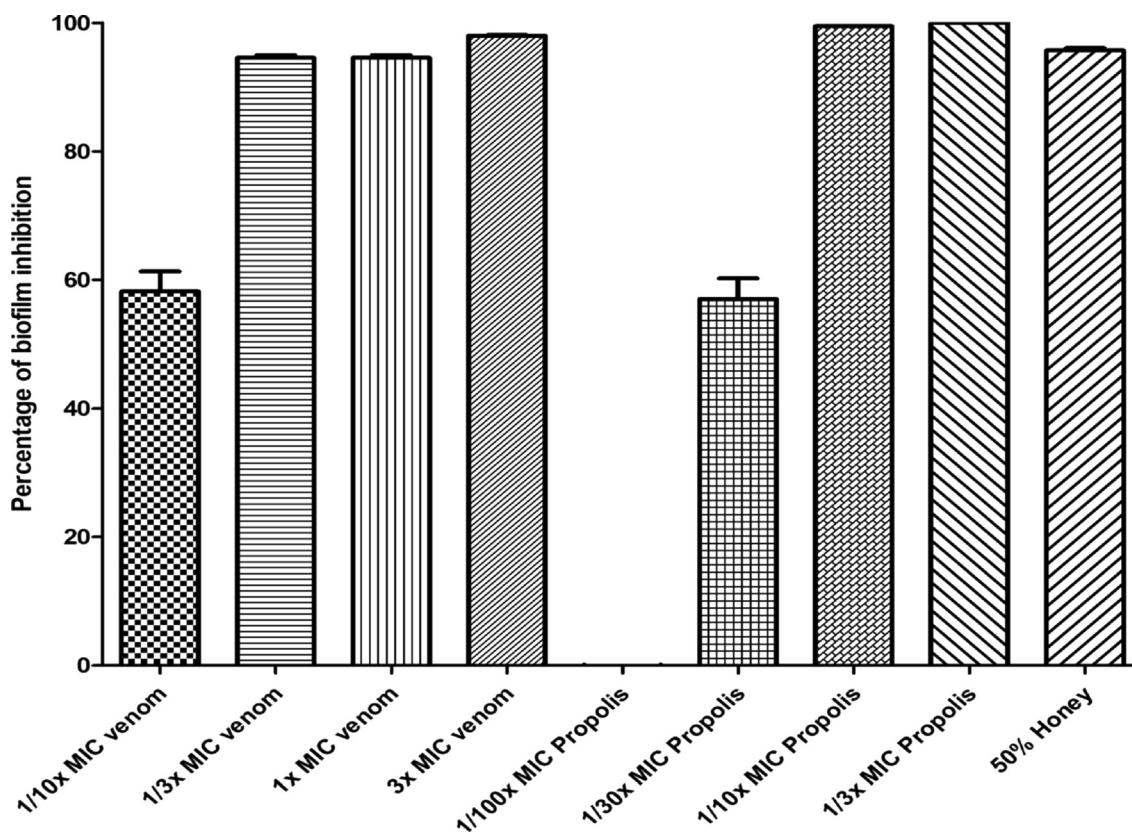


Fig. 7 Represents the effect of venom, propolis, and honey with different concentrations to inhibit the biofilm formation of the bacterial cocktail of MDRSA. The results of biofilm inhibition were carried out in triplicate (n = 3).

Melittin, isolated from bee venom, tested on *S. aureus* ATCC 29213 with a concentration of 1x MIC displayed the same pattern of persistence (Kenawy et al., 2019). In the present study,

10x MIC of bee venom against the cocktail of MDRSA also revealed a decrease in bacterial count relative to the control. Yet, secondary growth was also observed after a while. This

secondary growth can be eliminated by employing a higher concentration of the venom or by adding other antimicrobial agents to the venom to obtain a synergetic effect.

2.9. Biofilm inhibition and clearance

The ability of bee venom to inhibit biofilm formation was investigated. The results showed that the biofilm formation by the bacterial cocktail was decreased by 58.2%, 94.6%, and 98% when it was incubated with bee venom with concentrations of 1/10x MIC, 1x MIC, and 3x MIC, respectively, for 24 h at 37 °C (Fig. 7). However, the ability to eliminate the established *S. aureus* biofilm (biofilm clearance) was between 13.8% and 61.5% for bee venom with the concentration of 1x MIC and 3x MIC, respectively, for 24 h at 37 °C (Fig. 8). On the other hand, propolis decreased the ability of bacteria to form biofilm by 100%, 99.4%, and 57% at concentrations of 1/3x MIC, 1/10x MIC, and 1/30x MIC, respectively. However, it did not show any effect at 1/100x MIC. Nonetheless, propolis displayed a moderate biofilm clearance activity, as it revealed reduction by 45.5%, 38.96%, and 44.9% at concentrations of 1/3x MIC, 1/10x MIC, and 1/30x MIC respectively. The honey presents a high activity in inhibiting biofilm formation with 95.75% at 50% concentration. Yet, like propolis and venom, this activity was reduced in the case of biofilm clearance, and the result showed a 76.8% reduction with a 50% concentration.

The biofilm clearance and inhibition results showed a great potential of propolis to disrupt biofilms with a 100% reduction at a concentration of 1/3x MIC. Also, it was demonstrated that honey was the best option for biofilm clearance. It showed a decrease of 76.8% at 50% concentration compared to propolis and venom, which showed lower activity in reducing an already formed biofilm. According to the results by Grecka et al. (Grecka et al., 2019), an MIC of 128 µg/mL was able to eradicate biofilm by *S. aureus* compared to 45.5%, 38.96%, and 44.9% at concentrations of 1/3x MIC, 1/10xMIC, and 1/30xMIC, respectively. The previous results (Daikh et al., 2020) reported that the petroleum ether of propolis, chloroform extract of propolis, ethyl acetate, and methanol extracts displayed a biofilm inhibition activity ranging from 6.83% ± 0.78% to 80.72% ± 2.34. However, our study showed more activity as it varies from 100% to 57% with different concentrations.

2.10. Photodocumentary analysis of wound healing

A typical photograph of the non-treated and non-infected wound (G1), non-treated infected wound (G2), and the infected wound treated with Bee venom/Propolis/ chitosan hydrogel (G3) on days 0, 4, 7, 10, 14, and 17 post-surgery are offered in (Fig. 9). Moreover, the diagram of % wound closure is also supplied in (Fig. 10). The % wound closure of all wounds for different groups demonstrated that the

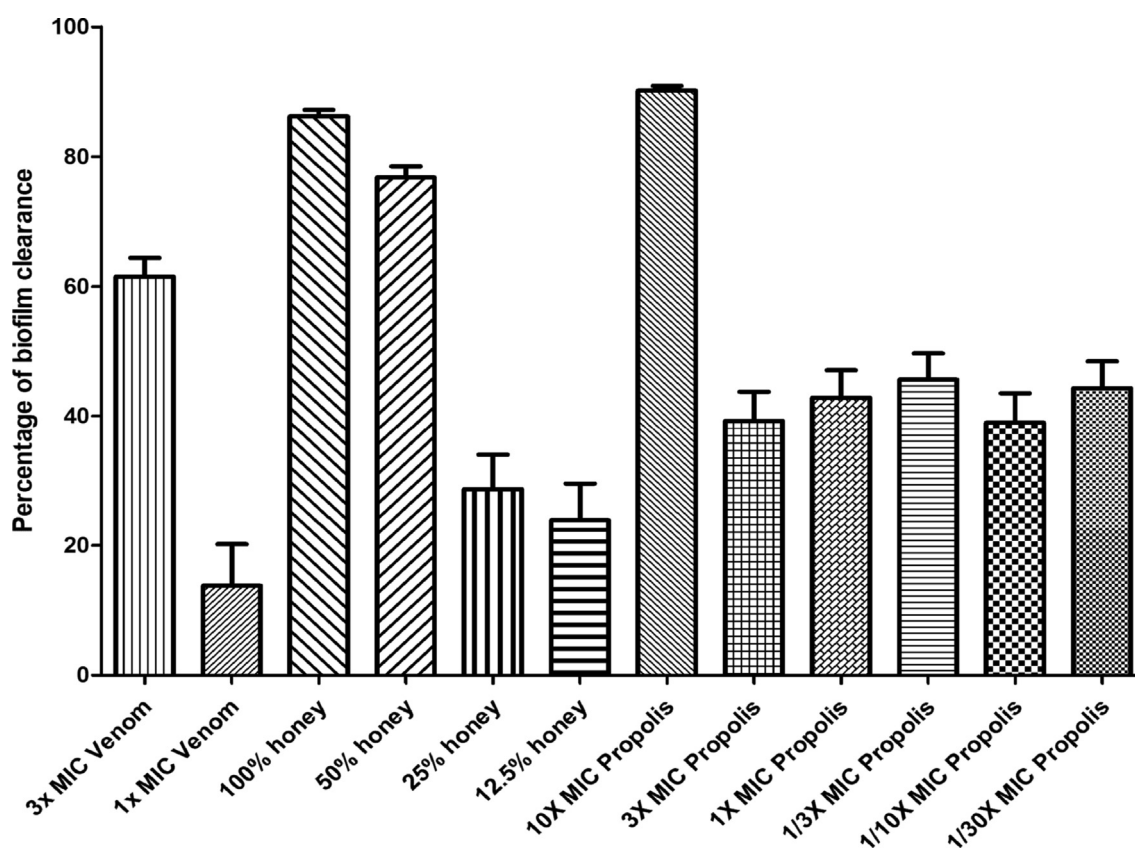


Fig. 8 Represents the effect of venom, propolis, and honey with different concentrations to clear the biofilm formed by a bacterial cocktail of MDRSA. The results of biofilm clearance were carried out in triplicate (n = 3).

