

## ORIGINAL RESEARCH ARTICLE

### *In vitro* evaluation of *Hyphaene thebaica* honey as a multitarget therapeutic product

Basheer Abu-Farich<sup>1</sup>, Mahmud Masalha<sup>2</sup>, Hadeel Hamarshi<sup>2</sup>,  
Asmae El Ghouizi<sup>1</sup>, Abderrazak Aboulghazi<sup>1</sup>, Mohammed El Ouassete<sup>3</sup>,  
Doha Weldali<sup>2</sup>, Badiaa Lyoussi<sup>1\*</sup>, and Bashar Saad<sup>2\*</sup>

<sup>1</sup>Laboratory of Natural Substances, Pharmacology, Environment, Modeling, Health, and Life Quality, Department of Biology, Faculty of Sciences Dhar El Mehraz, University Sidi Mohamed Ben Abdellah, Fez, Morocco

<sup>2</sup>Qasemi Research Center and Department of Biochemistry, Faculty of Medicine, Arab American University, Jenin, Palestine

<sup>3</sup>Laboratory of Microbial Biotechnology and Bioactive Molecules, Sciences and Technologies Faculty, Sidi Mohamed Ben Abdellah University, Fez, Morocco

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## Abstract

*Hyphaene thebaica* honey, commonly known as doum honey (DH), is widely utilized in the Mediterranean region due to its putative health benefits. However, the precise mechanisms underpinning these benefits remain obscure. This study sought to assess the anti-infective, anti-inflammatory, and anticancer properties of DH, and analyze its polyphenolic composition. The antibacterial effects of DH were tested against a range of multidrug-resistant Gram-positive and Gram-negative bacterial strains. In addition, we investigated the anti-inflammatory, antioxidant, and anticancer activities of DH in the MDA-MB-231 human breast cancer cell line. The phenolic compounds in DH were evaluated using quantitative high-performance liquid chromatography (HPLC). The model used to assess the anti-inflammatory properties was lipopolysaccharide (LPS)-activated macrophages. HPLC analysis revealed nine phenolic compounds in DH: Gallic acid, caffeic acid, carvacrol, p-coumaric acid, ellagic acid, kaempferol, pinobanksin, pinocembrin, and galangin. The minimum inhibitory concentration (MIC) values for DH varied between 0.19% and 0.78% w/w for the three Gram-positive strains tested and between 0.024% and 0.39% w/w for the four Gram-negative strains tested. Among all the bacterial strains tested, *Escherichia coli* was found to be the most susceptible, with an MIC of 0.024% w/w. Upon treating LPS-activated THP-1-derived macrophages with DH, the levels of nitric oxide were significantly diminished. Moreover, DH displayed a modest but significant cytostatic effect on the MDA-MB-231 cells. The most noticeable cytostatic impacts were observed at concentrations of 4 mg/mL and 2 mg/mL, resulting in a decrease in cell viability by 25% and 20%, respectively, compared to untreated control cells. A significant decline in the migration rate of MDA-MB-231 cells was observed following DH treatment compared to control cells ( $P < 0.05$ ). Our findings not only corroborate the well-established antibacterial properties of DH but also imply that its recognized anticancer advantages may be partially attributed to its antioxidant, anti-inflammatory, cytostatic, and antimigration effects.

**Keywords:** *Hyphaene thebaica*; Antibacterial; Antioxidant; Anticancer; Cytostatic; Anti-inflammatory; Antimigration

#### \*Corresponding authors:

Badiaa Lyoussi  
(lyoussi@gmail.com)  
Bashar Saad  
(Bashar@qsm.ac.il; Bashar.saad@aaup.edu)

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## 1. Introduction

Extensive research efforts have concentrated on exploring the therapeutic properties of a diverse array of natural compounds, which have been employed as natural remedies since ancient times and are generally regarded as having low toxicity. Their potential therapeutic benefits have attracted significant interest across various fields, including anti-inflammation, antimicrobial, and anticancer research.<sup>1</sup> A notable type of natural product is honey, a natural substance derived from nectar by honeybees. Honey has played a significant role in traditional medicine for centuries. Drawing from cultural beliefs, theoretical principles, and historical records, subjects from various civilizations, including the Greek, Roman, and Arab-Islamic societies, incorporate honey into their medical practices. In Arab-Islamic medicine, the medical value of honey is highly regarded owing to its therapeutic properties, and thus it is frequently used in wound care.<sup>1-4</sup> Different types of honey, many of which have been scientifically studied, are widely employed as natural remedies for maintaining health and addressing various illnesses.<sup>1,2</sup> Honey as well as its various constituents have been demonstrated to possess antibacterial, anti-inflammatory, antioxidant, antiproliferative, antitumor, antimetastatic, and anticancer properties.<sup>3-5</sup>

