Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

CHARACTERIZATION OF CHEMICAL PROFILING, CYTOTOXICITY, AND ANTIMICROBIAL ANALYSES OF CAROB HONEY FROM NORTHERN CYPRUS

(Ciri-Ciri Profil Kimia, Sitotoksik, dan Analisis, Antimikrob Madu Karob Dari Cyprus Utara)

Bashir Ado Ahmad[1,3,*], and Erkay Özgör[2,3]

[1]*Department of Bioengineering, Faculty of Engineering, Cyprus International University, Nicosia, North Cyprus, Mersin 10, Turkey*
[2]*Department of Molecular Biology and Genetics, Faculty of Arts and Sciences, Cyprus International University, Nicosia, North Cyprus, Mersin 10, Turkey*
[3]*Cyprus Bee and Bee Products Research Centre, Cyprus International University, Nicosia, North Cyprus, Mersin 10, Turkey*

[*]*Corresponding author*: *basharado88@gmail.com*

Received: 27 March 2024; Accepted: 11 June 2024; Published: 27 August 2024

Abstract

Currently, the preferred options (chemotherapy, radiotherapy, etc.) for the treatment of breast cancer indiscriminately attack healthy cells and cause hair loss. There is also a global concern about the rise of antibiotic resistance by infectious microorganisms. Therefore, searching for an alternative with fewer side effects and improved activity becomes paramount. This study explored the chemical components, cytotoxicity, and antimicrobial potentials of carob honey from Northern Cyprus. Gas chromatography-mass spectrometry was used for the identification of the chemical constituents. Well diffusion assay was used for antimicrobial assessment, and MTT assay was employed to determine the MDA-MB-231 cell viability. A total of 69 compounds, which include hydroxymethyl furfural, quercetin, and furan carboxaldehyde were detected in the honey. At 8000 µg/mL, the zones of inhibition are 13±1.7, 12±1.0, 11±1.7, 10±2.0, 8±1.0, 9±2.6, and 7±1 mm for *S. typhi, E. coli, K. pneumoniae, S. aureus, S. pneumoniae, A. niger* and *A. flavus* respectively. Only *E. coli* and *K. pneumoniae* responded at 2000 µg/mL. Carob honey also suppressed the proliferation of MDA-MB-231 in a concentration and time-dependent manner. At 300 µg/mL, 20 %, 50 % and 55 % of the MDA-MB-231 cells were inhibited after 24, 48 and 72 hours respectively. However, the overall cytotoxic effect of carob honey on the MDA-MB-231 cell line is substantially low.

Keywords: bioactivities, Carob honey, MTT assay, volatile constituents

Abstrak

Pada masa ini, pilihan rawatan yang disukai (kemoterapi, radioterapi, dan lain-lain) untuk rawatan kanser payudara menyerang sel-sel yang sihat tanpa diskriminasi dan menyebabkan keguguran rambut. Terdapat juga kebimbangan global mengenai peningkatan rintangan antibiotik oleh mikroorganisma berjangkit. Oleh itu, mencari alternatif dengan kesan sampingan yang lebih sedikit dan peningkatan aktiviti menjadi penting. Kajian ini meneroka komponen kimia, ketoksikan sitotoksik, dan potensi antimikrobial madu carob dari Cyprus Utara. Kromatografi gas-spektrometri jisim digunakan untuk mengenal pasti konstituen kimia. Ujian difusi telaga digunakan untuk penilaian antimikrobial, dan ujian MTT digunakan untuk menentukan daya hidup sel MDA-MB-231. Sebanyak 69 sebatian, termasuk hidroksimetil furfural, kuersetin, dan furan karboksaldehid telah dikesan dalam madu tersebut. Pada 8000 µg/mL, zon perencatan adalah 13±1.7, 12±1.0, 11±1.7, 10±2.0, 8±1.0, 9±2.6, dan 7±1 mm masing-masing untuk *S. typhi, E. coli, K. pneumoniae, S. aureus, S. pneumoniae, A. niger* dan *A. flavus*. Hanya *E. coli* dan *K. pneumoniae* yang memberi tindak balas pada 2000 µg/mL. Madu carob juga menekan percambahan MDA-MB-231 dengan cara bergantung kepada kepekatan dan masa. Pada 300 µg/mL, 20%, 50% dan 55% sel MDA-MB-231 dihalang selepas 24, 48 dan 72 jam masing-masing. Walau bagaimanapun kesan sitotoksik keseluruhan madu carob terhadap garis sel MDA-MB-231 adalah rendah.

Kata kunci: aktiviti bio, madu carob, ujian MTT, konstituen meruap

Introduction

Honey is a rich liquid substance used for centuries in human nutrition and medicine. It is enriched with water, sugar, minerals, vitamins, enzymes, organic acids, flavonoids, and phenols. These compounds are responsible for the remarkable biological properties of honey [1]. Some of these properties include; antioxidant [2], antibacterial [3], anti-diabetic [4], and anti-inflammatory [5] activities. The antibacterial potential of honey has been largely attributed to its high osmolarity, low pH, production of hydrogen peroxide, and bee defensing-1 formation. Many bacterial and fungal strains are vulnerable to honey [6-9], depending on its type and botanical origin [10]. Additionally, some honey such as Manuka honey and its flavonoids exhibit anti-proliferative activity against MDA-MB-231[11], similar to acacia honey from Malaysia against MCF-7 [12]. In addition, a dose-dependent cytotoxic activity of acacia honey against the MCF-7 cell line was reported by [13].

Over 2.2 million women have been diagnosed with breast cancer in 2020 alone, and it is expected that 2.6 million fresh cases may set in by 2030, which is quite alarming [14]. Triple-negative breast cancer (TNBC) accounts for 10-20% of all the breast cancers diagnosed in women, it lacks the expression of estrogen receptor (ER-), progesterone receptor (PR-), and human epidermal growth factor receptor (HER2) [14-16]. The absence of these receptors makes TNBC non-responsive to hormonal treatment and difficult to diagnose [17, 18]. MDA-MB-231 is a TNBC cell line used by researchers as a good model for studying potential breast cancer therapeutic agents [19]. The current treatment options for breast cancer such as chemotherapy and radiotherapy, have serious side effects on the patients. For instance, chemotherapeutic drugs may indiscriminately target healthy cells [20]. Therefore, the search for an alternative treatment for breast cancer is getting popular among the scientific community.