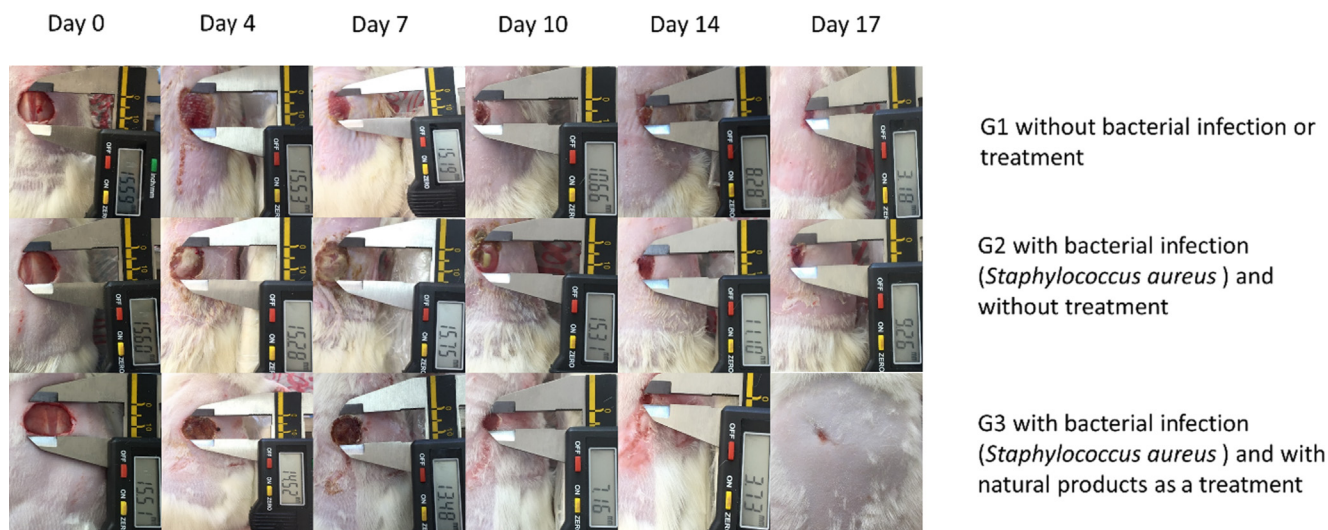


Fig. 9 Photodocumentary analysis of full-thickness excision wounds in rats at a fixed focal distance using digital photographs.

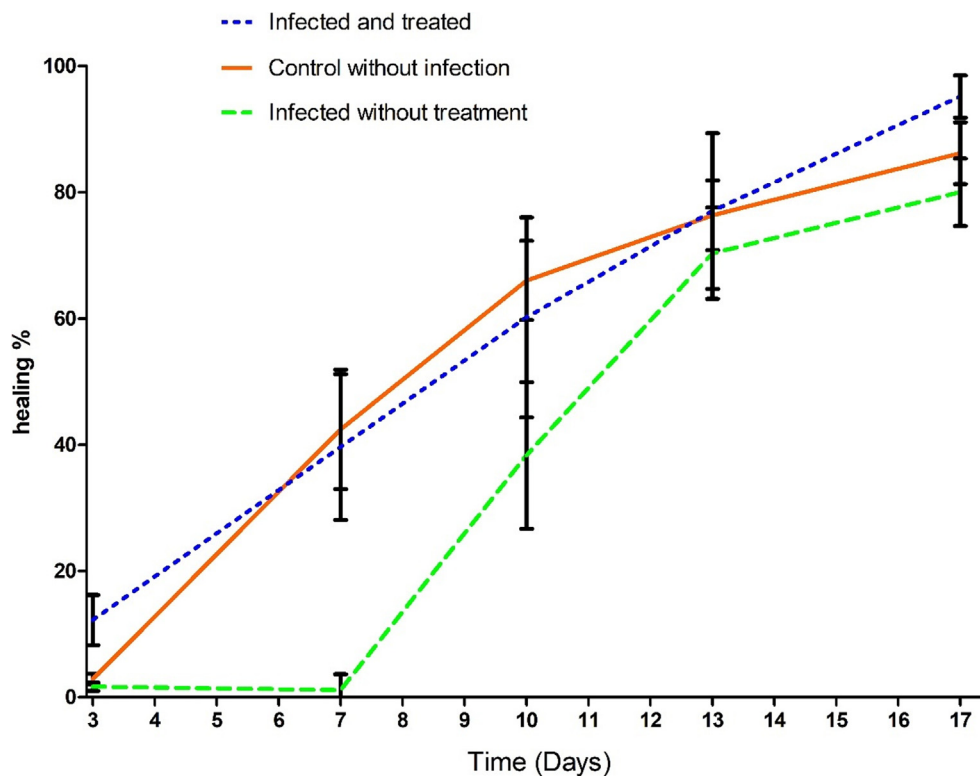


Fig. 10 The % wound closure of all wounds. The significance of the time in days on the wound healing was highly significant, $p < 0.0001$. In addition, the differences among the groups were highly effective, $p < 0.001$. the number of rats is 5 in each group from day 3 to day 10, and $n = 4$ until the end of the experiment.

topical application of Bee venom/Propolis/ chitosan hydrogel to the rounded area of full-thickness excision wound significantly accelerated the rate of wound healing. For example, starting from day 7, the G3 demonstrated a decrease in the wound area represented by a rise in the % wound closure ($42.3 \pm 19.2\%$) compared to the % wound closure of G2 ($1.1 \pm 5.0\%$). At the end of the experiment

(day 17), the G3 group acquired maximum % wound closure ($95.16 \pm 6.55\%$) followed by G1 ($86.18 \pm 9.56\%$). On the other hand, G2 showed a minimal % wound closure ($80 \pm 10.4\%$).

Wound infections are the leading cause of death in many patients, especially when they enter the bloodstream causing septicemia. Moreover, in many cases, wound infections lead

