Biological and Biomedical Journal

Journal homepage: https://bbj.journals.ekb.eg

Egyptian citrus honey as a promising antibacterial agent against multidrug-resistant *Staphylococcus aureus* with anti-oxidant and anti-inflammatory activities.

Khalid M. Swidan*, Mahmoud K. Ismail, Tarek M. Abdelghany

Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Egypt

ARTICLE INFO

Received: 12/12/2024
Accepted: 30/12/2024

***Corresponding author:**
Khalid Swidan, Ph.D
E-mail: khswidan2015@gmail.com
Mobile: 01022587445

P-ISSN: 2974-4334
E-ISSN: 2974-4324
DOI: 10.21608/BBJ.2024.344740.1065

ABSTRACT

The primary output of the apiary industry, whose heritage parallels that of humanity, is honey. Studies on the antibacterial effect of honey have shown that most bacterial pathogens remain sensitive to it, and it was hypothesized that a combination of antibiotics and honey would delay the development of Multi Drug Resistant (MDR) bacteria more effectively than antibiotics alone. The continued misuse of antibiotics provides strong selective pressure, favoring the emergence of antibiotic-resistant mutants. The objective of the current study is to examine the antibacterial role of citrus honey on resistant bacterial pathogens isolated from semen from patients in El Hussien Hospital. Phenolic compounds and flavonoids in the citrus honey extract (CHE) were analyzed using HPLC. Antioxidant action of the extract was done using the DPPH procedure. Moreover, the anti-inflammatory role of the CHE was done using a hemolysis assay. The safety of the extract was assessed by testing through the MTT method versus Vero cells as normal cells. Eleven various phenolic molecules with various concentrations could be seen in the extract. While separation of flavonoids compounds in citrus honey extract revealed the presence of eight different molecules with different levels. *Staphylococcus aureus* was identified as the most resistant bacterium from semen from Egyptian patients, and its identification was confirmed by molecular identification with the accession number PQ637167.1. The CHE had an antibacterial impact towards *S. aureus* with a diameter of 2.8±0.7 mm, as well as antioxidant, anti-inflammatory and cytotoxicity with levels of IC_{50}= 4.21 ±0.2 µg/ml, IC_{50}= 12.21 ±0.2 µg/ml, and CC_{50}=18.2±0.7 respectively. The present results suggest the possible *in vitro* pleiotropic impact of citrus honey for future pharmaceutical applications.

Keywords: Antioxidant, Anti-inflammatory, Citrus honey, Resistant bacteria, Toxicity assay

1. Introduction

Semen's nutrient-rich composition supports bacterial growth (Sepúlveda et al., 2016; Zhang et al., 2020). Bacteria found in semen are typically classified as either probiotics, which benefit mammals, or pathogens, which can cause infections. Probiotic bacteria help maintain semen integrity and longevity (Schulze et al., 2019; Farsimadan et al., 2020; Ďuračka et al., 2021). Pathogenic microorganisms can negatively affect reproductive function and semen quality. The impact of pathogenic bacteria and alterations in seminal plasma molecules on sperm composition are key areas of research

(Lundy et al., 2021; Liang et al., 2023; de Almeida Gomes et al., 2023).

Bacterial contamination of semen can reduce its quality, lowering sperm motility and shortening shelf life (Menezes et al., 2020; Miao et al., 2024). Additionally, bacteria in semen can infect the female reproductive tract, leading to early embryo implantation failure, fetal death, and birth defects, all of which may culminate in significant financial losses (McAnally et al., 2023).

Honey is the primary product of the apiary industry, with a history that dates back to humanity's earliest civilizations. Its medicinal benefits were documented in ancient Egyptian and Mediterranean therapies over five thousand years ago (Harutyunyan and Malfeito-Ferreira 2022). The bioactive compounds in honey, which vary among types, are responsible for its health benefits. Among the most significant bio-components found in the honey's makeup are polyphenols and flavonoids. Flavonoids and non-flavonoids make up polyphenols, a significant family of organically produced organic substances characterized by numerous phenol units. Honey contains various types of flavonoids and other polyphenolic compounds (Fratianni et al., 2023). Citrus honey is primarily manufactured in subtropical regions and countries of the Mediterranean (Pereira et al., 2020). This honey is pale, crystallizes rapidly, and has an odd flavor and scent. This honey is highly sought-after and economically lucrative due to these qualities linked to its biological features (Gao et al., 2020). These factors also promote the use of light honeys or fake substances in deception (Stefas et al., 2022). The novelty of this study lies in its focus on *S. aureus* isolated from semen samples using citrus honey extract. The chemical composition analysis revealed 11 phenolic and 8 flavonoid compounds through HPLC. The extract demonstrated antibacterial, antioxidant, and anti-inflammatory effects, along with high safety on normal cells, highlighting its potential for future pharmaceutical applications. This study aims to examine the antibacterial effects of citrus honey on antibiotic-resistant bacteria isolated from semen, as well as its vitro antioxidant, anti-inflammatory, and cytotoxic properties.

2. Materials and methods

Obtaining citrus honey and the process of extraction

The honey produced from Egyptian citrus was purchased from a beehive colonies manufacturing facility in the Elsharkya region and stored at 4°C in a well-ventilated and dimly lit environment to preserve its quality and prevent degradation of its bioactive compounds. The monofloral origin was confirmed by microscopically examining the honey for the most prevalent pollens (Ahmed and Othman, 2017). To prepare the extract, methanol was first brought to room temperature, then the mixture was rotated for 24 hours at 300 rpm to ensure efficient extraction of the honey's bioactive components (Babaei et al., 2017). Allowing it to reach room temperature enhances solubility, while rotation ensures maximum extraction efficiency.