Carob honey (CH) is made by honeybees that take nectar from the flowers of *Ceratonia siliqua L*, also called carob tree. It possesses numerous bioactive substances [21], however, the data regarding the effects of CH, originating from Northern Cyprus, on breast cancer and pathogenic microorganisms is limited. We therefore aim to evaluate the effect of Cypriot CH on the MDA-MB-231 cell line, and its antimicrobial activity on *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae, Salmonella typhi*, *Aspergillus niger,* and *Aspergillus flavus*.

Materials and Methods

Collection of CH

CH sample was obtained aseptically in December 2021, in the Kyrenia district of Northern Cyprus, an area rich in carob trees. The honey was brought to the lab in a dry and clean glass container and was authenticated by Assoc. Prof. Dr. Erkay Özgör of Cyprus bee and bee products research center, Cyprus International University.

Analysis of chemical composition of Cypriot CH using GC-MS

About 1g of CH extract was mixed with 10 mL ethanol. The mixture was filtered with filter paper (0.45 µm), and then with a syringe filter. The appropriate volume of the sample was transferred to the GC-MS vial for analysis. The method of [22] was adopted with minor modifications. The analysis was carried out using a gas chromatography coupled mass spectrometer. HP-5MS fused-silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) was employed for the analysis. The carrier gas was helium at a constant pressure of 14.5 psi. One microliter (1 µL) of the sample was injected using a splitless mode at an injector temperature of 250 ^{o}C. The oven temperature was ramped from 35 to 280 ^{o}C (1-minute hold) at a rate of 25 ^{o}C/min. The GC-MS interface temperature was set to 280 ^{o}C. Mass spectrometry mode was used during analytical scanning from 20-650 atomic mass units. The ion source temperature was set to 250 ºC. The blank was first injected, followed by the sample injection. The chromatograms obtained from the total ion count (TIC) were integrated without any correction for co-eluting peaks and the results were expressed as total abundance. All the peaks were identified based on mass spectral matching from the Wiley library.

Antimicrobial assay of CH: Sample preparation

Stock solution (10 mg/mL) of CH ethanol extract was prepared in dimethyl sulfoxide (DMSO (20% v/v)), from which the working concentrations were prepared (1000-8000 µg/mL). The prepared samples were stored at 4°C until use.

Microbial strains

The bacterial strains used are *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae,* and *Salmonella typhi,* and the fungi are *Aspergillus niger,* and *Aspergillus flavus.* The bacteria were maintained in Mueller-Hinton agar, while Sabouraud Dextrose agar was used to grow the fungi. The test isolates were inoculated in a sterile physiological saline and were compared to 0.5 Mcfarland standard to get 1.5×10^8 CFU/mL [23].

Well diffusion assay

Well labelled petri dishes containing appropriate agar were carefully and evenly covered with 1.5×10^8 CFU/mL microbial strains, using a sterile swab. Wells (6 mm) were made using cork-borer. The sample (50 µL) was then added to the appropriate wells. The plates were then incubated at 37 ºC for 24 hours, and the zone of inhibition was measured in mm [23]. Ciprofloxacin (30 µg) was used as a standard antibiotic for bacteria, and ketoconazole (200 µg) was used for fungi.

Cell culture

MDA-MB-231 was taken from the cell culture laboratory, Biotechnology Research Center, Cyprus International University. It was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco by Life Technologies™, USA) supplemented with 4mmol/L L-glutamine and 5% fetal bovine serum (FBS) and incubated at 37 °C, 5% CO_2 and 100% relative humidity. At 80% confluence, MTT assay was performed to assess the cell's viability [24] at various concentrations of CH.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cell viability assay

MTT assay as described by [25] was performed, with minor changes. The cells (1×10^4) were seeded in a 96-well plate and incubated overnight. Various concentrations (10 – 300 µg/mL) of CH were then added to the cells for 24–72 hr period. After the due period, MTT (5 mg/mL) was added to all the wells and incubated at 37 ºC for 3 hr. Formazan crystal produced by the live cells was dissolved by DMSO. The absorbance of the sample and control (only DMEM) were read using a multiwell plate reader (ELx800, Biotek Instruments) at 490 nm. The experiment was done in triplicate, and the cell viability percentage was determined from the formula;

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \tag{1}$$

Statistical analysis

Kruskal-Wallis and Mann-Whitney U tests were carried out to check for statistical significance between the test concentrations and the control drug. The significance level was set at $p<0.05$. The results were presented as mean±SD. The experiments were done in triplicate.

Results and Discussion

Chemical composition of Cypriot CH identified by GC-MS analysis

GC-MS is a technique that separates organic compounds. It uses gas chromatography to separate the components of a mixed substance, and a mass spectrometry, to decode the molecular structure of the relevant compound [26]. CH was prepared in ethanol and subjected to GC-MS analysis to determine its chemical components. After careful selection, a total of 69 compounds with at least 80% similarity match, upon data correlation on the WILEY 7 library, were detected. The GC-MS chromatogram showing the retention time and intensity is shown in Figure 1, and the molecular weights with the relative abundance (%) of each compound are shown in Table 1. Some of the detected compounds include; 2-Furancarboxaldehyde, 2-Furanmethanol, Dihydroxyacetone, 2-Furancarboxaldehyde, Benzene acetaldehyde, decanal, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 5-(Hydroxymethyl)furfural, benzene, (3-methylbutyl)-, acetaldehyde, hydrazine carboxamide, 2-(2-

methylcyclohexylidene)-, 1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester, 9-Octadecenamide, (Z)-, Quercetin 7,3',4'-trimethyl ether and Squalene.