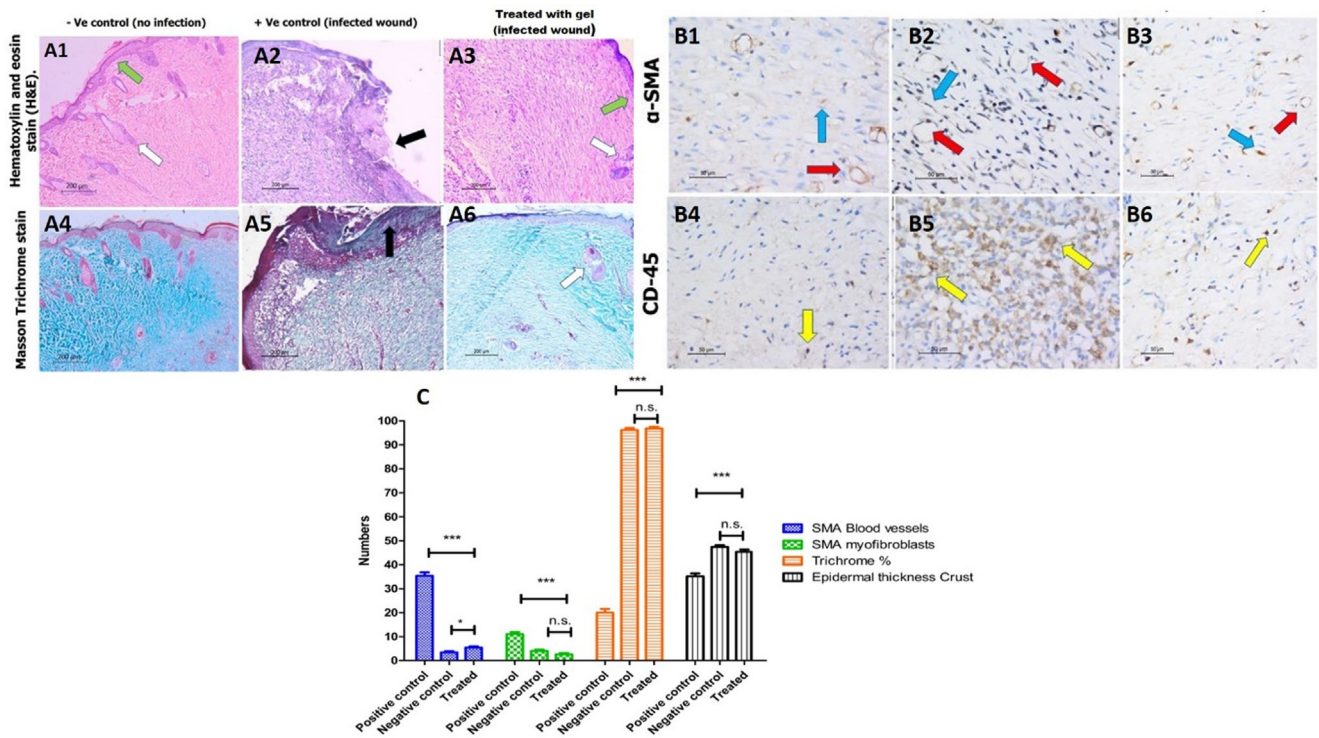


Fig. 11 Histological analysis of wounded skin section from different rat groups at day 17 post wounding. A1-3) hematoxylin and eosin (H&E) staining, $\times 100$ showing in A1) the non-infected, non-treated group epidermal re-epithelialization (green arrow) with the regeneration of the skin appendages (white arrow). A2) the non-treated group shows epidermal ulceration and crust formation (black arrow). A3) shows thick epidermal re-epithelialization (green arrow) with the regeneration of the skin appendages (white arrow). A4-6) Masson's trichrome staining $\times 100$ shows (A6) the remodeling phase of healing in the third group treated with the gel, similar to the (A4) non-infected group. In contrast, the healing process in (A5) the second group is delayed representing the inflammatory phase in the form of extensive inflammation and granulation tissue with the least amount of fibrous tissue, B1-3) immunohistochemistry staining for α -SMA, $\times 400$ highlights the blood vessels (red arrow) and myofibroblasts (blue arrow) of the granulation tissue representing the complete maturation of the wound healing with few residual amount of granulation tissue in groups (B1) without infection and (B3) infected and treated. In contrast, in (B2), the infected untreated group shows the highest granulation tissue. B4-6) immunohistochemistry for CD-45, $\times 400$, reveals the least amount of lymphocytes (yellow arrow) in (B4) the first and (B6) third groups, representing the healing process's completion. C) The comparison of all histological analyses and the results of the histological analysis were carried out five times ($n = 5$).

to further inflammation and severe tissue damage, the leading cause of delayed healing (Kenawy et al., 2019). Furthermore, bacterial resistance to many antimicrobial agents represents intimidation to public health and is mounting at an alarming rate (El-Deeb et al., 2020). Therefore, there is an urgent demand to evolve new drugs, especially from a natural source, suppressing microbial invasion at the wound site and minimizing the threat of bacterial resistance. In this context, numerous studies specified the efficiency of some natural biomaterials, including bee venom, propolis, and chitosan hydrogel, to accelerate the healing of infected wounds (Badr et al., 2016; Takzaree et al., 2016; Liu et al., 2018).

In this study, the therapeutic efficiency of (honey/bee venom/ propolis/chitosan hydrogel) was examined on rats to treat full-thickness wounds infected with *S. aureus* clinical isolate, which is resistant to multiple antibiotics, including Tetracycline (10 μ g) and Vancomycin (30 μ g). The presence of terpenes in the propolis with high concentration is one factor in accelerating wound healing (Salas-Oropeza et al., 2021). In addition, the active ingredients such as bee venom and propolis have a tested antibacterial activity; as a result, there is no evidence of infection in the wounds, unlike the clear bacterial

layer on infected wounds without treatment during the first 10 days as in Fig. 9.

From the former histopathological outcome, it is evident to us that among the *S. aureus* wound infected groups, superior healing was observed in the group treated with bee venom/ propolis/chitosan hydrogel, with the entire recovery process reaching the remodeling phase with complete restoration of the epidermal thickness and skin appendages regeneration, which was comparable to the sterile wound healing process with some superior features like the epidermal thickness, the collagen deposition and the minimal amount of the residual granulation tissue.

2.11. Histopathological analysis of wound healing

Histopathological photographs of wound tissues stained with both (H&E), Masson's trichrome (MTs), and immunostaining on day 17 are illustrated in (Fig. 11). The skin section from the G1 stained with (H&E) showed epithelial re-epithelialization, with epidermal thickness, $47 \pm 1.0 \mu$ m, with regenerated skin appendages $> 5/HPF$ (score 3). The dermis showed a remodeling phase of healing formed of collagen fibers mean $96 \pm 0.8\%$

of the wound area, evidenced by MTs, with scant residual granulation tissue formed of few dermal lymphocytes mean 7 ± 1.1 /HPF using CD45 IHC. In contrast, the mean blood vessels count was 4 ± 0.7 /HPF, and the myofibroblasts were 3.1 ± 0.4 /HPF using α -SMA IHC.

The skin specimens from the G 2 stained with (H&E) showed epidermal ulceration, with a thick crust of $33.6 \pm 1.2 \mu\text{m}$, lacking skin appendages (score 0). The dermis showed few amounts of fibrous tissue evidenced by MTs. The dermis was predominantly in the inflammatory phase with early proliferation phase of wound healing consisting of abundant acute inflammatory infiltrate, granulation tissue formed of newly-formed blood vessels, mean 35.2 ± 1.3 /HPF, the myofibroblasts count mean was 15.3 ± 2.4 /HPF using α -SMA IHC. With few dermal fibrous tissue fibers, the mean was $22.3 \pm 1.7\%$ of the total area and an increased number of lymphocytes 60 ± 2.3 /HPF using CD45 IHC.

The skin specimens from G3 stained with (H&E) showed epidermal re-epithelialization with a mean thickness of $45.3 \pm 0.9 \mu\text{m}$ with regenerated skin appendages more than 5/HPF (score 3). The healing process was in the remodeling healing phase; with the wound area formed mainly of collagen fibers, the mean area was $98.4 \pm 1.3\%$ (score 3), evidenced by MTs. The dermis revealed the scanty amount residual of the granulation tissue which was fully matured into collagen fibers, the mean count of the blood vessels was 6 ± 0.4 /HPF, and the mean number of myofibroblasts was 2.1 ± 0.4 /HPF using α -SMA IHC with decreased number of the lymphocytes, mean value was 6.3 ± 1.0 /HPF using CD45 IHC.

The augmented healing efficiency for this group might be attributed to the concomitant repression of cytokines associated with fibrosis by bee venom, minimizing the wound size and mounting the epithelial proliferation in a full-thickness excision wound model (Han et al., 2011). Also, the presence of propolis with its fundamental ingredient, flavonoids, accelerates wound healing due to their anti-inflammatory and antibacterial effects (Abu-Seida, 2015). It was previously reported that propolis contains caffeic acid phenethyl ester that possesses an immunosuppressive activity in T-cells responsible for the onset of various inflammatory diseases. Moreover, it also downregulates both interleukin- (IL-) 2 gene expression and IL-2 synthesis in stimulated T-cells (Lotfy, 2006). Hozzein et al. (Hozzein et al., 2015) reported that the topical application of propolis accelerates wound healing in diabetic mouse models via promoting TGF- β /Smad-mediated collagen synthesis. Several studies have reported the great value exerted by chitosan in boosting wound healing as its highly safe to mammalian cells, biocompatible, biodegradable, antimicrobial, and anti-inflammatory activity (Sabra et al., 2020; Tamer et al., 2018). The accelerated wound healing of chitosan is due to energizing the migration of mononuclear cells and polymorphonuclear (PMN), which boosts skin tissue regeneration and re-epithelialization (Hassan et al., 2021).

3. Materials and methods

3.1. Bacterial isolation

The bacterial samples (n = 11) of MDRSA were isolated from patients and provided to us by one of our collaborators. They were all streaked on Mannitol Salt Agar (MSA; Oxoid, Eng-

land) and left two days to grow at 37 °C. For the rest of the experiments, a day culture was prepared from the purified culture by incubating a single colony in Tryptone Soy Broth (TSB; Oxoid, England) at 37 °C.