HPLC analysis of different bioactive compounds

The study was performed using HPLC (Agilent 2200), which is made up of two LC pumps and a UV/Vis detector. C18 column (particle size of 5 µm, 120 x 4.80 mm). Chromatograms were gathered and examined using the Agilent ChemStation. Phenolic acids were separated using a mobile phase made up of two solvents: 0.4% methanol and phosphoric acid (50:50 v/v, isocratic mode). The instrument's wavelength was configured to 295 nm with the mobile phase, and the liquid flow rate was set to 1.5 mL/min for flavonoid analysis. The mobile phase consisted of a 50:50 v/v binary mixture of methanol and water that had been adjusted to pH 2.6 using phosphoric acid and had an isocratic flow rate of 1.4 mL min^{-1} (Bok et al., 2024).

Acquisition of semen specimens

The study was conducted in the Urology Department of El-Hussien Hospital, where participants were recruited and examined. A total of 200 participants were enrolled between January and June 2023, divided into 100 individuals in a reproductive well-being group and 100 in a patient group. Male infertile subjects served as controls for the seminal fluid bacteriological analysis, with semen samples collected from all participants. Each group

consisted of 100 participants to ensure statistical reliability and comparability. The patient group also included males with azoospermia or a combination of asthenozoospermia and oligozoospermia. Similarly, the control group consisted of 100 healthy male respondents, matched for age and reproductive status, who met the same eligibility criteria but showed no signs of infertility. Semen cultures were performed for all samples to identify bacterial pathogens. Ethical approval for the study was obtained from the International Islamic Institute for Population Development and Research at Al-Azhar University (Ethical no. AZF22720222). Further elaboration on the participant selection ensures methodological clarity and strengthens the study's credibility.

Isolation and identification of bacteria from seminal fluid

Bacteria were isolated and identified using seminal fluid specimens using the procedures outlined by Weng et al., (2014). The expanding colonies were then grown on nutrient agar plates after the research specimens were placed in nutrient broth, a subculture on solidified agar dishes using media like blood agar and MacConkey agar (Diamond, India). The pure bacterial strains were obtained by recultivation on nutrient-agar plates (Diamond, India) and then kept for 24 hours at 38°C (Pan et al., 2020) and then for another 24 hours at 38°C in an aerobic environment (Esmailkhani et al., 2018). The isolated strains' morphological characteristics were demonstrated using KOH and Gramm coloring (Folliero et al., 2022). Among the biochemical tests were oxidase and catalase analyses (Olana et al., 2023).

Finding the most resistant bacterial isolate

In accordance with the International Standard, every isolate's sensitivity to antibiotics was evaluated using Mueller-Hinton medium (Chand et al., 2022). After allowing the media to cool 40˚C, it was placed onto the glass plates till it was roughly 5 mm thick. The solidified discs were then allowed to stand at 38˚C for 12 to 20 minutes in order to release any remaining moisture. A sterile swab was dipped into the inoculum to inoculate the dishes. The swab was then firmly squeezed and rotated throughout the fluid level across the side of the tube wall to remove any remaining inoculum (Ngo et al., 2023). The swab was rubbed through the medium's interface, and then, after three sixty-degree rotations of the dish, it was enabled to slip over the topmost portion of the agar layer. The plate was coated and then allowed to air dry for a few minutes at the normal temperature. To prevent moisture buildup on the agar surface, the antibiotic discs were placed after the inoculation period of 15 minutes, and the plates of agar were subsequently incubated on their sides. Ten antibiotic discs were chosen using hot forceps, and before being placed in each dish, each was gently pressed from the bottom to guarantee sufficient contact with the bottom layer (Kumaresan et al., 2022).

Phylogenetic analysis and 16S RNA for the most resistant strain

Luria-Bertani broth (LB) was used to cultivate the bacterial isolates for a whole day. After three spinning cycles and recovery wash in 0.88% NaCl, the DNA fragments were extracted over five minutes at 16,000 g. Following the supplier's guidelines, genomic DNA was isolated using the Gene JET DNA genome sweeping kit (Abcam, UK). The forward primer 8F (5′-CTA GCC TAA CCG ATG CAA GTC-3′) and the reverse primer (5′-GGT CGG GTC GTACAA GGC-3′) were used for amplification. Cleaning and sequencing have been performed on the amplified PCR result. The basic sequencing information was modified using the Finch T.V. 1.6.0 program. The strain's 16S rRNA sequences were examined using the National Centre for Genetic Information's (NCBI) BLAST tool (MD, USA). ClustalW 2.2 was used to align many sequences. MEGA X was used to build the phylogenetic trees through the neighbor joining strategy (Malaluang et al., 2024).

Assessment of citrus honey's antibacterial properties and lowest inhibitory level

To evaluate the antibacterial capacity of citrus honey extract against the most resistant isolate of bacteria identified in semen, 100 µl of extract filtrate was added to the holes using the agar diffusion technique. At the end of the incubation period, the sites of inhibition were identified. For a range of test organisms, the optimum dosage

was produced in a series of dilution procedures and assessed (Wasfi et al., 2016).

Identification of citrus honey's antioxidant effects

The antioxidant examination, which relies on the exchange of electrons procedure, was performed to analyze the citrus honey specimen's capacity to scavenge free radicals using DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) (Diamond, India). At ambient temperatures, the samples in different dilutions were incubated with DPPH for half an hour. The color change of DPPH was measured using the spectroscopic technique at 580 nm. Ascorbic acid was used as a reference (Li et al., 2024).

Evaluation of citrus honey's anti-inflammatory properties

A membrane stabilization test was done to determine the anti-inflammatory qualities of citrus honey extract. A variety of sample levels were created, ranging from 100 to 1000 µg/ml. A liquid that was hypotonic was added to the acquired specimens. Purified water and indomethacin were employed as both positive and negative standards. The combination had been kept for two hours at 38°C after 500 µL of the specimens were added to the fresh erythrocyte solution (2.9%) in 0.7 mL of saline. The combination was then spun at 16,000 × g for 35 minutes at 10°C. At 580 nm, the substance's absorbency was determined (Luo et al., 2023).