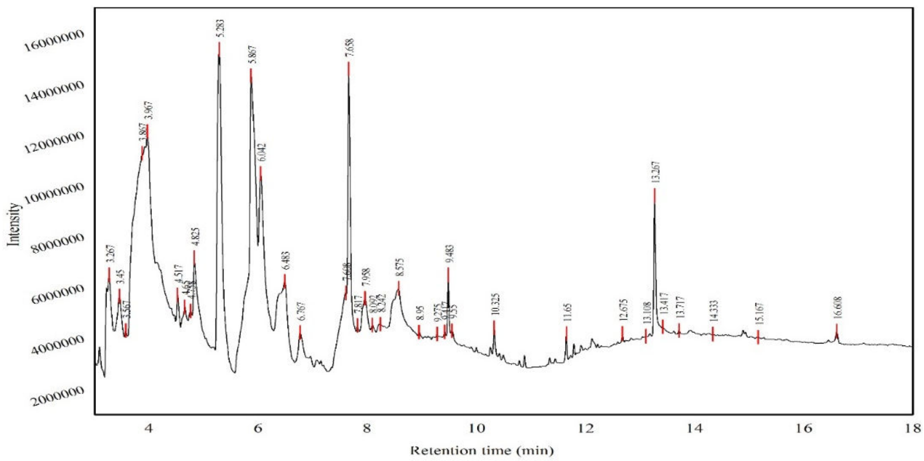

Figure 1. GC–MS chromatogram of the phytoconstituents present in the ethanol extract of carob honey

Butanoic acid, ethyl ester and furfural, detected in the CH are also present in carob fruit of Cyprus cultivars [27]. Moreover, acetaldehyde was also detected in the original *Apis mellifera* and *dorsata* honeys [28]. Linalool oxide detected in our CH was similarly found in carob tree honey samples from Portugal [29] and Morocco [30], and in the flower of the carob tree [31]. Furthermore, benzene acetaldehyde and decanal were also present in bell heather and lavender honey samples respectively [29].

Interestingly, some of the identified chemical components are pharmacologically active. For instance, 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-, 2-Furancarboxaldehyde, and 5-hydroxmethyl, also reported in marjoram honey, Trifolium honey, and citrus honey [32], have antioxidant, anti-inflammatory, and antifungal activities [33]. In addition, quercetin detected in the honey has remarkable antioxidant properties [34] besides its anticancer activity [35].

Antimicrobial Activity of CH

The zones of inhibition, measured in millimeters (mm), of the tested bacterial and fungal strains when exposed to antibiotics (ciprofloxacin, 30µg/disc and Ketoconazole, 200 µg/disc), and varying concentrations of CH (1000-8000 µg/mL) are shown in Tables 2 and 3, and in Figure 2. CH at 8000 µg/mL shows zones of inhibition in all the treated bacteria, with the highest activity against *S. typhi* (13±1.7 mm), and the lowest against *S. pneumoniae* (8±1.0 mm). CH at 4000 µg/mL show reduced inhibition against all the bacteria but *S. aureus* resisted the treatment. At 2000 µg/mL, only *K. pneumoniae* and *E. coli* responded with zones of 10±1.0 mm and 8±1.7 mm respectively. No effect observed at 1000 µg/mL. Generally, gram-negative bacteria show more sensitivity to CH than gram-positive bacteria. Gram-negative *E. coli and K. pneumoniae* respond to the treatment at 2000-8000 µg/mL, *S typhi* responds at 4000-8000 µg/mL and has the widest zone of inhibition. Although gram-positive *S. aureus* has a wider zone of inhibition than *S. pneumoniae*, it responds only at 8000 µg/mL, while the latter responds at both 8000 and 4000 µg/mL concentrations of CH.

Stronger antifungal activity was noted in *A. niger* than *A. flavus.* However, CH could not inhibit both the fungi at concentrations less than 8000 µg/mL. The antibacterial effect increases with an increase in CH concentration. This trend agrees with that of [36], who reported a dose-dependent antibacterial activity of Saudi honey from Asir region and [37], who also found that buckwheat and Manuka honeys exerted dose-dependent antibacterial activity.

Table 2. Antibacterial activity of CH in comparison with ciprofloxacin antibiotic

Carob Honey (CH) (µg/mL)/ Antibiotic (30 µg/disc)	**Zone of Inhibition (mm)**				
	Bacterial Strains				
	S. aureus	***E. coli***	***K. pneumoniae***	***S. pneumoniae***	***S. typhi***
Ciprofloxacin	16±2.0	21±4.0	25±2.6	15±2.0	16±3.0
CH (8000)	10±2.0*	12±1.0*	11±1.7*	8±1.0	13±1.7
CH (4000)	-	10±2.6*	11±2.0*	8±2.6	11±3.5
CH (2000)	-	8±1.7*	10±1.0*	-	
CH (1000)	-	-	-	-	-

Results are presented as mean±SD of zones of inhibition (mm). *p<0.05 vs control (antibiotic). (-) indicate the absence of antibacterial activity. p>0.05 for *S. pneumoniae* and *S. typhi* at 8000 and 4000 µg/mL vs positive control

Salmonella species cause salmonellosis, a lethal infection in both humans and animals [38]. *E. coli* is not only responsible for causing gastrointestinal infections but also infection of the urogenital tract [39]. *S. aureus* is implicated in the promotion of wound infection thus causing delayed wound healing [40]. It is the most virulent among the *Staphylococci* family, which causes lethal infections like meningitis, pneumonia and endocarditis [41].

Gram-negative bacteria develop resistance to many antibiotics due to their unique outer membrane structure. As a result, it is capable of causing serious infections in humans, especially immune-deficient individuals [42-44]. Although some studies have shown gram-positive bacteria as more susceptible to inhibition than gram-negative bacteria [45-49], our CH show better antibacterial activity against gram-negative strains, *S. typhi* at 8000 µg/mL (13±1.7 mm) and 4000 µg/mL (11±3.5 mm), *E. coli* (8000 µg/mL (12±1.0 mm), 4000 µg/mL (10±2.6 mm), and 2000 µg/mL (8±1.7 mm), and *K. pneumoniae* (8000 µg/mL (11±1.7 mm), 4000 µg/mL (11±2.0 mm), and 2000 µg/mL (10±1.0 mm) than the gram-positive bacterial strains.