3.2. Bacteria characterization

An oxidase test was conducted by adding N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to sterile distilled water, and then a single fresh colony was picked using sterile swap, where one drop of TMPD solution was added and observed for the color change to blue/purple. In addition, a single fresh colony was picked from each strain and smeared over a glass slide, then a drop of hydrogen peroxide was added and observed for the formation of bubbles. In addition, the fermentation of mannitol was confirmed by growing the bacterial isolates on MSA to observe the yellow colonies after incubation at 37 °C. Moreover, the confirmation of the bacterial isolates was confirmed via PCR-amplification using specific primers for *S. aureus*, *nuc* gene. The forward primer sequence used was (5'-GCGATTGATGGTGATACGGTT-3'), and the reverse sequence was (5'-AGCCAAGCCTTGACGAAC TAAAGC-3') with an expected product size of 267 base pairs (Brakstad et al., 1992). The colony PCR reaction has 26 cycles that were performed at denaturation temperature of 95 °C for 3 min; then 94 °C for 30 s; annealing at 55 °C for 30 s and final extension at 72 °C for 2 min. The ladder used was 100 bp length using an Applied Biosystems thermal cycler. The PCR product was run on a 1% (w/v) agarose gel to identify its size.

3.3. Antibiotic sensitivity test

A panel of antibiotics that are usually used to treat MDRSA was tested against the bacterial isolates to identify the sensitivity of isolates to selected antibiotics. The panel included a wide range of antibiotic classes including; β -lactam (Amoxicillin, 10 μL); Penicillins (Oxacillin, 1 μL); Aminoglycoside (Gentamicin, 30 μL); Glycopeptide (Vancomycin, 30 μL), Macrolide (Erythromycin, 15 μL); Tetracyclines (Tetracycline (10 μL), Doxycycline, 30 μL); Phenicol (Chloramphenicol, 30 μL); Oxazolidinones (Linezolid, 30 μL); Lincomycin (Clindamycin, 2 μL); Minobenzenesulfonamides (Sulfamethoxazole, 25 μL). The antibiotic disk diffusion results were compared to the National Committee for Clinical Standards guidelines (N.C. for C.L. Standards, 1999). Antibiotics used in this study were purchased from (Oxoid, England).

3.4. Honeybee venom collection and preparation

The venom used in this study was gifted to the center as a lyophilized powder from the faculty of Environmental Agricultural Sciences, Arish University, North Sinai, Arish, Egypt. The specific details on the venom extraction method were mentioned elsewhere (El-Bahnasy et al., 2017). The bee venom sample was saved at $-20 \text{ }^\circ\text{C}$ until needed.

3.5. Propolis collection and preparation

The propolis in this work was gifted to the center in its raw condition from the faculty of Environmental Agricultural

Sciences, Arish University, North Sinai, Arish, Egypt. For the preparation, the heated ethanol extraction method was followed with minor modifications. Briefly, 0.4 g from the row propolis was added to 10 mL of 80% Ethanol (HPLC grade) for 2 h in 60 °C, followed by incubation for another 2 h at 80 °C before centrifugation at 4000x g for 15 min undercooling (4 °C). The supernatant solution was withdrawn and filtered using the syringe filter with 0.45 µm pore size to ensure sterilization. Following the preparation, the yield was measured by the complete evaporation and weighing 1 mL extracted propolis. The tubes were covered with aluminum foil to prevent the sun rays during all extraction steps.

3.6. Honey hydrogel preparation

The commercial honey was prepared in a concentration of 70% via mixing it with 1% acetic acid solution followed by adding 1 mg of bee venom and 1.5% of ethanolic extract propolis in a sterile beaker under a magnet stirrer at 800 rpm for 1 h. The freshly prepared 2% chitosan and dissolved gelatin in sterile water were added to the previous mixture with glycerol, then continued stirring until the gel formed. After the physical gelatination was created due to the presence of chitosan and gelatin, the final concentrations in every mL of the gel are 7 mg gelatin, 300 mg honey, 70 µg bee venom, 140 mg glycerol, and 15 µL of extracted propolis. The glycerol was added to create a bacteriostatic environment (Stout and McKessor, 2012). The gel was stored undercooling (4 °C) for wound healing experiment.

3.7. In vitro antioxidant activity of propolis

Five different concentrations (10, 20, 40, 50, 60, and 80 µg/mL) of propolis extraction were prepared in methanol to evaluate the effect of 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging. Besides, five different concentrations (50, 40, 30, 20, 15, 10, and 5 µM) of Trolox standard were used as a reference. DPPH free radical assay was conducted according to Brand-Williams et al. (Brand-Williams et al., 1995) with modifications. Briefly, Aliquots of 100 µL from a methanolic solution of DPPH reagent (0.1%) were transferred to 100 µL of the propolis in 96 wells plate (n = 6). After 30 min of incubation at room temperature in the dark, the results were obtained from measuring the absorbance at 540 nm using microplate reader FluoStar Omega.

3.8. The silylation and gas chromatography/mass spectrometry (GC-MS) of the propolis extracts of propolis

For the silylation of the extracts, about 10 mg of the extract was added to both dry pyridine and bis (trimethylsilyl) trifluoroacetamide, then heated at 80 °C for 20 min, and investigated by GC-MS. The GC-MS analysis was done using TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), linked to a Thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer), equipped with a TR-5 MS column (30 m × 0.32 mm i.d., 0.25 µm film thickness). The temperature was programmed from 60 to 240 °C at a rate of 4 °C/min. Helium worked as a carrier gas, with a 1 mL/min flow rate. The split ratio of 1: 10 was used, and the injector temperature was set at 210 °C to dilute the samples (1:10 hex-

ane, v/v) of 1 µL of the propolis extraction. Ionization voltage was set at 70 eV by a spectral detection range of m/z 40–450. The identification was accomplished by AMDIS software, its retention indices (relative to n-alkanes C8-C22), and mass spectrum matching to Wiley spectral library collection and NSIT library database.

3.9. Protein analysis of the components of the natural products

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (Laemmli, 1970) with modifications. Briefly, bee venom, propolis, and honey were diluted in distilled water and then heated for 10 min at 90 °C after adding SDS sample loading buffer. The molecular weight marker 200 kDa was used to mark the bands. All samples were loaded in the SDS-PAGE gel (12%) and run parallel. The gel was obtained and stained with Coomassie blue solution dye. After that, the gel was washed to remove excess dye until the visualization of bands. (The results and discussion for this experiment are provided in supplementary data).

3.10. Cytotoxicity in human skin fibroblast for bee venom

The human skin fibroblast cell line, which was used in this experiment, its primary cells were obtained from foreskin circumcision operations at KAU hospital. Cells were cultured in DMEM media supplemented with streptomycin, penicillin, and fetal bovine serum (FBS). Cells were incubated in humidified, 5% (v/v) CO₂ atmosphere at 37 °C. Then, Sulforhodamine B (SRB) assay was done to determine the cytotoxic effect of the bee venom using the method described by Vichai et al. (Vichai and Kirtikara, 2006) with modifications. Briefly, aliquots of 100 µL cell suspension (5000 cells) were seeded in 96-well plates with complete media without venoms. After 24 h, cells were treated with another volume of media containing bee venom at different concentrations ranging from (0.01, 0.1, 1, 10, 100 µg/mL) and positive control incubated without venom. After 72 h of bee venom exposure, cells were fixed by discarding media and adding 150 µL of 10% Trichloroacetic acid (TCA) and was kept at 4 °C for 1 h to precipitate the proteins. The wells were washed 5 times with distilled water. 70 µL of SRB solution (0.4% w/v) were incubated in each well in a dark place at room temperature for 10 min to stain the proteins. The plate was rinsed 3 times with 1% acetic acid to omit unbounded dye and allowed to air-dry overnight. Then, 150 µL of 10 mM Tris-base solution was added to solubilize protein-bound SRB stain; the absorbance was determined at 540 nm using a BMG LABTECH®- FLUOstar Omega microplate reader (Ortenberg, Germany). The experiment was conducted in triplicates, and each well was visualized using converted microscopy with and without staining at 200x magnification.

3.11. Disc diffusion and well diffusion method

The disc diffusion method was conducted to predict the antibacterial activity of bee venom against 11 MDRSA isolates as described in (Surendra et al., 2011) with some modifications. The sterile TSB was used to prepare the overnight culture suspension followed by 1:10 dilution. Further, 200 µL was spread on a plate containing Tryptone Soy Agar (TSA) via a sterile

cotton swab. The surface of the medium was left to dry in a sterile area for 5 min, and sterile paper discs (6 mm in diameter) were added to them. Exactly, 10 μ L of various concentrations of bee venom and propolis (10 mg/mL, 3.3 mg/mL, 1.1 mg/mL, 0.37 mg/mL, 123 μ g/mL, 41.2 μ g/mL, and 13 μ g/mL) was used for each isolate and loaded on discs. The plates were incubated for 24 h at 37 °C. After that, the diameters of inhibition zones were measured.