Evaluation of citrus honey's cytotoxic impact

African green monkey cells (Vero cells) were utilized to test the extract's cytotoxic effects. Cells were exposed to the extract at concentrations ranging from 1000 to 15.63 µg/mL after 24 hours of adhesion until integration, and they were allowed to develop for 24 hours at 38°C. The fresh medium was inserted, and four hours later, at 38°C, 100 µL of MTT solution (6 mg/mL) was added. A microplate reader was used for determining intensity at 580 nm (Ahmed et al., 2022).

Analysis of statistics

Following a one-way ANOVA to determine whether the treatments were significant, a Tukey's post hoc test was conducted at $p < 0.05$ for the analysis of the information. The mathematical information analysis was conducted with GraphPad Prism 7.0.

4. Results

Chemical composition of citrus honey using HPLC

HPLC separation of phenolic compounds in citrus honey extract revealed the presence of 11 various molecules with various concentrations which were: Caffeic acid (19.16), Chorogenic acid (9.63), Cinnamic acid (9.16), Ellagic acid (4.09), Ferullic acid (4.70), Gallic acid (9.00), Coumaric acid (9.75), Sinapic acid (6.45), Syringic acid (9.33), Vanilic acid (9.64) and Hydeoxycinnamic acid (9.09) as shown in Figure 1, and Table 1).

Table 1. Various phenolic compounds with their concentrations and retention times in citrus honey extract separated by HPLC.

Retention Time	Molecule Name	Conc. (µg/ml)
3.0	Caffeic acid	19.16
5.0	Chorogenic acid	9.63
6.0	Cinnamic acid	9.16
8.0	Ellagic acid	4.09
9.0	Ferullic acid	4.70
11.0	Gallic acid	9.00
13.0	Coumaric acid	9.75
14.0	Sinapic acid	6.45
15.0	Syringic acid	9.33
16.2	Vanilic acid	9.64
17.5	Hydeoxycinnamic acid	9.09

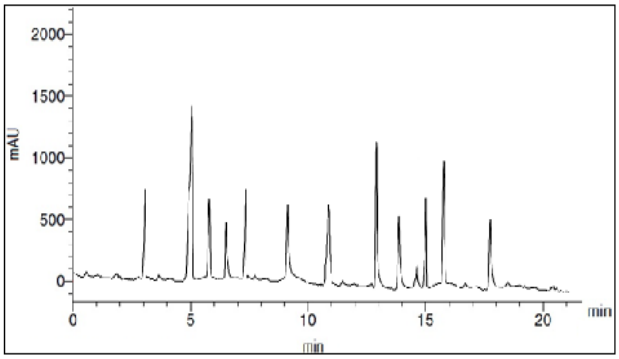

Figure 1. HPLC chromatogram showing various peaks for phenolic compounds exist in CHE.

While, HPLC separation of flavonoids in citrus honey extract revealed the presence of 8 different molecules with various levels which were 8 7-

OH flavone (12.11), Naringin (14.22), Rutin (11.12), Myricetin (5.79), Quercetin (12.20), Kamferol (16.22), Lutin (8.34), and Catechin (20.00) as illustrated in (Figure 2 and Table 2).

Table 2. Various flavonoids with their levels and corresponding retention times in CHE separated by HPLC.

Retention Time	Molecule Name	Conc. (µg/ml)
3.0	7-OH flavone	12.11
4.2	Naringin	14.22
5.3	Rutin	11.12
6.0	Myricetin	5.79
8.0	Quercetin	12.20
11.0	Kamferol	16.22
13.0	Lutin	8.34
15.0	Catechin	20.00

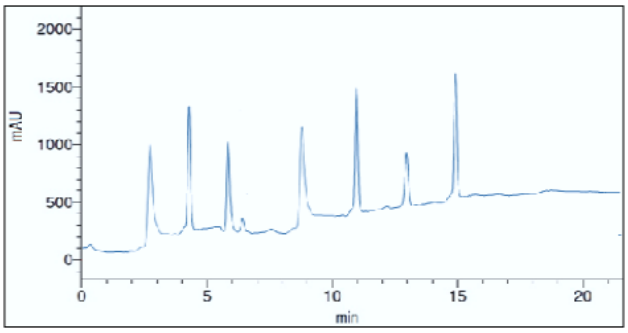

Figure 2. HPLC chromatogram showing various peaks for flavonoids exist in CHE.

Various isolated bacteria from semen

All 200 study subjects 100 infected and 100 normal controls—had their semen cultures performed. Only 60 of the 100 patients had a semen culture that tested positive for bacterial growth. As seen in Figure 3A, out of the 60 bacteriospermia patients, 34 (57%) had Gram-positive organisms, whereas only 26 (43%) had Gram-negative species. The most prevalent microbe in bacteriospermia was *S. aureus*, which made up 61% of the total. Gram-positive bacteria *S. aureus*, *B. subtilis*, and *E. faecalis* had isolate prevalence of 61%, 30.0%, and 9%, respectively. As shown in Figure 3B, the levels of the Gram-negative bacteria were 55.8%, 28.6%, 7.8%, and 7.8% for *P. aeruginosa*, *E. coli*, *P. mirabilis*, and *N. gonorrhoeae*, respectively. There was a significant distinction ($P < 0.05$) in the setting of the isolated bacteria between pyospermia (infected semen) and non-infected semen.