Among the traditional therapeutic uses of honey, its antimicrobial effects stand out as a prominent attribute. Antimicrobial resistance is globally acknowledged as a significant challenge to global health. This phenomenon is primarily associated with high morbidity, severe complications, and mortality rates attributed to multidrug-resistant bacteria, a rising public health challenge stemming from the scarcity or complete absence of effective drugs.<sup>6</sup> Thus, immediate measures are imperative to address the looming crisis of antibiotic resistance on both national and global scales. These measures could encompass enhancing public awareness, developing novel antibiotics, implementing antibiotic stewardship to control their usage, and exploring alternative approaches to antibiotics.<sup>7</sup> Alternative strategies to antibiotics may include the use of bacteriophages, antimicrobial peptides, and natural derivatives such as plant extracts, phytochemicals, and honey.<sup>8-13</sup> Antimicrobial efficacy of various forms of natural honey against an extensive array of bacteria has been documented,<sup>10</sup> corroborating their potential in treatment for infections instigated by multidrug-resistant bacteria. While the precise mechanism of natural honey's antimicrobial action remains unclear, it is generally accepted that its antimicrobial activity can be attributed to multiple underlying mechanisms.<sup>10</sup> Honey is believed to eliminate bacteria through one or more of the

following mechanisms: Disrupting or damaging bacterial cell membranes, inhibiting bacterial virulence factors, and preventing bacterial adhesion to target cells.<sup>14,15</sup> Contrary to other antimicrobial agents, honey has yet to be thoroughly investigated concerning its microbial resistance.<sup>16</sup>

Besides its antimicrobial properties, honey has been explored for its potential anticancer effects. Cancer, characterized by the uncontrolled proliferation of cells exhibiting potential malignancy, ranks among the primary causes of mortality globally.<sup>17</sup> Breast cancer holds the top position among all cancer types affecting women globally. Its etiology is complex, and influenced by various factors. The occurrence of events, such as pregnancy, breastfeeding, first menstruation, and menopause, along with their duration and the accompanying hormonal imbalances, play a crucial role in potentially inducing carcinogenic changes in the breast microenvironment. In Palestine, the incidence of breast cancer has surged in recent times, attributed partially to heightened awareness and screening efforts, as well as socioeconomic factors affecting lifestyle and dietary patterns. It stands as the most prevalent cancer among Palestinian women, constituting 32% of diagnoses in the West Bank and 18% in the Gaza Strip.<sup>18</sup> Existing cancer management strategies, such as surgery, chemotherapy, and radiotherapy, have significant limitations. In addition, cytotoxic drugs, though essential to treatment, present considerable challenges. Their accessibility and affordability are particularly problematic in developing regions. In addition, the utilization of these drugs often leads to a range of undesirable side effects and adverse reactions.<sup>19</sup> Consequently, a significant segment of the population prefers utilizing natural products. Despite its inherent limitations, natural products present several advantages over synthetic or standard drugs, including affordability, accessibility, and reduced side effects.<sup>3,2,19-21</sup> The escalating public interest in natural products can be attributed to the growing body of research underscoring its health benefits.

In addition, various types of honey have been shown to possess anti-inflammatory and antioxidant properties, which are vital for addressing chronic inflammation and mitigating the accumulation of reactive oxygen species (ROS) generated during inflammatory responses. Inflammation, a natural physiological reaction to harmful stimuli, such as allergens, infections, or injuries, can become a chronic condition due to prolonged exposure to certain factors. Chronic inflammation is linked to a wide range of health conditions, including allergies, metabolic disorders, cardiovascular diseases, cancer, and autoimmune disorders, making it a significant concern for global health.<sup>22</sup> The primary treatments for

inflammation encompass anti-inflammatory medications and immunosuppressants, which unfortunately can induce side effects such as gastrointestinal ulcers, cardiovascular toxicity, hormonal imbalances, and disruptions to the body's normal functions.<sup>23,24</sup> Therefore, it is of paramount importance to explore the incorporation of natural anti-inflammatory substances into treatment regimens to enhance pharmacological response and mitigate adverse effects. Recent studies have demonstrated promising results in utilizing natural products such as various types of honey for the treatment of ulcers.<sup>25</sup>

The antioxidant properties of honey play a critical role in lowering the levels of ROS generated during inflammatory processes. Elevated ROS levels are associated with the development of serious health conditions, including cardiovascular, muscular, metabolic, neurodegenerative, and cancer-related diseases. The antioxidant capacity of honey primarily stems from its phenolic compounds, especially flavonoids, which effectively neutralize ROS and interact with metals. Furthermore, flavonoids contribute to regulating enzymes, thereby boosting their antioxidant activity and influencing various biological functions.<sup>26–28</sup>

*Hyphaene thebaica* and Doum honey (DH) are extensively utilized throughout the Mediterranean region due to its health-enhancing properties, such as anti-infection, anti-inflammatory, and anticancer attributes.<sup>29,30</sup> Scientific evidence on the health advantages of DH remains largely unexplored, given that studies on this specific honey variety are not as comprehensive as those on the more frequently researched types such as Manuka honey. *H. thebaica* L., commonly known as the Doum plant, is a desert tree native to regions such as the Mediterranean region, Sub-Saharan Africa, and West India.<sup>29,30</sup> The precise cellular and molecular mechanisms underlying the traditionally asserted antibacterial, antioxidant, anti-inflammatory, and anticancer properties of the Palestinian DH remain largely unexplored. This study is designed to assess these effects and analyze the polyphenolic composition of DH.