Wells with 6-mm diameters were produced in TSA plates had streaked with cocktail MDRSA (ZCSA/02; 03; 04; 05; 06; 08; 14; 15; 16; 17; and 24) and filled with 75 μ L of 2% Chitosan gel, another well with 75 μ L of 70% honey was combined with 1 mg of propolis and venom, the last well with 75 μ L of 2% Chitosan gel with 70% honey and 1 mg propolis and venom then incubated at 37 °C for 24 h and the inhibition zone was measured.

3.12. MIC and MBC

The MIC values of the bee venom against each bacterial isolates and cocktail of bacteria were measured using a 96-well sterile microtiter plate F-bottom, according to the micro-well dilution method with modifications (Zgoda and Porter, 2001). Briefly, the bee venom and propolis were diluted in TSB at different concentrations 1:10 dilutions (0.3-1000 μ g/mL), and 20 μ L of bacterial suspension with 10^6 CFU/mL was added to reach a final volume of 200 μ L in each well. MIC was detected at the lowest concentration that inhibited the bacterial growth (clear well) after 24 h at 37 °C. Then, the MBC was determined by adding 10 μ L of clear-well samples into a fresh TSB for another 24 h. Then recording the lowest concentration of bee venom and propolis without detecting turbidity means the bee venom killed all bacteria in that concentration.

3.13. Antibacterial activity at 37 °C and 4 °C

The activity of bee venom and propolis against the cocktail of bacteria was evaluated with the concentration of 1x MBC. Four eppendorfs containing a fresh cocktail of bacteria in TSB with a concentration of 10^8 were used; the first two were incubated for 24 h at 37 °C and 4 °C without adding the bee venom. In the second two, the bee venom was added with the concentration of 1x MBC and incubated for 24 h at 37 °C and 4 °C. The bacterial count was observed using the serial dilution spotting technique.

3.14. Determination of time-kill curve

To measure the dynamic process of the bactericidal activity of bee venom, the time-kill curve method was conducted, as described previously (N.C. for C.L. Standards, 1999), with some modifications. Briefly, bee venom with 10x and 100x of MIC were tested against a cocktail of 11 MDRSA isolates. Cocktail of bacteria (control) and bacteria with 10x MIC (treatment 1) and 100x MIC of bee venom (treatment 2) were incubated at 37 °C, where 100 μ L aliquot of each was collected at different time points (2 h, 4 h, 6 h, and 24 h), serially diluted and spotted on TSA. The results were obtained in triplicate, and time-killing curves were plotted as colony counts (\log_{10} CFU/mL) versus time. The limit of detection for this experiment was 10^3 CFU/mL.

To compare the dynamic process of the bactericidal activity of bee venom and propolis, the time-kill curve method was conducted at 4 and 37 °C, as described previously. In addition, 1 mg of each propolis and venom was used at the time point of (0, 15, 45, 70 min).

3.15. Biofilm inhibition test

The ability of natural compounds to prevent the formation of biofilm was tested. First, 180 μ L of bacterial cocktail at log phase ($\leq 10^5$ CFU) were loaded in the 96-well microplate Flat-bottom, in which the bee venom and propolis were added with concentrations of 3x MIC, 1x MIC, 1/3x MIC, and 1/10x MIC for bee venom and 1x MIC, 1/3x MIC, and 1/10x MIC, 1/30x MIC for propolis in the presence of 50% honey. All treatments were added to the plat at which the final volume in each well reached 200 μ L. After 48 h incubation at 37° C, the planktonic bacteria were washed with sterile water, and then the plate was flipped down with shaking to remove the remaining unattached bacteria three times. To stain the biofilm mass, 200 μ L of 0.1% crystal violet solution was added to each well and incubated for 15 min; then, the plate was washed three times and left for 30 min to dry. To observe the results, 200 μ L of 30% acidic acid was added to each well and applied to a vis-spectrophotometer (Jenway 7200 visible) to report the absorbance change at 540 nm. In this experiment, both the bacterial cocktail has grown on a microplate without treatment. The treatments and media without the bacteria were utilized as positive and negative control (blank), respectively. 1x MIC of propolis is 100 μ g/mL and for the bee venom 10 μ g/mL, as a final concentration.

3.16. Biofilm clearance test

The cocktail of bacterial strains was incubated in the 96-well microplate F-bottom for 24 h at 37 °C. After incubation, the planktonic cells were withdrawn with sterile tips then the bee venom, propolis, and honey were added with concentrations of 3x MIC, 1x MIC, 1/3x MIC, and 1/10x MIC for bee venom, 10x MIC, 3x MIC, 1x MIC, 1/3x MIC, and 1/10x MIC, and 1/30x MIC for propolis, and 100%, 50%, 25%, and 12.5% for honey followed by further incubation for another 24 h. As previously described in the biofilm destruction test section, the biofilm results were observed.

3.17. Preparation of chitosan gel embedded natural product

The gel was prepared by dissolving 0.2 g of gelatin in 20 mL hot sterile deionized water. Then, 10 g of honey, 2 mg of bee venom, 4.2 g of glycerol, and 400 μ L of ethanolic propolis extract were added to the dissolved gelatin under magnetic stirring until the complete dissolving followed by UV sterilization for 15 min. Finally, 400 mg chitosan was mixed after adding 300 μ L of anhydrous acetic acid to provide an environment suitable for chitosan.

3.18. In vivo wound healing

The chitosan/ propolis/bee venom/honey mixture can be applied locally to the wounded skin area using antimicrobial gel to examine its healing potential on the full-thickness skin infected wound rat model. Fifteen Wistar male rats (8 weeks

old), weighing approximately 180–200 g, were utilized in the *in vivo* animal study. The Animal Care Committee of the Alexandria University (ALEXU-IACUC) approved all experimental procedures, with an official approval for the surgical protocol (AU-IACUC-14/210601-3-4). Every rat was housed in an individual crate and given access to a standard laboratory diet and mineral water ad libitum under striped ecological conditions. According to our previously published research, the surgery was performed (Mohamed et al., 2021; Shalaby et al., 2020; El-Aassar et al., 2020). The animals were randomly divided into three groups comprising five animals in each group at which group one applied sterile gauze (no wound infection), group 2 applied sterile gauze to *S. aureus* infected wound, and group 3 applied 100 μ L of hydrogel mixture (0.7 mg gelatin, 30 mg honey, 7 μ g bee venom, 14 mg glycerol, and 1.5 μ L of extracted propolis) to *S. aureus* infected wound.

The animals were anesthetized by an intramuscular injection of xylazine (10 mg/kg) and ketamine (60 mg/kg) then the back hair at the dorsal areas was removed using an electric shaver. The shaved skin was cleaned via 70% ethanol before induction of a full-thickness wound of about 15 mm in diameter with the help of dissecting scissors and sterile forceps (one wound /rat). Wound infection was completed by inoculating the wound with *S. aureus* (10^8 CFU/mL) for two hours before applying the treatment. Both sterile gauze and hydrogel containing (gelatine/chitosan/propolis/bee venom/ honey) were applied to cover the wound area and replaced with a new one on the observation day. On days 3, 7, 10, 14, and 17 after surgery, the changes in the wound areas were examined by taking the digital images, and the % contraction of each wound was calculated using the following equation.

Wound area reduction (%) = [(Wound area at the day 0 – Wound area at the given day) / Wound area at the day 0] \times 100 (Werner et al., 1994).

At the end of the experiment (day 17), the full thickness of the skin was eradicated and fixed in 10 % formaldehyde for histopathological investigations.

3.19. Histological Examination:

On day 17, the rats were sacrificed, followed by removing the tissue from the wound bed and its surrounding healthy skin to assess the wound healing; all specimens were fixed in 10 % formaldehyde and embedded in paraffin blocks for histopathological examination. The skin sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Leica, Germany). Histological examination of the whole wound area was done, and the mean value of the percentage of the fibrous tissue and epidermal thickness was quantified using Imagej, v1.53 (Maryland, USA). The skin appendages were scored (no skin appendages 1, few < 5/wound are 2 and greater than 5/ wound area 3). The skin sections were also stained with Masson Trichrome staining (MTs). Imagej, color deconvolution v1.53 (Maryland, USA) was used to quantify the percentage area of fibrosis in each specimen (Park et al., 2015; Abramov et al., 2007).