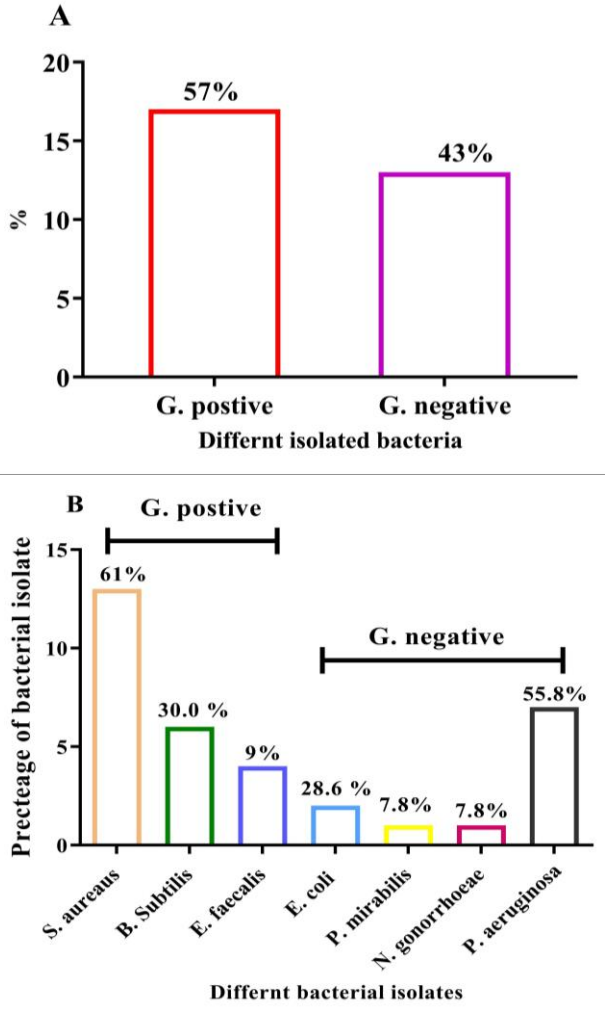

Figure 3. A: Various percentages of bacteria in infected semen according to Gram staining; B: Different levels of bacterial species in both Gram positive and Gram negative bacteria.

Assessment of antibiotics and sensitivity assay towards various isolated bacteria

The antibiotic sensitivity assessments were carried out using the Kirby-Bauer disc method of diffusion and determined the lowest inhibitory level for each bacterial isolate to determine the most commonly used antibacterial medications for the treatment of pyospermia. While some Gram positive bacteria were tolerant to some of the utilized medicines, all Gram negative bacteria were vulnerable to the majority of the antibiotics. Additionally, as shown in Table 3 and Figure 4. *S. aureus* was shown to be the most prevalent and resilient bacterium in the samples that were gathered.

Table 3. Assay for various antibiotics and their sensitives towards bacterial isolates collected from semen study participants

Isolates	E	DA	CEC	NOR	AM	AMC	OX	TE	FOX	TN
G. positive bacteria										
S. aureaus	R	R	R	R	R	R	R	R	R	R
B. Subtilis	1.7 ± 0.2	1.8 ± 0.2	2.1 ± 0.2	2.2 ± 0.2	2.1 ± 0.2	2.1 ± 0.2	1.8 ± 0.2	1.7 ± 0.2	1.8 ± 0.3	1.8 ± 0.1
E. faecalis	1.8 ± 0.1	2.1 ± 0.1	2.2 ± 0.1	R	3.1 ± 0.1	2.1 ± 0.3	2.1 ± 0.1	2.1 ± 0.2	1.7 ± 0.2	1.7 ± 0.1
G. negative bacteria										
E. coli	1.8 ± 0.3	2.7 ± 0.1	1.8 ± 0.2	R	1.9 ± 0.2	1.8 ± 0.2	1.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.2	1.9 ± 0.2
P. mirabilis	1.7 ± 0.1	1.0 ± 0.2	R	1.7 ± 0.2	1.4 ± 0.2	2.1 ± 0.2	R	1.8 ± 0.1	1.9 ± 0.3	R
P. aeruginoa	3.1 ± 0.1	3.0 ± 0.3	2.1 ± 0.3	2.4 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	3.0 ± 0.2	3.0 ± 0.3
N. gonorrhoeae	1.8 ± 0.1	R	1.4 ± 0.2	1.6 ± 0.1	1.9 ± 0.1	2.3 ± 0.1	1.6 ± 0.1	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.2

Zones of Inhibitions were represented by mm, outcomes are inserted as means ± SD, R: Resistant, E: Erythromycin, DA: clindamycin, CEC: Cefaclor, NOR: Norfloxacin, AM: Amoxicillin, AMC: Amoxicillin-clavulanic acid, OX: Oxacillin, TE: tetracycline, FOX: cefoxitin, TN: Ciprofloxacin.

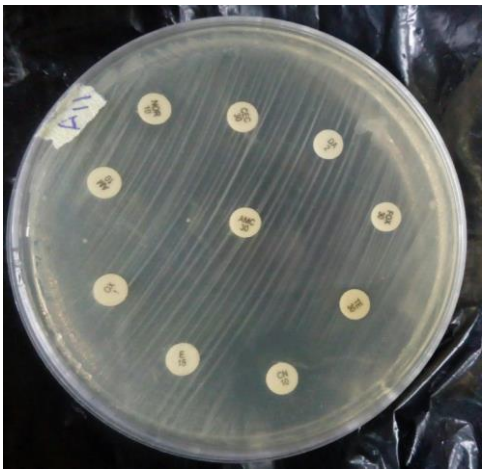

Figure 4. Antibiotics sensitivity pattern in the most resistant bacterial strain isolated from semen

S. aureus identification through genetics

The most resistant bacterial isolate strain was identified using 16 RNA *as Staphylococcus aureus* and was deposited in the gene bank with the accession number PQ637167.1 (https://www.ncbi.nlm.nih.gov/nuccore/PQ637167

It shared 98.60% of its similarities with the isolated in the gene bank. Besides, its phylogenetic tree could be seen in Figure 5.