## 2. Materials and methods

### 2.1. DH sample collection and preparation

The DH samples, which were collected from the Jordan Valleys in 2021, were obtained from a reputable supplier known as “Honey Spring,” situated in Tulkarem in the Northern West Bank. Following their purchase, these samples were sealed in cans for preservation and stored under ambient conditions in a moisture-free environment. They remained in this unopened state until further experimental procedures.

### 2.2. Assessment of antibacterial activities

The microbial strains used in the current investigation were *Pseudomonas aeruginosa* strain 27853, *Staphylococcus aureus* strain BAA-1026, *Escherichia coli* strain 25922, *Streptococcus* strain 49619, *Klebsiella quasipneumoniae* strain 700603, *Haemophilus influenzae* strain 49247, and *Bacillus subtilis* strain 6633, which were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of DH samples in 96-well, flat-bottomed micro-titration plates, were determined as described previously.<sup>31</sup>

### 2.3. Cell culture

The MDA-MB-231 cell line (European Collection of Authenticated Cell Cultures [ECACC] catalog no. 92020424) is supplied by the ECACC, which is part of the UK Health Security Agency and the THP-1 human monocytic cell line (ATCC: TIB-202) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Biological Industries, Israel) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 µg/mL streptomycin to maintain optimal growth conditions.

### 2.4. Evaluation of cytotoxic and cytostatic effects

In the cytotoxicity experiments, 20,000 MDA cells per 100 µL of media were seeded in 96-well plates and incubated for 24 h. Subsequently, they were exposed to 0 – 4000 µg/mL of DH for 24 h. The cytotoxic impacts were then evaluated using an MTT assay.

In the cytostatic experiments, 5,000 MDA cells per 100 µL of media were seeded in 96-well plates and incubated for 24 h. These cells were then exposed to a range of honey concentrations, from 0 to 4000 µg/mL, over a period of 72 h. The MTT assay was employed to assess the cytostatic effects, following the methodology outlined by Abu-Farich *et al.*<sup>32</sup>

### 2.5. Scratch assay

MDA cells were seeded at a density of 400,000 cells per well in a 12-well plate (Corning Costar Corporation, Corning, NY, USA) with 2.5 mL of culture medium and incubated for 24 h in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. After incubation, monolayer formation in each well was confirmed under a microscope. Scratches were then created across the monolayers using sterile 200 µL pipette tips. The culture medium was removed, and each well was washed 4 times with DMEM devoid of serum and additives. Following this, 4 mL of either 4 mg/mL DH solution or culture medium (for untreated control) was added to the

wells in triplicate. Images were taken immediately after the scratches were made and subsequently every 24 h for a total of 48 h.

## 2.6. Determination of nitric oxide (NO) production

The efficacy of DH samples in inhibiting the formation of NO radicals in lipopolysaccharide (LPS)-stimulated macrophages derived from THP-1 cell line was ascertained utilizing the Griess reagent, in accordance with a previously outlined methodology.<sup>33</sup>

## 2.7. Quantitative high-performance liquid chromatography (HPLC) determination of phenolic compounds in DH

The presence of polyphenols in DH was analyzed by means of quantitative HPLC, as previously described.<sup>31</sup>

## 2.8. Determination of total antioxidant capacity

The total antioxidant activity (TAC) was quantified by utilizing the phosphomolybdenum technique, as delineated by Prieto *et al.*<sup>34</sup> The experimental procedure was executed in triplicate, with the resultant values expressed as ascorbic acid equivalents, measured in milligrams per gram of dry weight.

## 2.9. Determination of total polyphenols content (TPC) and total flavonoids content

TPC and total flavonoid content (TFC) were determined as previously described.<sup>32</sup>

## 2.10. Evaluation of free radical scavenging activity

The free radical scavenging activity was determined using the microdilution 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, with slight modifications, as previously described.<sup>35,36</sup>

## 2.11. Statistical analysis

The error limits mentioned and depicted error bars indicate basic standard deviations of the average. Typically, numerical outcomes are provided with precision only up to the least significant digit. In comparing various samples, results were deemed statistically significant when  $P < 0.05$  (using Student's t-test for unpaired samples).

# 3. Results and discussion

## 3.1. Antibacterial properties

Due to the intensifying global challenge of antimicrobial resistance, there exists an exigency to explore alternative methods and substances to supplant antibiotics. This immediate need is especially critical considering the scarce availability of effective antibiotics to tackle multidrug-resistant bacteria,<sup>37</sup> leading to a notable increase in the