3.20. Immunohistochemical (IHC) staining and interpretation:

IHC of all sections was done using the Avidin-Biotin Peroxidase method (Hsu et al., 1981). CD45 (Ready to use pri-

mary antibody, mouse anti-human, monoclonal antibody, P0042; Leica Biosystems, USA) and α -SMA (Ready to use primary antibody, mouse anti-human, monoclonal antibody, P0943; Leica Biosystems, USA) were used to stain the lymphocytes, blood vessels, and myofibroblasts respectively. The antibodies were added to each section using the Bond-Max fully automated immunostainer (Leica Biosystems, USA). In each IHC run, rat tonsil was used as the positive control for CD45 antibody and leiomyoma was used as the positive control for α -SMA antibody. Negative controls were also included in each run. The quantification of the IHC of CD45 and α -SMA was done, in each slide, using the quantitative-image analysis (Leica microsystems, Switzerland).

3.21. Statistical analysis

All experiments were repeated triplicates, and the data were presented in the form of mean \pm standard deviation (SD). All statistical analyses and graphs were generated using GraphPad Prism v5 software. Both T-test and ANOVA tests were conducted during the study to evaluate the significance $p < 0.05$.

4. Conclusion

In conclusion, our results demonstrate that propolis and bee venom are effective against MDRSA isolated from clinical wounds and can work as a potential alternative to antibiotics. In addition, propolis is found to have antioxidant activity (40.07 ± 2.18 μ g/mL), which can protect the cells against the damage of oxidation. On the other hand, Bee venom has a cytotoxic effect with IC_{50} 18.3 μ g/mL. Thus, at a low concentration (not exceeding 10 μ g/mL), the venom will be safe to be used on the cells. In addition, propolis and bee venom are efficient for inhibiting and clearing the biofilm of the bacterial cocktail. Based on the presented results, combining those natural products in a gel can be a promising agent against wound infection in the future.

Data availability

The data used to support the findings of this study are included within the supplementary information file.

Funding

This research was funded by the Egyptian Sciences and Technology Development Fund (STDF), grant #41909.

CRedit authorship contribution statement

Abdallah S. Abdelsattar: Conceptualization, Methodology, Writing – review & editing, Writing – original draft. **Salsabil Makky:** Methodology, Writing – review & editing. **Rana Nofal:** Methodology, Writing – original draft. **Mariam Hebshy:** Methodology. **Mona M. Agwa:** Methodology, Writing – review & editing. **Rania G. Aly:** Methodology, Writing – review & editing. **Mohamed Y. Abo El-Naga:** Writing – review & editing. **Yehia A. Heikal:** Writing – review & editing. **Mohamed S. Fayez:** Methodology, Writing – review & editing. **Nouran Rezk:** Methodology, Writing – review & editing. **Ayman El-Shibiny:** Conceptualization, Supervision, Methodology, Validation, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors would like to thank Sara Aziz, Heba Sami, Amara Elsayed, Anan Safwat, and Kareem Essam for their help in this work.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Animal Care Committee of the Alexandria University (ALEXU-IACUC) (AU-IACUC-14/210601-3-4 and 01 June 2021).

References

- Abd El Hady, F.K., Hegazi, A.G., 2002. Egyptian propolis: 2. Chemical composition, antiviral and antimicrobial activities of East Nile Delta propolis. *Zeitschrift Für Naturforsch. C.* 57, 386–394.
- Abramov, Y., Golden, B., Sullivan, M., Botros, S.M., Miller, J.J.R., Alshahrour, A., Goldberg, R.P., Sand, P.K., 2007. Histologic characterization of vaginal vs. abdominal surgical wound healing in a rabbit model. *Wound Repair Regen.* 15, 80–86.
- Abu-Seida, A.M., 2015. Effect of propolis on experimental cutaneous wound healing in dogs. *Vet. Med. Int.* 2015.
- Alni, R.H., Tavasoli, F., Barati, A., Badarbani, S.S., Salimi, Z., Babaeekhou, L., 2020. Synergistic activity of melittin with mupirocin: A study against methicillin-resistant *S. Aureus* (MRSA) and methicillin-susceptible *S. Aureus* (MSSA) isolates, Saudi. *J. Biol. Sci.* 27, 2580–2585.
- Al-Waili, N., Hozzein, W.N., Badr, G., Al-Ghamdi, A., Al-Waili, H., Al-Waili, T., Salom, K., 2015. Propolis and bee venom in diabetic wounds; a potential approach that warrants clinical investigation. *African J. Tradit. Complement. Altern. Med.* 12, 1–11.
- Badr, G., Hozzein, W.N., Badr, B.M., Al Ghamdi, A., Saad Eldien, H. M., Garraud, O., 2016. Bee venom accelerates wound healing in diabetic mice by suppressing activating transcription factor 3 (ATF 3) and inducible nitric oxide synthase (iNOS) mediated oxidative stress and recruiting bone marrow derived endothelial progenitor cells. *J. Cell. Physiol.* 231, 2159–2171.
- Bakdash, A., Almohammadi, O.H., Taha, N.A., Abu-Rumman, A., Kumar, S., 2018. Chemical composition of propolis from the Baha region in Saudi Arabia. *Czech J. Food Sci.* 36.
- Boda, F.A., Mare, A., Szabó, Z.I., Berta, L., Curticapean, A., Dogaru, M., Man, A., 2019. Antibacterial activity of selected snake venoms on pathogenic bacterial strains. *Rev. Rom. Med. Lab.* 27, 305–317.
- Boly, R., Lamkami, T., Lompo, M., Dubois, J., Guissou, I., 2016. DPPH free radical scavenging activity of two extracts from *Agelanthus dodoneifolius* (Loranthaceae) leaves. *Int. J. Toxicol. Pharmacol. Res.* 8, 29–34.
- Brakstad, O.G., Aasbakk, K., Maeland, J.A., 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the nuc gene. *J. Clin. Microbiol.* 30, 1654–1660.
- Brand-Williams, W., Cuvelier, M.-E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity, *LWT-Food. Sci. Technol.* 28, 25–30.
- Brito Pereira Bezerra Martins, A.O., Albuquerque Siebra, A.L., de Moraes Oliveira, C.D., Leite, G.O., Melo Coutinho, H.D., Athayde, M.L., Bolognon, A.A., Andrade, J.C., Oliveira, L.R., Kerntopf, M.R., 2018. Antimicrobial, gastroprotective and healing effect of the hydroalcoholic extract of *Astronium fraxinifolium*. *Lett. Drug Des. Discov.* 15, 325–334.
- Cheesman, M.J., Ilanko, A., Blonk, B., Cock, I.E., 2017. Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? *Pharmacogn. Rev.* 11, 57.
- Chen, X., Zhang, L., Wu, Y., Wang, L., Ma, C., Xi, X., Bininda-Emonds, O.R.P., Shaw, C., Chen, T., Zhou, M., 2018. Evaluation of the bioactivity of a mastoparan peptide from wasp venom and of its analogues designed through targeted engineering. *Int. J. Biol. Sci.* 14, 599.
- Christov, R., Bankova, V., Hegazi, A., Abd El Hady, F., Popov, S., 1998. Chemical composition of Egyptian propolis. *Zeitschrift Für Naturforsch. C.* 53, 197–200.
- Cunha, I., Sawaya, A.C.H.F., Caetano, F.M., Shimizu, M.T., Marcucci, M.C., Dreza, F.T., Povia, G.S., Carvalho, P. de O., 2004. Factors that influence the yield and composition of Brazilian propolis extracts. *J. Braz. Chem. Soc.* 15, 964–970.
- da Silveira Regueira-Neto, M., Relison Tintino, S., Pereira da Silva, A. R., do Socorro Costa, M., de Moraes Oliveira-Tintino, C.D., Augusti Boligon, A., Menezes, I.R.A., de Queiroz Balbino, V., Melo Coutinho, H.D., 2019. Comparative analysis of the antibacterial activity and HPLC phytochemical screening of the Brazilian red Propolis and the resin of *Dalbergia ecastaphyllum*. *Chem. Biodivers.* 16.
- Daikh, A., Segueni, N., Dogan, N.M., Arslan, S., Mutlu, D., Kivrak, I., Akkal, S., Rhouati, S., 2020. Comparative study of antibiofilm, cytotoxic activity and chemical composition of Algerian propolis. *J. Apic. Res.* 59, 160–169.
- El-Aassar, M.R., Ibrahim, O.M., Fouda, M.M.G., El-Beheri, N.G., Agwa, M.M., 2020. Wound healing of nanofiber comprising Polygalacturonic/Hyaluronic acid embedded silver nanoparticles: In-vitro and in-vivo studies. *Carbohydr. Polym.* 238, 116175.
- El-Bahnasy, S.A., Mahfouz, H.M., El-Shibiny, A.A., El-Bassiony, M. N., 2017. Effect of some factors on honey bee venom production from different Strains, Sinai. *J. Appl. Sci.* 6, 59–66.
- El-Deeb, N.M., Abo-Eleneen, M.A., Al-Madboly, L.A., Sharaf, M. M., Othman, S.S., Ibrahim, O.M., Mubarak, M.S., 2020. Biogenically Synthesized Polysaccharides-Capped Silver Nanoparticles: Immunomodulatory and Antibacterial Potentialities Against Resistant *Pseudomonas aeruginosa*. *Front. Bioeng. Biotechnol.* 8, 643.
- El-Guendouz, S., Aazza, S., Lyoussi, B., Bankova, V., Popova, M., Neto, L., Faleiro, M.L., Miguel, M. da G., 2018. Moroccan propolis: A natural antioxidant, antibacterial, and antibiofilm against *Staphylococcus aureus* with no induction of resistance after continuous exposure. *Evidence-Based Complement. Altern. Med.* 2018.
- Elnakady, Y.A., Rushdi, A.I., Franke, R., Abutaha, N., Ebaid, H., Baabbad, M., Omar, M.O.M., Al Ghamdi, A.A., 2017. Characteristics, chemical compositions and biological activities of propolis from Al-Bahah, Saudi Arabia. *Sci. Rep.* 7, 1–13.
- El-Seedi, H., El-Wahed, A., Yosri, N., Musharraf, S.G., Chen, L., Moustafa, M., Zou, X., Al-Mousawi, S., Guo, Z., Khatib, A., 2020. Antimicrobial Properties of *Apis mellifera*'s Bee Venom. *Toxins (Basel)*. 12, 451.
- Fadl, A.-E.-W., 2018. Antibacterial and antibiofilm effects of bee venom from (*apis mellifera*) on multidrug-resistant bacteria (MDRB). *Al-Azhar. J. Pharm. Sci.* 58, 60–80.
- Fernandes Júnior, A., Balestrin, E.C., Betoni, J.E.C., Orsi, R. de O., da Cunha, M. de L.R. de S., Montelli, A.C., 2005. Propolis: anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. *Mem. Inst. Oswaldo Cruz.* 100, 563–566.
- Galdiero, E., Siciliano, A., Gesuele, R., Di Onofrio, V., Falanga, A., Maione, A., Liguori, R., Libralato, G., Guida, M., 2019. Melittin inhibition and eradication activity for resistant polymicrobial