A similar result was reported in honey bee honey, in which a higher zone of inhibition was obtained in gram-negative *E.coli* (26±0mm), *S. typ*hi (14±0mm) and *K. pneumoniae* (17±0mm) than gram-positive *S. aureus* [50]. Similar studies confirmed a better inhibitory effect of honey against *E. coli* than *S. aureus* [51, 52]. Moreover, gram-negative *E. coli* and *S. typhi* were found to be more susceptible to ethanol extract from raw and processed honey than gram-positive *S. aureus* [52]. Furthermore, *E. coli* showed the highest sensitivity to Dharm-1 honey (80%) with a zone of inhibition of 15.33 ± 1.1 mm [36]. A study conducted on Malaysian *Trigona* honey revealed a higher zone of inhibition in *E. coli* and *S. typhi* than *S. aureus* [53]. Algerian honeys were, however, found to have better activity against gram-positive bacteria with a zone of inhibition ranging between 14 to 38 mm than gram-negative bacteria with a zone of inhibition ranging between 8 to 28 mm [54]. In the same vein, honey sample from Adamawa state of Nigeria showed that gram-positive *S. aureus* has the highest susceptibility (13.6 mm) followed by gram-negative *K. pneumoniae* (12.8 mm) and *E. coli* (12.4 mm) [55].

The proposed mechanism of destruction caused by honey on gram-negative bacteria is the destruction of the microbial cell wall and lipopolysaccharide which leads to increased cellular permeability and ultimately cell lysis [56]. In addition, many bioactive components contribute to the antibacterial properties of honey, which include flavonoids and polyphenols [57-59]. Some important components detected in the Cypriot CH may be responsible for the recorded antimicrobial activities. These include; benzene acetaldehyde [60], 2, 3 dihydro-3,5-di-hydroxy-4H-pyran-4-one [61], 5-hydroxymethylfurfural [62], and quercetin [63]. Increased honey acidity, due to the presence of phenolic acids, adds to the honey's flavor, and boosts its antibacterial activity [57]. Generally, the type and amount of bioactive compounds in honey depends on the geographical location, botanical origin, processing and storage conditions [64].

Table 3. Antifungal activity of carob honey against *Aspergillus niger* and *Aspergillus flavus* and Ketoconazole

Carob Honey (CH) (µg/mL)/ Antibiotic (200 µg/disc)	**Zone of Inhibition (mm) Fungal Strains**	
	Aspergillus flavus	***Aspergillus niger***
Ketoconazole	16±4.3	15±1.7
CH (8000)	7±1*	9±2.6*
CH(4000)	-	-
CH(2000)	-	-
CH(1000)	-	-

Results are presented as mean±SD of zones of inhibition (mm). *p<0.05 vs control (antibiotic). (-) indicate the absence of antifungal activity

According to our results, *A. niger* is more sensitive to the tested CH than *A. flavus*. However, the antifungal inhibition occurred only at 8000 µg/mL and no inhibition was recorded at lower concentrations (4000-1000 µg/mL). Similar to our findings, the effects of ethanol, methanol, and ethyl acetate extracts of processed honey against *A. niger*, have shown 11, 15 and 10.5 mm inhibition diameters for the respective extracts [65]. Related research revealed the antifungal activity of Romanian honey against *Aspergillus spp*, with an inhibition diameter from 7 to 12 mm [66]. These findings conformed to the studies on honey samples from Nigeria, India and Pakistan [67-69].

Honeys have generally been shown to exhibit antifungal properties against diverse species of fungi [70]. Our study shows that Cypriot CH has potential antimicrobial activity, which may be attributed to its phytoconstituents. The antimicrobial activities of honey are ascribed to the wide range of bioactive components, such as phenolic acids, flavonoids, and other biomolecules. Phenolic compounds are the notable components responsible for most biological activities of honey. Their antimicrobial role is mainly due to their ability to cause protein denaturation [71].

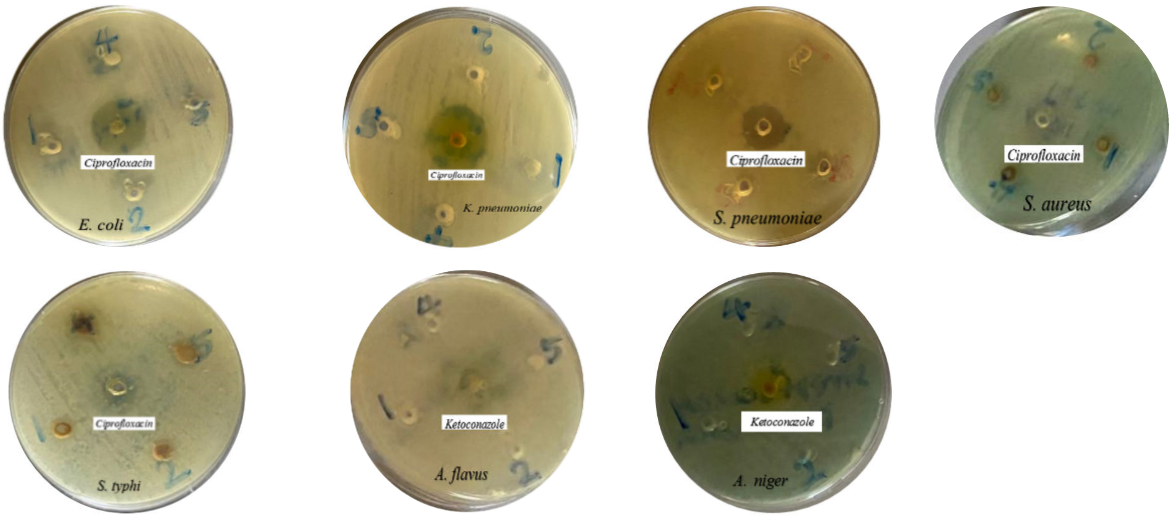

Figure 2. Zones of inhibition of carob honey at 1=8000 µg/mL, 2=4000 µg/mL, 4=2000 µg/mL, 5=1000 µg/mL

Effect of CH on MDA-MB-231 cell viability

Figure 3 below shows a concentration and time-dependent anti-proliferative activity of CH. MDA-MB-231 was treated with CH at five different concentrations (10, 25, 75, 150 and 300 µg/mL). The cell viability (%) was found to decrease according to the concentration applied and the time taken for sample activity. After 24 hours of treatment, the cells showed 80% viability with 20% inhibited. After 48 hours, the viable cells were 50% with 50% attenuated and after 72 hours the viable cells were found to be 45%, indicating 55% inhibition. However, the IC_{50} values are substantially high, 300 µg/mL and 70 µg/mL after 48 and 72hr respectively.