demand for diverse natural products that demonstrate antibacterial properties and function through mechanisms that are different from traditional drugs. Honey is one such product that has attracted considerable interest.<sup>38,39</sup> Honey has long been valued in traditional medicine for its wound-healing abilities and as a complementary therapy for cancer and other health conditions. Across the globe, many cultures have relied on honey for its medicinal and nutritional properties. It has been used to address a wide range of ailments, such as eye disorders, bronchial asthma, tuberculosis, hepatitis, throat infections, hemorrhoids, eczema, wounds, and ulcers, while also serving as a beneficial dietary supplement.<sup>40</sup> Honey is recognized for its diverse beneficial properties, including antioxidant, antimicrobial, anti-inflammatory, antiproliferative, anticancer, and antimetastatic effects. Research has highlighted its potential in managing various conditions, such as wounds, diabetes, cancer, and disorders affecting the cardiovascular, neurological, and gastrointestinal systems.<sup>5-8</sup> Its antibacterial properties, in particular, have been extensively studied, with findings confirming that natural honey exhibits broad-spectrum antibacterial activity, even against drug-resistant pathogens.<sup>41,42</sup> Hence, honey is recognized as a potential bioactive natural substance demonstrating promising efficacy against pathogenic bacteria in the treatment of various bacterial infections. Given that diverse types of honey exhibit a broad spectrum of antibacterial activity, this activity seems to be unrelated to antibiotic susceptibility or resistance, and it appears improbable that pathogenic bacteria would develop resistance to honey.<sup>43,44</sup>

The antibacterial efficacy of honey is attributed to a multitude of factors. Its substantial viscosity, which is primarily a result of its high sugar concentration and minimal water content, establishes a protective shield that aids in thwarting infections. In addition, its mild acidity coupled with the presence of hydrogen peroxide bolsters its antimicrobial prowess.<sup>45,46</sup> Numerous scientific studies have explored the effects of honey on various bacterial species, revealing that its antibacterial effectiveness can vary widely. Different microorganisms show varying levels of sensitivity to specific types and concentrations of honey. Comprehensive reviews have examined honey's antibacterial properties, tracking the growth patterns of different bacteria under exposure to varying concentrations of honey.<sup>47,48</sup>

This study assessed the antibacterial effects of DH employing the MIC and MBC techniques. Tetracycline and kanamycin against antimicrobial resistance in both Gram-positive and Gram-negative bacteria were taken as positive controls (Table 1).



Table 1. MIC and MBC values of tetracycline and kanamycin

Pathogenic microorganisms	MIC (µg/mL)		MBC (µg/mL)		MBC/MIC ratio	
	Tetracycline	Kanamycin	Tetracycline	Kanamycin	Tetracycline	Kanamycin
<i>Pseudomonas aeruginosa</i>	0.07	0.85	0.07	1.7	1	2
<i>Staphylococcus aureus</i>	0.02	0.11	0.04	0.11	2	1
<i>Escherichia coli</i>	0.03	0.47	0.06	0.94	2	2
<i>Bacillus subtilis</i>	0.78	0.39	1.56	0.78	2	2
<i>Haemophilus influenzae</i>	0.024	0.048	0.024	0.048	1	1
<i>Streptococcus pneumoniae</i>	1.56	0.78	3.12	1.56	2	2
<i>Klebsiella pneumoniae</i>	0.19	0.048	0.19	0.096	1	2

Abbreviations: MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration.

The assessment of MIC and MBC is universally acknowledged as an efficient and relatively cost-effective method for evaluating the efficacy of various antimicrobial substances. DH demonstrated a favorable impact in eradicating all examined bacterial strains. The MIC and MBC values of DH against the tested bacterial strains are stated in Table 2.

The MIC values for the DH fluctuated between 0.19% and 0.78% w/w for the three Gram-positive strains and between 0.024% and 0.39% w/w for the four Gram-negative strains. Among all the bacterial strains examined, *E. coli* was identified as the most susceptible with an MIC of 0.024% w/w, whereas *S. pneumoniae* was the most resistant with an MIC of 0.78% w/w (Table 2).

The ratio of MBC to MIC can offer valuable insights into the nature of an antimicrobial agent, specifically whether it is bacteriostatic (halts bacterial growth) or bactericidal (kills bacteria). If the MBC is not significantly greater than the MIC (typically MBC/MIC  $\leq$  4), the antimicrobial agent is deemed bactericidal, as it kills bacteria at concentrations near the inhibitory concentration. Conversely, if the MBC is significantly higher than the MIC, the agent is classified as bacteriostatic, as it primarily inhibits bacterial growth rather than killing the bacteria. The DH's ratio of MBC to MIC against all examined bacteria consistently exhibited an approximate increase of up to two-fold over their MIC values. This implies that DH honey exerts a bactericidal effect on all bacteria tested (Table 2).

The MIC values for *E. coli* recorded in this study were lower than the previously documented 0.52 – 1.0% range.<sup>31,47–49</sup> The antimicrobial effects of honey primarily stem from hydrogen peroxide, with additional contributions from non-peroxide components such as phenolic acids and flavonoids, which enhance its antibacterial and antioxidant properties.<sup>50,51</sup> Research indicates that these antibacterial effects can vary depending on the phytogeographical origin of the honey, influencing the production of various

Table 2. MIC and MBC of DH against various bacterial strains

Pathogenic microorganisms	MIC (% w/w)	MBC (% w/w)	MBC/MIC ratio
<i>Pseudomonas aeruginosa</i>	0.39	0.78	2
<i>Klebsiella pneumoniae</i>	0.096	0.096	1
<i>Escherichia coli</i>	0.024	0.048	2
<i>Haemophilus influenzae</i>	0.048	0.048	1
<i>Streptococcus pneumoniae</i>	0.78	1.56	2
<i>Bacillus subtilis</i>	0.38	0.76	2
<i>Staphylococcus aureus</i>	0.19	0.19	1