- biofilm isolated from a dairy industry after disinfection. *Int. J. Microbiol.* 2019.
- Grecka, K., Kuś, P.M., Okińczyc, P., Worobo, R.W., Walkusz, J., Szveda, P., 2019. The anti-staphylococcal potential of ethanolic polish propolis extracts. *Molecules* 24, 1732.
- Grecka, K., Xiong, Z.R., Chen, H., Pełka, K., Worobo, R.W., Szveda, P., 2020. Effect of Ethanol Extracts of Propolis (EEPs) against Staphylococcal Biofilm—Microscopic Studies. *Pathogens* 9, 646.
- Halcón, L., Milkus, K., 2004. Staphylococcus aureus and wounds: a review of tea tree oil as a promising antimicrobial. *Am. J. Infect. Control* 32, 402–408.
- Han, S.M., Kim, J.M., Hong, I.P., Woo, S.O., Kim, S.G., Jang, H.R., Pak, S.C., 2016. Antibacterial activity and antibiotic-enhancing effects of honeybee venom against methicillin-resistant staphylococcus aureus. *Molecules* 21, 79.
- Han, S., Lee, K., Yeo, J., Kim, W., Park, K., 2011. Biological effects of treatment of an animal skin wound with honeybee (*Apis mellifera*. L) venom. *J. Plast. Reconstr. Aesthetic Surg.* 64, e67–e72.
- Hannan, A., Batool, A., Qamar, M.U., Khalid, F., 2015. Propolis as an antibacterial agent against clinical isolates of *mdr-acinetobacter baumannii*. *J. Ayub Med. Coll. Abbottabad* 27, 216–219.
- Hassan, M.E., Shehata, H.A., Fahmy, A., Badr, M., Tamer, T.M., Omer, A.M., 2021. Development of biodegradable poly (vinyl alcohol)/chitosan cross linked membranes for antibacterial wound dressing applications., *Jordan. J. Biol. Sci.* 14.
- Hegazi, A.G., Abd El Hady, F.K., 2001. Egyptian propolis: 1-antimicrobial activity and chemical composition of Upper Egypt propolis. *Zeitschrift Für Naturforsch. C* 56, 82–88.
- Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S., Ciofu, O., 2010. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35, 322–332.
- Hozzein, W.N., Badr, G., Al Ghamdi, A.A., Sayed, A., Al-Waili, N.S., Garraud, O., 2015. Topical application of propolis enhances cutaneous wound healing by promoting TGF-beta/Smad-mediated collagen production in a streptozotocin-induced type I diabetic mouse model. *Cell. Physiol. Biochem.* 37, 940–954.
- Hsu, S.-M., Raine, L., Fanger, H.X., 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29, 577–580.
- Jami, A.L.A.A., Fathi, B., Jamshidi, A., Zolfagharian, H., Zare, M.A., 2010. Investigation of the antibacterial effect of venom of the Iranian snake *Echis carinatus*.
- Jouvenaz, D.P., Blum, M.S., MacConnell, J.G., 1972. Antibacterial Activity of Venom Alkaloids from the Imported Fire Ant, *Solenopsis invicta* Buren. *Antimicrob. Agents Chemother.* 2, 291–293.
- Kenawy, E., Omer, A.M., Tamer, T.M., Elmeligy, M.A., Eldin, M.S.M., 2019. Fabrication of biodegradable gelatin/chitosan/cinnamaldehyde crosslinked membranes for antibacterial wound dressing applications. *Int. J. Biol. Macromol.* 139, 440–448.
- Ko, S.J., Kim, M.K., Bang, J.K., Seo, C.H., Luchian, T., Park, Y., 2017. *Macropis fulvipes* venom component Macropin exerts its antibacterial and anti-biofilm properties by damaging the plasma membranes of drug resistant bacteria. *Sci. Rep.* 7, 1–14.
- Kortenska, V.D., Velikova, M.P., Yanishlieva, N.V., Totzeva, I.R., Bankova, V.S., Marucci, M.C., 2002. Kinetics of lipid oxidation in the presence of cinnamic acid derivatives. *Eur. J. Lipid Sci. Technol.* 104, 19–28.
- Ku, Y.H., Kang, J.H., Lee, H., 2020. Effect of Bee Venom on an Experimental Cellular Model of Alzheimer's Disease. *Am. J. Chin. Med.* 1–17.
- Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. *Food Chem.* 84, 329–339.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Liu, H., Wang, C., Li, C., Qin, Y., Wang, Z., Yang, F., Li, Z., Wang, J., 2018. A functional chitosan-based hydrogel as a wound dressing and drug delivery system in the treatment of wound healing. *RSC Adv.* 8, 7533–7549.
- Lotfy, M., 2006. Biological activity of bee propolis in health and disease. *Asian Pac J Cancer Prev.* 7, 22–31.
- Mi, F.L., Wu, Y.B., Shyu, S.S., Schoung, J.Y., Bin Huang, Y., Tsai, Y. H., Hao, J.Y., 2002. Control of wound infections using a bilayer chitosan wound dressing with sustainable antibiotic delivery. *J. Biomed. Mater. Res. An Off. J. Soc. Biomater. Japanese Soc. Biomater. Aust. Soc. Biomater. Korean Soc. Biomater.* 59, 438–449.
- Mohamed, R., El-Beheri, N.G., Agwa, M.M., Eltaher, H.M., Alse-qely, M., Sadik, W.S., El-Khordagui, L., 2021. Antibiotic-free combinational hyaluronic acid blend nanofibers for wound healing enhancement. *Int. J. Biol. Macromol.* 167, 1552–1563.
- Molan, P.C., 1999. The role of honey in the management of wounds. *J. Wound Care.* 8, 415–418.
- N.C. for C.L. Standards, A.L. Barry, 1999. Methods for determining bactericidal activity of antimicrobial agents: approved guideline, National Committee for Clinical Laboratory Standards Wayne, PA.
- Neto, M.S.R., Tintino, S.R., da Silva, A.R.P., do Socorro Costa, M., Boligon, A.A., Matias, E.F.F., de Queiroz Balbino, V., Menezes, I. R.A., Coutinho, H.D.M., 2017. Seasonal variation of Brazilian red propolis: Antibacterial activity, synergistic effect and phytochemical screening. *Food Chem. Toxicol.* 107, 572–580.
- Ong, S.-Y., Wu, J., Moochhala, S.M., Tan, M.-H., Lu, J., 2008. Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties. *Biomaterials* 29, 4323–4332.
- Oryan, A., Alemzadeh, E., Moshiri, A., 2018. Potential role of propolis in wound healing: Biological properties and therapeutic activities. *Biomed. Pharmacother.* 98, 469–483.
- Park, I.-S., Chung, P.-S., Ahn, J.C., 2015. Enhancement of ischemic wound healing by spheroid grafting of human adipose-derived stem cells treated with low-level light irradiation. *PLoS ONE* 10, e0122776.
- Pawde, S.M., Deshmukh, K., 2008. Characterization of polyvinyl alcohol/gelatin blend hydrogel films for biomedical applications. *J. Appl. Polym. Sci.* 109, 3431–3437.
- Pereira, P.S., de Lima, M. do C.A., Neto, P.P.M., de Moraes Oliveira-Tintino, C.D., Tintino, S.R., de Alencar Menezes, I.R., de Oliveira, J.F., Marchand, P., Coutinho, H.D.M., do Desterro Rodrigues, M., reira et al. 2019. Thiazolidinedione and thiazole derivatives potentiate norfloxacin activity against NorA efflux pump over expression in *Staphylococcus aureus* 1199B strains. *Bioorg. Med. Chem.* 27, 3797–3804.
- Picoli, T., Peter, C.M., Zani, J.L., Waller, S.B., Lopes, M.G., Boesche, K.N., de Oliveira Hübner, S., Fischer, G., 2017. Melittin and its potential in the destruction and inhibition of the biofilm formation by *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* isolated from bovine milk. *Microb. Pathog.* 112, 57–62.
- Rybak-Chmielewska, H., Szczęśna, T., 2004. HPLC study of chemical composition of honeybee (*Apis mellifera* L.) venom. *J. Apic. Sci.* 48, 103–109.
- Saarai, A., Sedlacek, T., Kasparkova, V., Kitano, T., Saha, P., 2012. On the characterization of sodium alginate/gelatin based hydrogels for wound dressing. *J. Appl. Polym. Sci.* 126, E79–E88.
- Sabra, S., Ragab, D.M., Agwa, M.M., Rohani, S., 2020. Recent advances in electrospun nanofibers for some biomedical applications. *Eur. J. Pharm. Sci.* 144, 105224.
- Said, S.A., Khan, S.A., Ahmad, I., Ali, H.S., 2006. Chemical composition of Egyptian and UAE propolis. *Pak. J. Pharm. Sci.* 19, 58–61.
- Salada, J.-A.-T., Balala, L.M., Vasquez, E.A., 2015. Phytochemical and antibacterial studies of *Lantana camara* L. leaf fraction and essential oil. *Int. J. Sci. Res. Publ.* 5, 1–5.