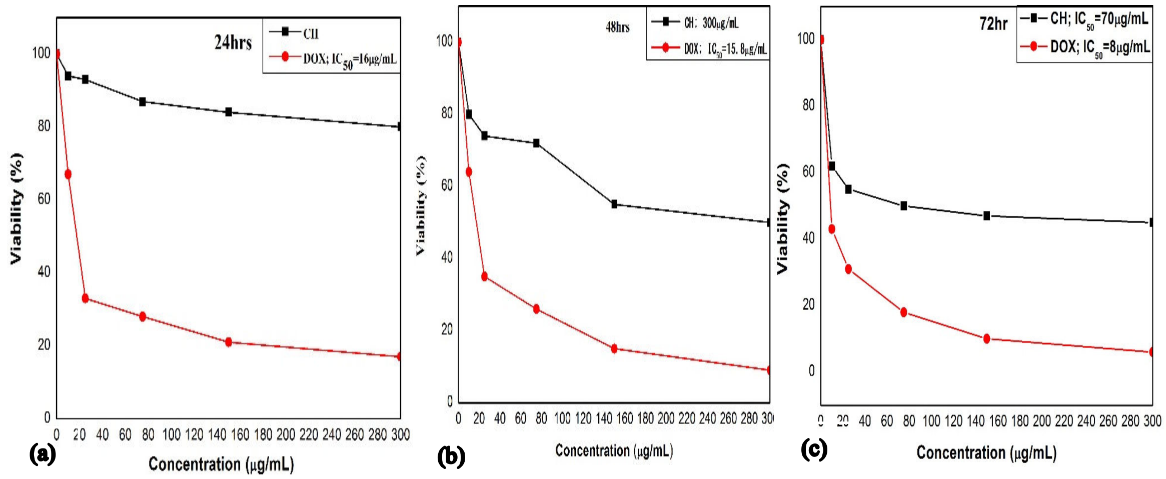

Figure 3. MDA-MB-231 inhibition at different CH concentrations, after A) 24 B) 48 and C) 72 hr. CH=Carob honey. DOX=Doxorubicin

Breast cancer poses a great threat to women due to difficulties in prognosis and uncertainties of the available treatments. Among the breast cancer types, TNBC is the most aggressive and it does not respond to hormonal or HER2 receptor therapy. Although chemotherapy, radiotherapy and surgical procedures have been used to treat it, huge risks are associated with the processes [72]. Honey is a natural dietary ingredient that has been used for centuries in treating many medical conditions. Honey's bioactive components have been linked to antitumor effects at various stages of cancer [73]. Biological activities such as anti-inflammation, antioxidant, immune-modulation and anti-proliferation are all reported in honey [74], hence honey is a great candidate to exploit in search of alternative cancer therapies.

The cytostatic effects of honey can be attributed to its ample phenolic compounds, which possess antitumor and antimicrobial activities [73, 75]. In addition, organic acids, vitamins and trace minerals contribute to the anticancer and antimicrobial effects of honey [69, 76-77]. For example, Manuka honey inhibits the proliferation of MCF-7 breast cancer cell line in a concentration-dependent manner, and this effect correlates with its phenolic compounds [78]. Tualang honey was used as a promoting agent with tamoxifen, and the combination was found to have a considerable cytotoxic activity on MDA-MB-231 and MCF-7 cell lines [79]. Cedar and pine honey from Turkey also have inhibitory effects on MCF-7, MDA-MB-231 and SKBR3 cell lines at high dose [80]. Chestnut honey and cedar honeys were reported to exert 50% inhibition on the MCF-7 cell line. Cedar honey could only exert this inhibition at a higher concentration [81]. Treatment of Sidr and wild honeys changed the appearance of the MDA-MB-231 breast cancer cell line after 48 hr indicating their cytotoxic effects against the breast cancer cell [82]. The differences in the anticancer activities of various honeys may be due to geographical locations, storage conditions, differences in the amount and type of minerals, amino acids, hydrogen peroxide and phenolic contents [83].

Conclusion

The current research assessed the chemical compositions, and antimicrobial and breast cancer inhibitory potentials of CH from Northern Cyprus. Some of the bioactive components detected are quercetin, furan methanol, squalene and 4H-Pyran-4-one. CH inhibited the growth of both gram-negative and gram-positive bacteria as well as the fungi used in this research. CH also exhibited a dose and time-dependent anti-proliferative effect against the MDA-MB-231 breast cancer cell line. These biological activities may be due to the presence of various bioactive compounds detected in the honey in the GC-MS analysis. Further research on carob honey safety, toxicity, and other pharmacological potentials is highly recommended.

Acknowledgements

This research got technical support from the technical staff of the cell culture laboratory, biotechnology research center, Cyprus International University.

Likewise, the microbiology technicians of the microbiology department, Bayero University Kano.