Abbreviations: MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration, DH: Doum honey.

compounds.<sup>52,53</sup> Recent studies have also identified other antimicrobial components, such as the bee-derived peptide defensin-1, 5-hydroxymethylfurfural, and methylglyoxal, as well as phenolic compounds, including flavonoids.<sup>54,55</sup>

Various varieties of honey from the Eastern Mediterranean region have demonstrated significant inhibitory effects against both Gram-positive and Gram-negative bacteria, including methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* strains.<sup>38</sup> However, compared to other types of honey, studies examining the bioactive properties of DH are limited. This study aimed to address this gap by evaluating the phytochemical composition of DH, as well as its antibacterial, antioxidant, anti-inflammatory, and anticancer properties. The findings revealed broad-spectrum antibacterial activity against all tested Gram-positive and Gram-negative bacteria. Honey has also been shown to be effective against drug-resistant pathogens, including MRSA, drug-resistant hemolytic Streptococci, and vancomycin-resistant Enterococci.<sup>39,55</sup> Furthermore, honey from various global regions has been found to exhibit similar or even greater potency compared to

Manuka honey. Despite this, there are only a few studies exploring the bioactive potential of traditional Palestinian honey.<sup>31,32,47,52</sup>

### 3.2. Effects of DH on NO levels

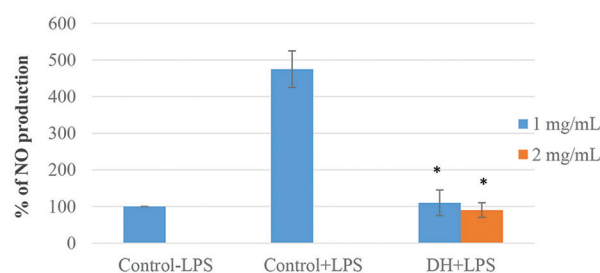
NO, a transient bioactive free radical, functions as a signaling molecule in numerous physiological and pathological processes. The overproduction of NO serves as an indicator of the severity and stage of both acute and chronic inflammation.<sup>56</sup> Following the incubation of TPH-1-derived macrophages with LPS, there was an approximately five-fold increase in NO production compared to the control (Figure 1), observed at non-toxic concentrations of DH (Figure 2). Notably, the levels of NO were significantly reduced by DH, with a reversion to the baseline levels observed in untreated cells.

Numerous studies have previously indicated that honey has the capacity to downregulate inducible NO synthase expression and NO production.<sup>57-60</sup> The varying phenolic composition of honey may elucidate the differences in their nitrogen oxides suppression capabilities, as literature has suggested a link between these compounds and NO inhibition.<sup>59,60</sup> A study on Moroccan *Euphorbia* honey revealed a strong negative correlation between the phenolic compounds in honey and the NO scavenging activity of the honey samples.<sup>59</sup> This implies that a higher phenolic content in the honey leads to more effective NO inhibition activity. Another study affirmed that honey possesses anti-inflammatory properties, which could be partially attributed to the inhibition of NO release, indicating that the chemical constituents of honey, including its phenolic compounds, may contribute to this inhibitory effect.<sup>60</sup>

However, it is important to note that the specific chemical constituents of honey that contribute to this effect and the precise mechanisms involved may differ depending on the type of honey, necessitating further research. While these studies suggest a correlation, they do not establish a direct cause-and-effect relationship. The correlation signifies a relationship between the two factors, but it does not definitively prove causality.

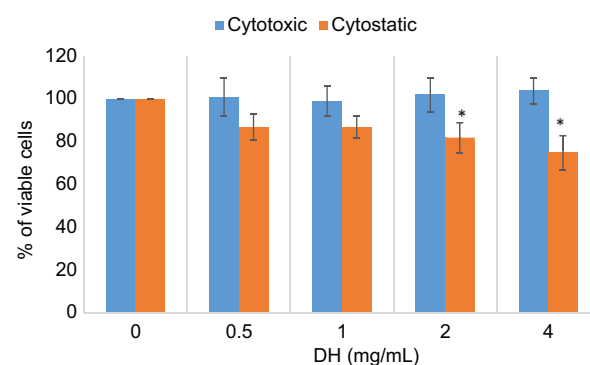
### 3.3. Cytotoxic and cytostatic effects of DH in cells from the MDA cell line

Cancer cells are characterized by uncontrolled growth, a central focus for both traditional chemotherapy and emerging therapeutic strategies. DNA damage can lead to growth arrest at the G0/G1 or G2/M phases or may even initiate apoptosis. Many chemotherapy agents are designed to disrupt the cell cycle, particularly during the S and M phases. Studies have extensively documented honey's ability to induce cell cycle arrest at the G0/G1



**Figure 1.** NO production by LPS-activated THP-1-derived macrophages after 72 h of incubation with 1 mg/mL and 2 mg/mL of DH. Data are presented as mean  $\pm$  SD from three independent experiments performed in triplicate. Comparisons were made between Control + LPS and DH + LPS groups relative to Control - LPS, which was normalized to 100%. \* $P < 0.05$  compared to Control + LPS.