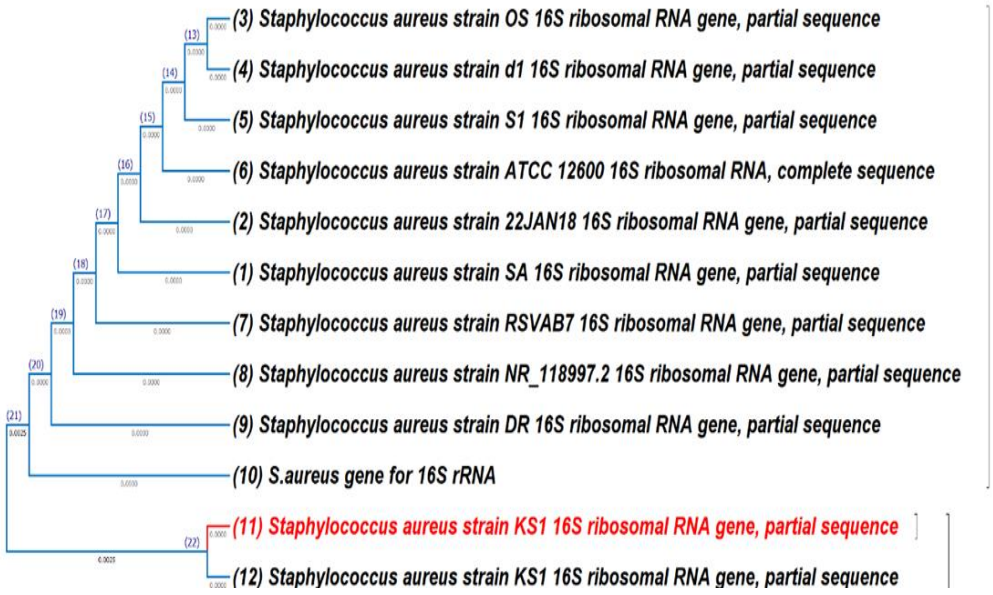

Figure 5. Phylogenetic tree for the most resistant bacterial strain isolated from semen (*S. auraeus*).

Antimicrobial properties of citrus honey towards the most resistant bacterial strain

The CHE demonstrated promising antibacterial activity against the most resistant bacterial isolate from semen, with an inhibition zone of 2.8 ± 0.7 mm and a minimal inhibitory concentration (MIC) of 125 ± 0.4 µg/ml (Figure 6). The antibiotic sensitivity profile of the bacterial isolate was tested against several antibiotics, including erythromycin, clindamycin, cefaclor, norfloxacin, amoxicillin, amoxicillin-clavulanic acid, oxacillin, tetracycline, cefoxitin, and ciprofloxacin. Among these, erythromycin exhibited the most effective antibacterial action. This highlights the potential of citrus honey extract as a complementary or alternative treatment, particularly against strains resistant to commonly used antibiotics.

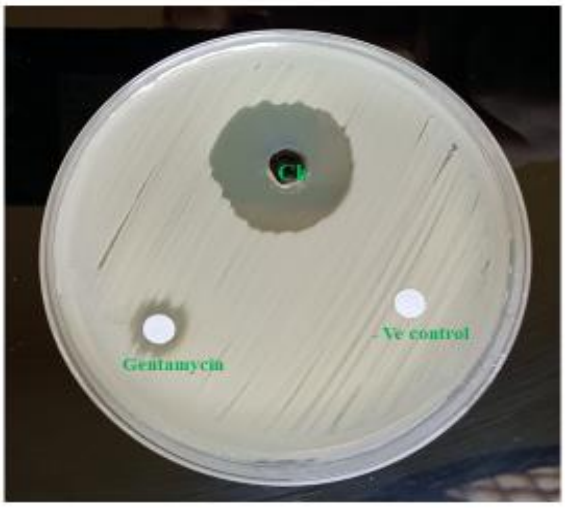

Figure 6. Agar diffusion assay for detection of antibacterial impact of citrus honey extract towards resistant *S. auraeus* isolated from semen (Ch: citrus honey extract; Gentamycin as positive control; -ve control: methanol as solvent applied for CHE)**.**

Antioxidant of citrus honey

The antioxidant value of ascorbic acid as a standard antioxidant was determined with a value of IC_{50} =2.52±0.7 µg/ml, and the antioxidant level for citrus honey extract was determined at IC_{50}= 4.21 ±0.2 µg/ml, revealing its promising antioxidant impact as shown in (Figure 7). Where, IC_{50}, or the half-maximal inhibitory concentration, is a quantitative measure used in pharmacology and biochemistry to indicate the effectiveness of a substance in inhibiting a specific biological or biochemical function. It represents the concentration of a substance (such as a drug, inhibitor, or extract) required to inhibit 50% of a given activity, such as enzyme activity, cell viability, or free radical scavenging in assays.

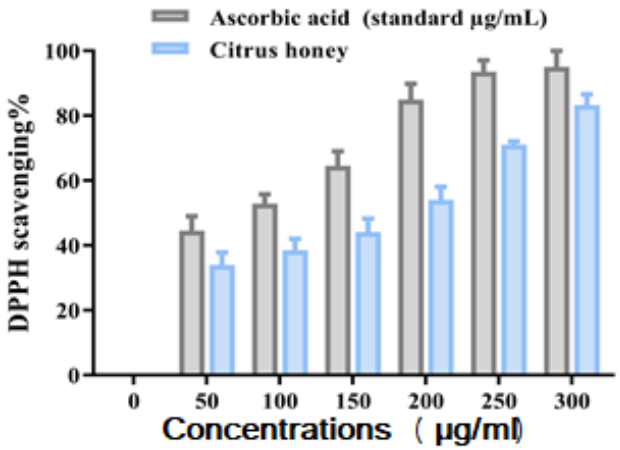

Figure 7. Determination of antioxidant impact of CHE using DPPH assay (Data are represented as means ± SD).

Anti-inflammatory of citrus honey

The anti-inflammatory value of indomethacin as a standard anti-inflammatory was determined as with a value of IC_{50} =7.42±0.7 µg/ml, and the anti-inflammatory level for citrus honey extract was determined at IC_{50}= 12.21 ±0.2 µg/ml, revealing its promising anti-inflammatory impact as depicted in (Figure 8).