References

1. White, J. W. (1978) Honey. In: Chichester C. O. (ed) Advances in Food Research. Academic Press, pp 287-374.
2. Chua, L. S., Rahaman, N. L. A., Adnan, N. A. and Eddie Tan, T. T. (2013). Antioxidant activity of three honey samples in relation with their biochemical components. *Journal of Analytical Methods in Chemistry,* 2013: 313798.
3. Arawwawala, L. and Hewageegana, H. (2017). Health benefits and traditional uses of honey: A review. *Journal of Apitherapy*, 2017: 9-14.
4. Al-Waili, N. S. (2004). Natural honey lowers plasma glucose, c-reactive protein, homocysteine, and blood lipids in healthy, diabetic, and hyperlipidemic subjects: comparison with dextrose and sucrose. *Journal of Medicinal Food,* 7(1): 100-107.
5. Markelov, V. and Trushin, M. (2006). Bee venom therapy and low dose naltrexone for treatment of multiple sclerosis. *Nepal Journal of Neuroscience,* 3(2): 71-77.
6. Jenkins, R., Burton, N. and Cooper, R. (2014). Proteomic and genomic analysis of methicillin-resistant *Staphylococcus Aureus* (Mrsa) exposed to manuka honey in vitro demonstrated down-regulation of virulence markers. *Journal of Antimicrobial Chemotherapy,* 69(3): 603-615.
7. Lusby, P. E., Coombes, A. L. and Wilkinson, J. M. (2005). Bactericidal activity of different honeys against pathogenic bacteria. *Archives of Medical Research,* 36(5): 464-467.
8. Manyi-Loh, C. E., Clarke, A. M., Munzhelele, T., Green, E., Mkwetshana, N. F. and Ndip, R. N. (2010). Selected South African honeys and their extracts possess in vitro anti-helicobacter pylori activity. *Archives of Medical Research,* 41(5): 324-331.
9. Irish, J., Carter, D. A., Shokohi, T. and Blair, S. E. (2006). Honey has an antifungal effect against candida species. *Medical Mycology,* 44(3): 289-291.
10. Chauhan, A., Pandey, V., Chacko, K. and Khandal, R. (2010). Antibacterial activity of raw and processed honey. *Electronic Journal of Biology,* 5(3): 58-66.
11. Aryappalli, P., Shabbiri, K., Masad, R. J., Al-Marri, R. H., Haneefa, S. M., Mohamed, Y. A., Arafat, K., Attoub, S., Cabral-Marques, O. and Ramadi, K. B. (2019). Inhibition of tyrosine-phosphorylated stat3 in human breast and lung cancer cells by manuka honey is mediated by selective antagonism of the Il-6 receptor. *International Journal of Molecular Sciences,* 20(18): 4340.
12. Salleh, M. A. M., Eshak, Z. and Ismail, W. I. W. (2017). Acacia honey induces apoptosis in human breast adenocarcinoma cell lines (Mcf-7). *Jurnal Teknologi,* 79(4): 9882.
13. Hamadou, W. S., Bouali, N., Alhejaili, E. B., Soua, Z., Patel, M., Adnan, M., Siddiqui, A. J., Abdel-Gadir, A.-M., Sulieman, A. M. E., Snoussi, M. and Badraoui, R. (2023). Acacia honey-derived bioactive compounds exhibit induction of p53-dependent apoptosis in the Mcf-7 human breast cancer cell line. *Pharmacognosy Magazine,* 19(1): 144-155.
14. Siegel, R. L., Miller, K. D., Fuchs, H. E. and Jemal, A. (2022). Cancer statistics, 2022. *CA Cancer Journal for Clinicians,* 72(1): 7-33.
15. Siegel, R. L., Miller, K. D., Wagle, N. S. and Jemal, A. (2023). Cancer statistics, 2023. *CA Cancer Journal for Clinicians,* 73(1): 17-48.
16. Hinçal, E., Taneri, B., Taneri, U. and Djamgoz, M. B. (2008). Cancer incidence in North Cyprus (1990-2004) relative to european rates. *Asian Pacific Journal for Cancer Prevention,* 9(4): 725-732.
17. Siddharth, S. and Sharma, D. (2018). Racial disparity and triple-negative breast cancer in African-American women: A multifaceted affair between obesity, biology, and socioeconomic determinants. *Cancers (Basel),* 10(12): 514.
18. Bianchini, G., De Angelis, C., Licata, L. and Gianni, L. (2022). Treatment landscape of triple-negative breast cancer - expanded options, evolving needs. *Nature Reviews Clinical Oncology,* 19(2): 91-113.
19. Barman, S. K., Zaman, M. S., Veljanoski, F., Malladi, C. S., Mahns, D. A. and Wu, M. J. (2022). Expression profiles of the genes associated with