Abbreviations: LPS: Lipopolysaccharide; NO: Nitric oxide; DH: Doum honey.



**Figure 2.** Cytotoxic and cytostatic effects were assessed on MDA cells using MTT Assay following treatment with honey samples ranging from 0 to 4 mg/mL for 24 h (for cytotoxic effects) and 72 h (for cytostatic effects). The absorbance of the MTT formazan was quantified at 570 nm utilizing an ELISA reader. Cell viability was computed as the percentage ratio of absorbance of honey-treated cells relative to untreated cells. Data represent the mean  $\pm$  SD from three independent experiments conducted in triplicate. \* $P < 0.05$  compared to the untreated control.

Abbreviations: DH: Doum honey; ELISA: Enzyme-linked immunosorbent assay; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide.

phase in various cancer cell lines, such as bladder (T24, 253 J, RT4, and MBT-2), colon (HCT-15 and HT-29), and human melanoma (A375) cells.<sup>61-63</sup> The cytostatic effects of different types of honey have been highlighted in recent research,<sup>64,65</sup> which has focused on identifying components in honey that can inhibit cancer cell proliferation without eliciting cytotoxicity. For instance, an analysis of bioactive honey samples has identified phenolic compounds such as rosmarinic acid, tannic acid, caffeic acid, coumaric acid, gallic acid, ferulic acid, syringic acid, catechin, and pyrogallol.<sup>62</sup>

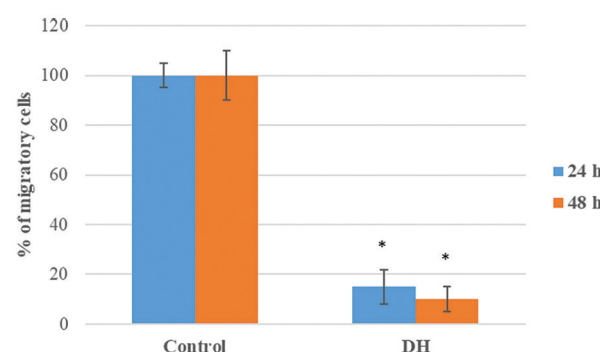
Figure 2 delineates the cytostatic and cytotoxic effects of DH samples on MDA cells. MDA cells are widely utilized models for breast cancer research, offering a relevant

platform to evaluate potential therapeutic interventions. They exhibit characteristics similar to those of aggressive breast cancer phenotypes. Notably, no cytotoxic effects were observed across all tested concentrations. DH exhibited a modest yet significant cytostatic activity on MDA cells. The most pronounced cytostatic effects were noted at concentrations of 4 mg/mL and 2 mg/mL, leading to a reduction in cell viability by 25% and 20%, respectively, compared to untreated control cells. These findings are consistent with prior studies, underscoring that the influence of honey can vary based on its type.<sup>65-68</sup> Furthermore, our results corroborate numerous studies that have highlighted the cytostatic properties of honey on diverse cancer cells, suggesting that these effects are modulated by the phenolic content.<sup>65-68</sup> Recent findings by Imtara *et al.*<sup>66</sup> revealed the cytotoxic effects of various honeys from Morocco and Palestine on human colon adenocarcinoma (HCT-116) and breast cancer (MCF-7) cell lines. Their study highlighted a strong positive correlation between the concentration of antioxidant components such as phenols, flavonoids, and flavonols and the cytostatic effects observed in MCF-7 cells. In addition, an inverse relationship was identified between the levels of syringic and tannic acids and the cytostatic activity in HCT-116 cells. Similarly, research has demonstrated the cytotoxic effects of Sardinian STH honey<sup>51</sup> and Manuka honey from New Zealand<sup>69</sup> on HCT-116 and metastatic colon epithelial adenocarcinoma cells, with reduced cytotoxicity observed in non-cancerous cells. Furthermore, Chinese jujube honey was found to exert cytotoxic effects on HepG2 liver cancer cells.<sup>69,70</sup>

### 3.4. Effect of honey samples on cell migration of MDA cells

Cancer is characterized not only by the unregulated and accelerated proliferation of cells but also by the invasive and metastatic nature of these proliferating cells. It is well recognized that the tendency of breast cancer cells to metastasize to other tissues significantly increases the mortality risk.<sup>71</sup>

Here, we performed a scratch assay to assess the impact of DH, administered at a dose of 4 mg/mL, on migration of MDA cells. As illustrated in Figure 3, treatment with DH significantly reduced the migration rate of MDA cells compared to the untreated controls ( $P < 0.05$ ). This reduction in tumor cell migration may partly result from the DH's cytostatic properties, as evidenced by its inhibition of cell migration and its cytostatic effects on MDA cells (Figure 2). Notably, the more pronounced suppression of cell migration relative to its cytostatic effects suggests the involvement of additional cellular and molecular mechanisms underlying DH's beneficial effects.