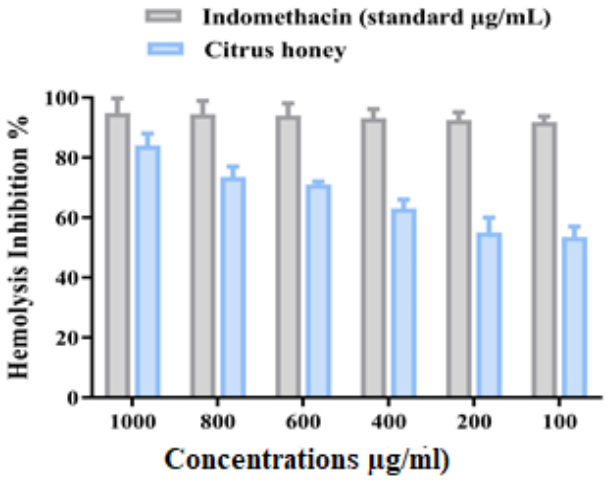

Figure 8. Detection of anti-inflammatory role of CHE using hemolysis method (Outcome are drawn as means ± SD).

Cytotoxicity of citrus honey

The cytotoxic impact of CHE was tested using the MTT assay, revealing its potent effect on normal cells with CC_{50}=18.2±0.7 µg/ml, as illustrated in Figure (9).

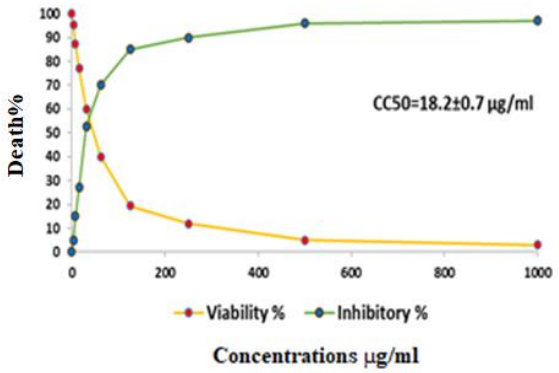

Figure 9. Assessment of toxicity impact of CHE towards normal cells by MTT method (Outcome are drawn as means ± SD).

5. Discussion

Natural antimicrobial medicines have been studied for years as a potential replacement for the pharmacological antibiotics currently in use in order to address the growing issue of bacterial multidrug resistance (Cheesman et al., 2017). The rising prevalence of MDR bacteria in patients and infections associated with healthcare (HAIs) is a major worldwide health concern (Raman et al., 2018). Bacterial tolerance is typically followed by biofilm formation, active antibiotic expulsion via the efflux pumps, and changes in the production of proteins in the membrane's outer layer (Uruén et al., 2020).

The present study examined the various bacteria isolated from semen and Gram positive bacteria were the most predominant bacteria in the study group, where *S. aureus* was reported as the most resistant bacteria isolated in this healthcare facility. The majority of Polish ejaculates include two or three pathogenic bacteria (Gączarzewicz et al., 2016), with the most commonly isolated bacterial *taxa being Staphylococcus, Streptococcus, and Pseudomonas*. In a semen extender, sperm and bacteria fight over resources; spermatozoa may be harmed by metabolic waste products from thriving bacteria and lipopolysaccharides produced from the cell walls of dead bacteria (Lenický et al., 2021). The overall bacterial population in cold prolonged semen rises with storage, and sperm cell membrane and acrosome stability are similarly disrupted (Hensel et al., 2020). The presence of bacteria and sperm characteristics, including motility and survival, were found to be negatively correlated in another investigation (Ngo et al., 2023).

Liquid chromatographic separation in this study revealed the presence of 11 phenolic compounds and eight flavonoids with various levels in Egyptian citrus honey. In accordance with various investigators who report the presence of poly phenols and flavonoids in citrus honey which is responsible for its biological activities (Lawag et al., 2022; Lawang et al., 2023; Aburayyan et al., 2024).

In this study, citrus honey showed a promising antibacterial action towards *S. aureus*, which was multidrug resistant bacterial strain isolated from semen in males with an age range of 40 to 60 years in El-Hussein Hospital. Besides, it showed promising antioxidant and anti-inflammatory properties with minimal action versus the tested normal cell line. The curative properties of honey against diseases, which are among the major health difficulties mostly in western countries, have been confirmed by *in vitro* and *in vivo* research conducted in recent years. Besides, bacterial biofilm development additionally impacts bacterial growth of amyloid structures, which are employed to reinforce the biofilm matrix and are clinically linked to protein unravelling and neurodegenerative sickness (Miller et al., 2021). In the past few years, a particular amount of bacterial infections has been linked to a greater likelihood of neurodegeneration (Murray et al., 2022). Raising antioxidant levels may help prevent these neurological disorders. The citrus honey also contains polyphenols, flavonoids, and vitamins, including vitamin C, which have anti-inflammatory and antioxidant properties as reported by many researchers (Swingier et al., 2022; Fratianni et al., 2023).

Conclusion

The bacterial strain *Staphylococcus aureus* (accession number PQ637167.1) was identified as the most resistant strain among male patients aged 40 to 60 at El-Hussein Hospital. Citrus honey extract demonstrated significant

antimicrobial efficacy against this resistant strain, along with notable antioxidant and anti-inflammatory properties. Furthermore, its high safety profile for normal cells suggests its potential for broader applications. Future large-scale utilization of this extract could be feasible following comprehensive validation through pre-clinical and clinical studies.

5. Reference

Aburayyan WS, Seder N, Al-fawares OL, Fararjeh A, Majali IS, Al-Hajaya Y 2024. Characterization of Antibiofilm and Antimicrobial Effects of Trigona Stingless Bee Honey Compared to Stinging Bee Centaurea hyalolepis and Citrus Honeys. Journal of Evidence-Based Integrative Medicine.;29:2515690X241271978.