- Salas-Oropeza, J., Jimenez-Estrada, M., Perez-Torres, A., Castell-Rodriguez, A.E., Becerril-Millan, R., Rodriguez-Monroy, M.A., Jarquin-Yañez, K., Canales-Martinez, M.M., 2021. Wound Healing Activity of α -Pinene and α -Phellandrene. *Molecules* 26, 2488.
- Samanci, T., Kekeçođlu, M., 2019. Comparison of commercial and Anatolian bee venom in terms of chemical composition. *Uludađ Arcılık Derg.* 19, 61–68.
- Santos-Buelga, C., González-Paramás, A.M., 2017. Chemical composition of honey. In: *Bee Prod. Biol. Prop.*. Springer, pp. 43–82.
- Serra, R., Grande, R., Butrico, L., Rossi, A., Settimio, U.F., Caroleo, B., Amato, B., Gallelli, L., de Francis, S., 2015. Chronic wound infections: the role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Expert Rev. Anti. Infect. Ther.* 13, 605–613.
- Shalaby, M., Agwa, M., Saeed, H., Khedr, S.M., Morsy, O., El-Demellawy, M.A., 2020. Fish scale collagen preparation, characterization and its application in wound healing. *J. Polym. Environ.* 28, 166–178.
- Shebeeb, A.K., Ghanim, A.F., Awayid, H.S., 2020. Antibacterial Activity Of Bee Venom Against Multidrug Resistance *Staphylococcus Aureus* From Milk Of Cow And Buffaloes. *Int. J. Res. Pharm. Sci.* 11, 2177–2182.
- Sobral, F., Sampaio, A., Falcão, S., Queiroz, M.J.R.P., Calheta, R. C., Vilas-Boas, M., Ferreira, I.C.F.R., 2016. Chemical characterization, antioxidant, anti-inflammatory and cytotoxic properties of bee venom collected in Northeast Portugal. *Food Chem. Toxicol.* 94, 172–177.
- Sowndhararajan, K., Kang, S.C., 2013. Free radical scavenging activity from different extracts of leaves of *Bauhinia vahlii* Wight & Arn., Saudi. *J. Biol. Sci.* 20, 319–325.
- Stewart, P.S., 2002. Mechanisms of antibiotic resistance in bacterial biofilms. *Int. J. Med. Microbiol.* 292, 107–113.
- Stout, E.I., McKessor, A., 2012. Glycerin-based hydrogel for infection control. *Adv. Wound Care.* 1, 48–51.
- Surendra, N.S., Jayaram, G.N., Reddy, M.S., 2011. Antimicrobial activity of crude venom extracts in honeybees (*Apis cerana*, *Apis dorsata*, *Apis florea*) tested against selected pathogens, African. *J. Microbiol. Res.* 5, 2765–2772.
- Szweda, P., Kot, B., 2017. Bee products and essential oils as alternative agents for treatment of infections caused by *S. aureus*. In: *Front. Staphylococcus Aureus*, IntechOpen.
- Takzaree, N., Hadjiakhondi, A., Hassanzadeh, G., Rouini, M.R., Manayi, A., 2016. Synergistic effect of honey and propolis on cutaneous wound healing in rats.
- Tamer, T.M., Valachová, K., Hassan, M.A., Omer, A.M., El-Shafeey, M., Eldin, M.S.M., Šoltés, L., 2018. Chitosan/hyaluronan/edaravone membranes for anti-inflammatory wound dressing: In vitro and in vivo evaluation studies. *Mater. Sci. Eng. C.* 90, 227–235.
- Tang, F., Lv, L., Lu, F., Rong, B., Li, Z., Lu, B., Yu, K., Liu, J., Dai, F., Wu, D., 2016. Preparation and characterization of N-chitosan as a wound healing accelerator. *Int. J. Biol. Macromol.* 93, 1295–1303.
- Uawonggul, N., Thammasirirak, S., Chaveerach, A., Arkaravichien, T., Bunyatratchata, W., Ruangjirachuporn, W., Jearranaipreame, P., Nakamura, T., Matsuda, M., Kobayashi, M., 2007. Purification and characterization of Heteroscorpine-1 (HS-1) toxin from *Heterometrus laoticus* scorpion venom. *Toxicon* 49, 19–29.
- Ueno, H., Yamada, H., Tanaka, I., Kaba, N., Matsuura, M., Okumura, M., Kadosawa, T., Fujinaga, T., 1999. Accelerating effects of chitosan for healing at early phase of experimental open wound in dogs. *Biomaterials* 20, 1407–1414.
- Ueno, H., Mori, T., Fujinaga, T., 2001. Topical formulations and wound healing applications of chitosan. *Adv. Drug Deliv. Rev.* 52, 105–115.
- Vichai, V., Kirtikara, K., 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat. Protoc.* 1, 1112–1116.
- Wehbe, R., Frangieh, J., Rima, M., El Obeid, D., Sabatier, J.M., Fajloun, Z., 2019. Bee venom: Overview of main compounds and bioactivities for therapeutic interests. *Molecules* 24. <https://doi.org/10.3390/molecules24162997>.
- Werner, S., Breeden, M., Hübner, G., Greenhalgh, D.G., Longaker, M.T., 1994. Induction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse. *J. Invest. Dermatol.* 103, 469–473.
- Zgoda, J.R., Porter, J.R., 2001. A convenient microdilution method for screening natural products against bacteria and fungi. *Pharm. Biol.* 39, 221–225.
- Zoghi, N., Fouani, M.H., Bagheri, H., Nikkha, M., Asadi, N., 2021. Characterization of minocycline loaded chitosan/polyethylene glycol/glycerol blend films as antibacterial wound dressings. *J. Appl. Polym. Sci.* 138, 50781.
- Zolfagharian, H., Mohajeri, M., Babaie, M., 2016. Bee venom (*Apis Mellifera*) an effective potential alternative to gentamicin for specific bacteria strains: Bee venom an effective potential for bacteria. *J. Pharmacopuncture.* 19, 225.