**Figure 3.** Effect of honey samples on MDA cell migration. The results reflect the extent of wound closure after 24 and 48 h of treatment with honey samples. Each data point was determined based on the initial wound size at time 0 h and was normalized to the untreated control, which was set as 100%. Data represent the mean  $\pm$  SD from three independent experiments conducted in triplicate. \* $P < 0.05$  compared to the untreated control.

Abbreviation: DH: Doum honey.

These findings are consistent with previous studies that have reported the inhibitory effects of various honey types and isolated phytochemicals, such as resveratrol, kaempferol, and epigallocatechin-3-gallate, on the migration of colorectal cancer and oral squamous cell carcinoma cells. To the best of our knowledge, this is the first study to demonstrate the antimetastatic properties of Palestinian honey on MDA breast cancer cell lines.<sup>72-74</sup> Metastasis, the most lethal aspect of cancer, involves intricate processes<sup>75</sup> and a wide array of molecules,<sup>76</sup> including matrix metalloproteinases (MMPs), integrins, cadherins, plasminogen activators, PI3Ks, small GTPases (*e.g.*, Rho, Rac, Cdc42), phospholipase C, and focal adhesion kinases. Although the effects of honey on cancer metastasis remain underexplored, an *in vivo* study using wildflower honey from Croatia revealed a significant reduction in metastasis when administered before tumor cell inoculation in CBA mice and Y59 rats.<sup>77</sup>

Beyond the cytostatic effects of honey samples, the suppression of MMPs is probably a mechanism behind their beneficial properties. MMPs are proteases critical for extracellular matrix degradation and are highly expressed in metastatic cells.<sup>78</sup> Gallic acid, a phenolic compound, has been shown to reduce the gelatinolytic activity of MMP-2 and MMP-9, potentially via modulation of the nuclear factor kappa B (NF- $\kappa$ B) pathway.<sup>63</sup> Furthermore, several studies suggest that honey can inhibit both the expression and nuclear translocation of NF- $\kappa$ B *in vivo* and *in vitro* settings.<sup>79-81</sup> Honey has also been demonstrated to decrease the enzymatic activity of MMP-2 and MMP-9.<sup>82</sup> For example, Fir honey was found to inhibit human keratinocyte migration by downregulating MMP-9 expression.<sup>82,83</sup> Similarly, quercetin, a flavonoid present

in honey, has been reported to suppress the expression of MMP-2 and MMP-9 in PC3 prostate cancer cells.<sup>84,85</sup>

### 3.5. Total polyphenols, flavonoids, and antioxidant capacity

Honey serves as a robust source of natural antioxidants, which play a crucial role in protecting against the effects of oxidizing agents on both food preservation and human health. It contributes to the reduction of health risks such as heart disease and cancer and prevents the deterioration of the immune system, cataracts, and various inflammatory processes.<sup>8,20,25</sup> As presented in Table 3, the TPC values were found to be  $101.04 \pm 22.46$  mg Eq GA/100 g of honey. This value surpasses the TPCs reported in ten Palestinian honey samples from diverse geographical regions,<sup>47</sup> which ranged between  $26.96 \pm 0.71$  mg/100 g and  $70.73 \pm 0.71$  mg/100 g. These findings are akin to those discovered in thyme honey from Morocco.<sup>86</sup> The TFC values were recorded as  $4.13 \pm 0.31$  mg Eq Q/100 g of honey (Table 3), which aligns with the values reported for other Palestinian honey samples.<sup>47</sup>

The ability of DH to neutralize DPPH free radicals, represented as  $IC_{50}$ , was evaluated (Table 3). The observed  $IC_{50}$  ( $4.40 \pm 0.71$  mg/mL) was lower than the values previously reported for other Palestinian samples, indicating a correlation between the  $IC_{50}$  of DPPH free radicals and the values of polyphenolic compounds, as well as the TFC.<sup>47,87,88</sup> As mentioned above, the samples from DH exhibited a higher TPC compared to other Palestinian honey samples, which could account for the lower  $IC_{50}$  values observed in DH compared to other honey samples.

The determined TAC was found to be  $3.05 \pm 0.12$  g Eq AA/100 g of DH (Table 3). Similar values were found in other Palestinian honey samples that have similar TPC.<sup>47</sup>

### 3.6. Identification of phenolic compounds of DH by HPLC

A growing body of research, both *in vitro* and *in vivo*, has highlighted the anti-carcinogenic properties of plant-derived polyphenols on tumor cells. These properties

include the inhibition of angiogenesis and metastasis, anti-proliferative effects, anti-inflammatory action, and the promotion of apoptosis. Recently, a wide range of novel polyphenolic compounds with potential anticancer activity have been identified globally. Some of these compounds show promise as anticancer agents, capable of treating or preventing cancer growth by targeting various stages of cancer development, such as initiation, promotion, and progression.<sup>89</sup>