Ahmed HY, Kareem SM, Atef A, Safwat NA, Shehata RM, Yosri M, Youssef M, Baakdah MM, Sami R, Baty RS, Alsubhi NH 2022. Optimization of supercritical carbon dioxide extraction of *saussurea costus* oil and its antimicrobial, antioxidant, and anticancer activities. Antioxidants. 30;11(10):1960.

Ahmed S, Othman NH 2017. The anti-cancer effects of Tualang honey in modulating breast carcinogenesis: An experimental animal study. BMC Complementary and Alternative Medicine.;17:1-1.

Babaei S, Rahimi S, Torshizi MA, Tahmasebi G, Miran SN 2016 . Effects of propolis, royal jelly, honey and bee pollen on growth performance and immune system of Japanese quails. In Veterinary Research Forum (Vol. 7, No. 1, p. 13). Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Bok VV, Šola I, Rusak G, Budisavljević A, Nguyen R, Ludwig-Müller J, Maleš Ž. 2024. Phenolic content and antioxidant activity of Croatian and German honey. Acta Pharm.

Chand N, Pande M, Tyagi S, Sirohi AS, Mahajan S, Kumar S, Sharma A 2022. Antibiogram of Microorganisms Isolated from Fresh and Frozen Semen of Crossbred Frieswal Bulls. Cryoletters. 1;43(6):322-7.

Cheesman MJ, Ilanko A, Blonk B, Cock IE 2017. Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution?. Pharmacognosy reviews.;11(22):57.

de Alcantara Menezes T, Mellagi AP, da Silva Oliveira G, Bernardi ML, Wentz I, da Rosa Ulguim R, Bortolozzo FP 2020. Antibiotic-free extended boar semen preserved under low temperature maintains acceptable in-vitro sperm quality and reduces bacterial load. Theriogenology. 1; 149:131-8.

de Almeida Gomes I, Monteiro PB, de Moura GA, Santos NO, Fontanezi CT, de Souza SV, Teixeira CA 2023. Microbiota and seminal quality: A systematic review. JBRA assisted reproduction.; 27(3):507.

Ďuračka M, Belić L, Tokárová K, Žiarovská J, Kačániová M, Lukáč N, Tvrdá E 2021.Bacterial communities in bovine ejaculates and their impact on the semen quality. Syst. Biol. Reprod. Med.; 67:438–449.

Esmailkhani A, Akhi MT, Sadeghi J, Niknafs B, Bialvaei AZ, Farzadi L, Safadel N 2018. Assessing the prevalence of *Staphylococcus aureus* in infertile male patients in Tabriz, northwest Iran. Int J Reprod Biomed.;16(7):469-474.

Farsimadan M, Motamedifar M 2020. Bacterial infection of the male reproductive system causing infertility. J. Reprod. Immunol.;142:103183.

Folliero V, Santonastaso M, Dell'Annunziata F, De Franciscis P, Boccia G, Colacurci N, De Filippis A, Galdiero M, Franci G 2022. Impact of *Escherichia coli* Outer Membrane Vesicles on Sperm Function. Pathogens.;11(7):782.

Fratianni F, Amato G, d'Acierno A, Ombra MN, De Feo V, Coppola R, Nazzaro F 2023. *In vitro* prospective healthy and nutritional benefits of different Citrus monofloral honeys. Sci Rep.;13(1):1088.

Gaczarzewicz D, Udala J, Piasecka M, Blaszczyk B, Stankiewicz T 2016. Bacterial contamination of boar semen and its relationship to sperm quality preserved in commercial extender containing gentamicin sulfate. Polish Journal of Veterinary Sciences.;19(3).

Gao Y, Xue A, Li X, Huang X, Ning F, Zhang X, Liu T, Chen H, Luo L 2020. Analysis of

chemical composition of nectars and honeys from Citrus by extractive electrospray ionization high resolution mass spectrometry, LWT, Volume 131, 109748..

Harutyunyan M, Malfeito-Ferreira M 2022. The rise of wine among ancient civilizations across the Mediterranean basin. Heritage. 2;5(2):788-812.

Hensel B, Jakop U, Scheinpflug K, Mühldorfer K, Schröter F, Schäfer J, Greber K, Jung M, Schulze M 2020. Low temperature preservation of porcine semen: Influence of short antimicrobial lipopeptides on sperm quality and bacterial load. Scientific Reports. 6;10(1):13225.

Kumaresan G, Gangwar C, Mishra AK, Kumar A, Kharche SD, Singh NP, Pachoori A. 2022. Occurrence, molecular characterization and antimicrobial-resistance pattern of Staphylococcus species isolates from buck semen. Arch Microbiol.;204(2):135.

Lawag IL, Islam MK, Sostaric T, Lim LY, Hammer K, Locher C 2023. Antioxidant activity and phenolic compound identification and quantification in western australian honeys. Antioxidants. 12;12(1):189.

Lawag IL, Lim LY, Joshi R, Hammer KA, Locher C. A 2022. comprehensive survey of phenolic constituents reported in Monofloral honeys around the globe. Foods. 15;11(8):1152.

Lenický M, Slanina T, Kačániová M, Galovičová L, Petrovičová M, Ďuračka M, Benko F, Kováč J, Tvrdá E 2021. Identification of bacterial profiles and their interactions with selected quality, oxidative, and immunological parameters of turkey semen. Animals. 14;11(6):1771.

Li W, Zan Y, Wu T, Yang S, Liu L, Li S, Dai P, Gao J 2024. Impact of chlorantraniliprole on honeybees: Differential sensitivity and biological responses in *Apis mellifera* and *Apis cerana*. Science of The Total Environment. 20;957:177417.

doi: 10.1016/j.scitotenv.2024.1.