zinc homeostasis in normal and cancerous breast and prostate cells. *Metallomics,* 14(8): 038.
20. Li, C. I., Daling, J. R. and Malone, K. E. (2003). Incidence of invasive breast cancer by hormone receptor status from 1992 to 1998. *Journal of Clinical Oncology,* 21(1): 28-34.
21. El-Haskoury, R., Kriaa, W., Lyoussi, B. and Makni, M. (2018). Ceratonia siliqua honeys from Morocco: Physicochemical properties, mineral contents, and antioxidant activities. *Journal of Food and Drug Analysis,* 26(1): 67-73.
22. Nurul Syazana, M. S., Gan, S. H., Halim, A. S., Shah, N. S., Gan, S. H. and Sukari, H. A. (2013). Analysis of volatile compounds of Malaysian Tualang (*Koompassia excelsa*) honey using gas chromatography mass spectrometry. *African Journal of Traditional Complementary and Alternative Medicines,* 10(2): 180-188.
23. Humphries, R. M., Fang, F. C., Aarestrup, F. M. and Hindler, J. A. (2012). In vitro susceptibility testing of fluoroquinolone activity against salmonella: recent changes to clsi standards. *Clinical Infectious Diseases,* 55(8): 1107-1113.
24. Fraser, S. P., Diss, J. K., Chioni, A. M., Mycielska, M. E., Pan, H., Yamaci, R. F., Pani, F., Siwy, Z., Krasowska, M., Grzywna, Z., Brackenbury, W. J., Theodorou, D., Koyutürk, M., Kaya, H., Battaloglu, E., De Bella, M. T., Slade, M. J., Tolhurst, R., Palmieri, C., Jiang, J., Latchman, D. S., Coombes, R. C. and Djamgoz, M. B. (2005). Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis. *Clinical Cancer Research,* 11(15): 5381-5389.
25. Isbilen, O., Rizaner, N. and Volkan, E. (2018). Anti-proliferative and cytotoxic activities of allium autumnale P. H. Davis (Amaryllidaceae) on human breast cancer cell lines Mcf-7 and Mda-Mb-231. *BMC Complementary and Alternative Medicines,* 18(1): 30.
26. Radovic, B. S., Careri, M., Mangia, A., Musci, M., Gerboles, M. and Anklam, E. (2001). Contribution of dynamic headspace GC–MS analysis of aroma compounds to authenticity testing of honey. *Food Chemistry,* 72(4): 511-520.
27. Krokou, A., Stylianou, M. and Agapiou, A. (2019). Correction To: Assessing the volatile profile of carob tree (*Ceratonia siliqua* L.). *Environmental Science and Pollution Research,* 26: 35375-35376.
28. Damayanti, N. W., Sahlan, M., Lischer, K., Hermansyah, H., Kusumoputro, B. and Pratami, D. K. (2019) Comparison of original honey (Apis Sp and Tetragonula Sp) and fake honey compounds in indonesia using gas chromatography-mass spectrometry (GC-MS). In: AIP Conference Proceedings. AIP Publishing
29. Machado, A. M., Antunes, M., Miguel, M. G., Vilas-Boas, M. and Figueiredo, A. C. (2021). Volatile profile of portuguese monofloral honeys: Significance in botanical origin determination. *Molecules,* 26(16): 4970.
30. Ouradi, H., Hanine, H., Fauconnier, M.-L., Kenne, T., Rizki, H., Ennahli, S. and Hssaini, L. (2021). Determination of physico-biochemical proprieties and composition in volatile constituents by solid phase micro-extraction of honey samples from different botanical and geographical origins in Morocco. *Journal of Apicultural Research,* 60(1): 84-98.
31. Custódio, L., Serra, H., Nogueira, J. M. F., Gonçalves, S. and Romano, A. (2006). Analysis of the volatiles emitted by whole flowers and isolated flower organs of the carob tree using HS-SPME-GC/MS. *Journal of Chemical Ecology,* 32 929-942.
32. Montaser, M., Sayed, A. M., Bishr, M. M., Zidan, E. W., Zaki, M. A., Hassan, H. M., Mohammed, R. and Hifnawy, M. S. (2023). GC-MS analysis of honeybee products derived from medicinal plants. *Beni-Suef University Journal of Basic and Applied Sciences,* 12 (1): 1-12.
33. Awasum, C. A., Fotzo, S. L. M., Ndukum, J. A., Genesis, C. and Zoli, A. (2015). Gas chromatography-mass spectroscopy analysis and chemical composition of Ngaoundere, Cameroon honey. *American Journal of Bioscience and Bioengineering,* 3(5): 33-36.
34. Srimathi Priyanga, K. and Vijayalakshmi, K. (2017). Investigation of antioxidant potential of quercetin and hesperidin: An *in vitro* approach. *Asian Journal of Pharmaceutical and Clinical Research,* 10 83-86.
35. Hisaka, T., Sakai, H., Sato, T., Goto, Y., Nomura, Y., Fukutomi, S., Fujita, F., Mizobe, T., Nakashima, O. and Tanigawa, M. (2020). Quercetin suppresses proliferation of liver cancer cell lines in vitro. *Anticancer Research,* 40(8): 4695-4700.

36. Ghramh, H. A., Khan, K. A. and Alshehri, A. M. A. (2019). Antibacterial potential of some saudi honeys from asir region against selected pathogenic bacteria. *Saudi Journal of Biological Sciences,* 26 (6): 1278-1284.
37. Deng, J., Liu, R., Lu, Q., Hao, P., Xu, A., Zhang, J. and Tan, J. (2018). Biochemical properties, antibacterial and cellular antioxidant activities of buckwheat honey in comparison to manuka honey. *Food Chemistry,* 252: 243-249.
38. Pal, M., Teashal, B. M., Gizaw, F., Alemayehu, G. and Kandi, V. (2020). Animals and food of animal origin as a potential source of salmonellosis: A review of the epidemiology, laboratory diagnosis, economic impact and public health significance. *American Journal of Microbiological Research,* 8(2): 48-56.
39. Aibinu, I., Aednipekun, E. and Odugbemi, T. (2004). Emergence of quinolone resistance amongst *Escherichia coli* strains isolated from clinical infections in some Lagos State Hospitals, in Nigeria. *Nigerian Journal of Health and Biomedical Sciences,* 3(2): 73-78.
40. Caldwell, M. D. (2020). Bacteria and antibiotics in wound healing. *Surgical Clinics of North America,* 100 (4): 757-776.
41. David, M. Z. and Daum, R. S. (2010). Community-associated methicillin-resistant *Staphylococcus aureus:* Epidemiology and clinical consequences of an emerging epidemic. *Clinical Microbiology Reviews,* 23(3): 616-687.
42. Miller, S. I. (2016). Antibiotic resistance and regulation of the gram-negative bacterial outer membrane barrier by host innate immune molecules. *mBio,* 7(5): 1541.
43. Gupta, V. and Datta, P. (2019). Next-generation strategy for treating drug resistant bacteria: Antibiotic hybrids. *Indian Journal of Medical Research,* 149(2): 97-106.
44. Exner, M., Bhattacharya, S., Christiansen, B., Gebel, J., Goroncy-Bermes, P., Hartemann, P., Heeg, P., Ilschner, C., Kramer, A., Larson, E., Merkens, W., Mielke, M., Oltmanns, P., Ross, B., Rotter, M., Schmithausen, R. M., Sonntag, H. G. and Trautmann, M. (2017). Antibiotic resistance: What is so special about multidrug-resistant gram-negative bacteria? *GMS Hygiene and Infection Control,* 12: 290.
45. Ewnetu, Y., Lemma, W. and Birhane, N. (2013). Antibacterial effects of *Apis mellifera* and stingless bees honeys on susceptible and resistant strains of *Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae* in Gondar, Northwest Ethiopia. *BMC Complementary and Alternative Medicine,* 13: 269.
46. Brown, E., O'Brien, M., Georges, K. and Suepaul, S. (2020). Physical characteristics and antimicrobial properties of *Apis mellifera, Frieseomelitta nigra and Melipona favosa* bee honeys from apiaries in trinidad and tobago. *BMC Complementary Medicine and Therapies,* 20(1): 85.
47. Chanchao, C. (2009). Antimicrobial activity by *Trigona laeviceps* (stingless bee) honey from Thailand. *Pakistan Journal of Medical Sciences,* 25(3): 364-369.
48. Omar, S., Mat-Kamir, N. F. and Sanny, M. (2019). Antibacterial activity of malaysian produced stingless-bee honey on wound pathogens. *Journal of Sustainability Science and Management,* 14: 67-79.
49. Zainol, M. I., Mohd Yusoff, K. and Mohd Yusof, M. Y. (2013). Antibacterial activity of selected malaysian honey. *BMC Complementary and Alternative Medicine,* 13: 129.
50. Ng, W. J., Sit, N. W., Ooi, P. A., Ee, K. Y. and Lim, T. M. (2020). The antibacterial potential of honeydew honey produced by stingless bee (*Heterotrigona itama*) against antibiotic resistant bacteria. *Antibiotics (Basel),* 9 (12): 871.
51. Al-Naama, R. T. (2009). Evaluation of in-vitro inhibitory effect of honey on some microbial isolate. *Journal of Bacteriology Research,* 1(6): 64-67.
52. Tuksitha, L., Chen, Y.-L. S., Chen, Y.-L., Wong, K.-Y. and Peng, C.-C. (2018). Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak). *Journal of Asia-Pacific Entomology,* 21(2): 563-570.
53. Shalsh, F. J., Khalaf, A. M., Hafiz, M. and Ali, I. I. (2021). Antibacterial properties of local malaysian Trigona Sp. honey towards different of pathogenic bacteria *in vitro*. *Diyala Agricultural Sciences Journal,* 13(1): 1-9.
54. Bouacha, M., Besnaci, S. and Boudiar, I. (2023). Comparative study of the antibacterial activity of