We employed HPLC analysis to isolate phenolic compounds with potential anticancer properties from the honey samples under investigation. It is important to note that the polyphenolic composition of honey can vary depending on the source of pollination, as well as geographic and climatic factors.<sup>47,90,91</sup> Studies on the Palestinian honey samples collected from various regions and floral sources revealed phenolic content ranging from 26.96 to 70.73 mg equivalent per gram of honey.<sup>47,66</sup> Table 4 outlines the concentrations (in mg/g) of several phenolic compounds identified in DH, including nine key compounds: Gallic acid, caffeic acid, carvacrol, p-coumaric acid, ellagic acid, kaempferol, pinobanksin, pinocembrin, and galangin. Many of these compounds are known for their potential health benefits, including anticancer effects. For example, caffeic acid, a phenolic compound found in various natural sources such as honey,<sup>47</sup> exhibits a range of biological activities such as antioxidant, anti-inflammatory, anticancer, and antidiabetic properties.<sup>92,93</sup> The therapeutic effects of caffeic acid are thought to be mediated through the repression and inhibition of transcription and growth factors.<sup>94</sup>

P-coumaric acid, a hydroxycinnamic acid found in cereal grains, fruits, and vegetables, has demonstrated a range of health benefits, including antioxidant, antidiabetic,

**Table 4. Concentrations of constituent polyphenolic compound and total polyphenols content in DH**

Phenolic compounds	Concentration in mg/g DH
Gallic acid	0.37
Caffeic acid	0.68
Carvacrol	0.42
P-coumaric acid	5.03
Ellagic acid	5.22
Kaempferol	11.88
Pinobanksin	5.00
Pinocembrin	2.02
Galangin	4.09
TPC	34.71

Abbreviation: TPC: Total polyphenols content, DH: Doum honey.

**Table 3. Free radical scavenging activity, TFC, TPC, and TAC of DH samples**

Parameter	Value
Free radical scavenging activity (DPPH $IC_{50}$ ; mg/mL)	$4.40 \pm 0.71$
TFC (mg Eq Q/100 g of DH)	$4.13 \pm 0.31$
TPC (mg Eq GA/100 g of DH)	$101.04 \pm 22.46$
TAC (g Eq AA/100 g of DH)	$3.05 \pm 0.12$

Abbreviations: DPPH: 2,2-diphenyl-1-picrylhydrazyl; TAC: Total antioxidant activity; TFC: Total flavonoids content; TPC: Total polyphenols content, DH: Doum honey.



anti-inflammatory, antiplatelet, antiulcer, and anticancer effects. It has been shown to inhibit growth and induce apoptosis in certain colon cancer cells and to prevent cancer in a short-term animal model.<sup>95</sup> In addition, this study examined the antibacterial activity of p-coumaric acid, suggesting its potential use in treating microbiome-related inflammation or cancer.<sup>95</sup> P-coumaric acid was found in relatively high quantities in the honey samples (Table 4).

Carvacrol, a compound present in plants such as oregano and thyme, has demonstrated medicinal potential, particularly against various types of cancer cells. Copper complexes, along with other organometallic compounds, are also recognized as potent anticancer agents, effective against a variety of cancer types, including lung and leukemia cells. Copper, being an endogenous metal, is non-toxic to normal cells, contributing to its therapeutic value.<sup>96</sup>

Pinocembrin has been shown to inhibit the viability, migration, and invasiveness of colorectal cancer cells while reducing the expression of MMP-2 and N-cadherin, and promoting the expression of E-cadherin and beta-lactamase-like protein.<sup>97,98</sup>

In a study by Ho *et al.*,<sup>79</sup> the levels of gallic acid, a compound with known antioxidant, anti-inflammatory, antimutagenic, anticancer, and cardioprotective properties, were found to vary in two types of Malaysian honey. Gallic acid, characterized by its trihydroxylated phenolic structure, has been identified in several honey samples.<sup>47,66</sup>

## 4. Conclusion

This study aimed to evaluate the biological effects of DH, focusing on its antibacterial activity as well as its antioxidant, anti-inflammatory, cytotoxic, cytostatic, and antimigration effects on MDA human breast cancer cells. A quantitative HPLC analysis identified nine phenolic compounds across three honey samples. The results obtained affirm the antibacterial attributes of DH and attribute its potential anticancer advantages to its anti-inflammatory, antioxidant, cytostatic, and antimigration effects. These effects are strongly linked to the presence of specific polyphenols, including carvacrol, pinocembrin, chrysin, protocathechuic acid, rutin, and salicylic acid.

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## Conflict of interest

Bashar Saad is the Editorial Board Member of this journal and the Guest Editor of this special issue but was not in any way involved in the editorial and peer-review process conducted for this paper, directly or indirectly. Separately, other authors declared that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

## Author contributions

*Conceptualization:* Badiaa Lyousi, Bashar Saad

*Formal analysis:* Basheer Abu-Farich, Bashar Saad

*Investigation:* Hadeel Hamarshi, Doha Weldali, Abderrazak

Aboulghazi, Mohammed El Ouassete

*Methodology:* Basheer Abu-Farich, Mahmud Masalha

*Writing—original draft:* Bashar Saad

*Writing—review & editing:* All Authors

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

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## Availability of data

Data are available upon reasonable request to the corresponding author.

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