Liang J., Wu T., Wang T., Ma Y., Li Y., Zhao S., Guo Y., Liu B. 2023. *Moringa oleifera* leaf ethanolic extract benefits cashmere goat semen quality via improving rumen microbiota and metabolome. Front. Vet. Sci.; 10:1049093.

Lundy SD, Sangwan N, Parekh NV, Selvam MK, Gupta S, McCaffrey P, Bessoff K, Vala A, Agarwal A, Sabanegh ES, Vij SC 2021. Functional and taxonomic dysbiosis of the gut, urine, and semen microbiomes in male infertility. European urology. 1;79(6):826-36.

Luo Y, Guo Y, Zhao W, Khalifa SAM, El-Seedi HR, Su X, Wu L. 2023. Total Lipid Extracts of Honeybee Drone Larvae Are Modulated by Extraction Temperature and Display Consistent Anti-Inflammatory Potential. Foods.;12(22):4058.

Malaluang P, Niazi A, Guo Y, Nagel C, Guimaraes T, Rocha A, Aurich C, Morrell JM. 2024. Bacterial diversity in semen from stallions in three European countries evaluated by 16S sequencing. Vet Res Commun.;48(3):1409-1421.

McAnally BE, Smith MS, Wiegert JG, Palanisamy V, Chitlapilly Dass S, Poole RK 2023. Characterization of boar semen microbiome and association with sperm quality parameters. Journal of Animal Science. 1;101:skad243.

Miao X, Zhao Y, Zhu L, Zeng Y, Yang C, Zhang R, Lund AK, Zhang M 2024. The Equilibrium of Bacterial Microecosystem: Probiotics, Pathogenic Bacteria, and Natural Antimicrobial Substances in Semen. Microorganisms. 7;12(11):2253. doi: 10.3390/microorganisms12112253.

Miller AL, Bessho S, Grando K, Tükel Ç 2021. Microbiome or infections: amyloid-containing biofilms as a trigger for complex human diseases. Frontiers in Immunology. 26;12:638867.

Murray ER, Kemp M, Nguyen TT 2022. The microbiota–gut–brain axis in Alzheimer's disease: a review of taxonomic alterations and potential avenues for interventions. Archives of Clinical Neuropsychology. 37(3):595-607.

Ngo C, Suwimonteerabutr J, Prapasarakul N, Morrell JM, Tummaruk P. 2023. Bacteriospermia and its antimicrobial resistance in relation to boar sperm quality during short-term storage with or without

antibiotics in a tropical environment. Porcine Health Manag.;9(1):21.

Olana S, Mazzilli R, Santino I, Martinelli D, Zamponi V, Macera M, Salerno G, Mazzilli F, Faggiano A, Gianfrilli D 2023. Sperm culture and bacterial susceptibility to antibiotics in a large andrological population: prevalence and impact on seminal parameters. International Microbiology.;26(1):69-79.

Pan F, Xiao X, Guo J, Song Y, Li H, Patel DP, Spivak AM, Alukal JP, Zhang X, Xiong C, Li PS 2020. No evidence of severe acute respiratory syndrome–coronavirus 2 in semen of males recovering from coronavirus disease 2019. Fertility and sterility. 1;113(6):1135-9..

Pereira JR, Campos AN, de Oliveira FC, Silva VR, David GF, Da Silva JG, Nascimento WW, Silva MH, Denadai ÂM 2020. Physical-chemical characterization of commercial honeys from Minas Gerais, Brazil. Food Bioscience. 1;36:100644.

doi.org/10.1016/j.fbio.2020.100644.

Raman G, Avendano EE, Chan J, Merchant S, Puzniak L 2018. Risk factors for hospitalized patients with resistant or multidrug-resistant *Pseudomonas aeruginosa* infections: a systematic review and meta-analysis. Antimicrobial Resistance & Infection Control. 7:1-4.

Schulze M, Czirják GÁ, Müller K, Bortfeldt R, Jung M, Jakop U 2019. Antibacterial defense and sperm quality in boar ejaculates. Journal of Reproductive Immunology. 1;131:13-20.

Sepúlveda L, Bussalleu E, Yeste M, Bonet S 2016. Effect of *Pseudomonas aeruginosa* on sperm capacitation and protein phosphorylation of boar spermatozoa. Theriogenology. 1;85(8):1421-31.

Stefas D, Gyftokostas N, Kourelias P, Nanou E, Tananaki C, Kanelis D, Liolios V, Kokkinos V, Bouras C, Couris S 2022. Honey discrimination based on the bee feeding by Laser Induced Breakdown Spectroscopy. Food Control. 1;134:108770.

Szwajgier D, Baranowska-Wójcik E, Winiarska-Mieczan A, Gajowniczek-Ałasa D 2022. Honeys as possible sources of cholinesterase inhibitors. Nutrients. 20;14(14):2969.

Uruén C, Chopo-Escuin G, Tommassen J, Mainar-Jaime RC, Arenas J 2020. Biofilms as promoters of bacterial antibiotic resistance and tolerance. Antibiotics. 23;10(1):3.

Wasfi R, Elkhatib WF, Khairalla AS. 2016. Effects of Selected Egyptian Honeys on the Cellular Ultrastructure and the Gene Expression Profile of *Escherichia coli*. PLoS One.;11(3): e0150984.

Weng SL, Chiu CM, Lin FM, Huang WC, Liang C, Yang T, Yang TL, Liu CY, Wu WY, Chang YA, Chang TH 2014. Bacterial communities in semen from men of infertile couples: metagenomic sequencing reveals relationships of seminal microbiota to semen quality. PloS one. 23;9(10):e110152..

Zhang J, Liu H, Yang Q, Li P, Wen Y, Han X, Li B, Jiang H, Li X 2020. Genomic sequencing reveals the diversity of seminal bacteria and relationships to reproductive potential in boar sperm. Frontiers in microbiology. 4;11:1873.