algerian honeys and manuka honey toward pathogenic bacteria from burn wound infections. *Mikrobiolohichnyi Zhurnal,* 85(2): 26-36.
55. Isa, H., Sa'id, A. S. and Adaora, O. J. (2020). Evaluation of antibacterial activity of different honey samples on *Staphylococcus aureus, Klebsilla pneumoniae and Escherichia coli* isolated from wounds. *Science,* 5(1): 107-111.
56. Brudzynski, K. and Sjaarda, C. (2014). Antibacterial compounds of canadian honeys target bacterial cell wall inducing phenotype changes, growth inhibition and cell lysis that resemble action of β-lactam antibiotics. *PLoS One,* 9(9): e106967.
57. Bouacha, M., Besnaci, S., Boudiar, I. and Al-kafaween, M. A. (2022). Impact of storage on honey antibacterial and antioxidant activities and their correlation with polyphenolic content. *Tropical Journal of Natural Product Research,* 6(1): 34-39.
58. Bucekova, M., Jardekova, L., Juricova, V., Bugarova, V., Di Marco, G., Gismondi, A., Leonardi, D., Farkasovska, J., Godocikova, J., Laho, M., Klaudiny, J., Majtan, V., Canini, A. and Majtan, J. (2019). Antibacterial activity of different blossom honeys: New findings. *Molecules,* 24(8): 1573.
59. Cebrero, G., Sanhueza, O., Pezoa, M., Báez, M. E., Martínez, J., Báez, M. and Fuentes, E. (2020). Relationship among the minor constituents, antibacterial activity and geographical origin of honey: A multifactor perspective. *Food Chemistry,* 315: 126296.
60. Api, A. M., Belsito, D., Botelho, D., Bruze, M., Burton, G. A., Jr., Buschmann, J., Dagli, M. L., Date, M., Dekant, W., Deodhar, C., Francis, M., Fryer, A. D., Jones, L., Joshi, K., La Cava, S., Lapczynski, A., Liebler, D. C., O'Brien, D., Patel, A., Penning, T. M., Ritacco, G., Romine, J., Sadekar, N., Salvito, D., Schultz, T. W., Sipes, I. G., Sullivan, G., Thakkar, Y., Tokura, Y. and Tsang, S. (2018). Rifm Fragrance Ingredient Safety Assessment, Benzeneacetaldehyde, 3,4-Dimethyl-, Cas Registry Number 68844-97-3. *Food and Chemical Toxicology,* 122: 664-669.
61. Shukla, R., Banerjee, S. and Tripathi, Y. B. (2018). Antioxidant and antiapoptotic effect of aqueous extract of *Pueraria tuberosa* (Roxb. Ex Willd.) Dc. on streptozotocin-induced diabetic nephropathy in rats. *BMC Complementary and Alternative Medicine,* 18 (1): 156.
62. Rajkumari, J., Borkotoky, S., Reddy, D., Mohanty, S. K., Kumavath, R., Murali, A., Suchiang, K. and Busi, S. (2019). Anti-quorum sensing and anti-biofilm activity of 5-hydroxymethylfurfural against *Pseudomonas Aeruginosa* pao1: insights from *in vitro, in vivo* and *in silico* studies. *Microbiological Research,* 226: 19-26.
63. Nguyen, T. L. A. and Bhattacharya, D. (2022). Antimicrobial activity of quercetin: an approach to its mechanistic principle. *Molecules,* 27(8).
64. Gambogou, B., Khadimallah, H., Bouacha, M. and Ameyapoh, Y. A. (2018). Antibacterial activity of various honey monofloral and polyfloral from different regions of algeria against uropathogenic gram-negative bacilli. *Journal of Apitherapy,* 4(1):1-8.
65. Ogidi, O. I., Adigwe, P. C., Enenebeaku, U. E. and Ayebaboagha, M. N. (2019). Determination of antimicrobial susceptibility of ethanol, methanol, and acetate extracts of processed honey. *Journal of Apitherapy,* 7(1): 1-5.
66. Vică, M. L., Glevitzky, M., Dumitrel, G. A., Bostan, R., Matei, H. V., Kartalska, Y. and Popa, M. (2022). Qualitative characterization and antifungal activity of romanian honey and propolis. *Antibiotics (Basel),* 11 (11): 552.
67. Ahmad, K., Khalil, A. T., Somayya, R., Khan, F. N., Shah, A. R., Ovais, M., and Shinwari, Z. K (2017). Potential antifungal activity of different honey brands from Pakistan: A quest for natural remedy. *African Journal of Traditional, Complementary and Alternative Medicines,* 14 18-23.
68. Anyanwu, C. (2012). Investigation of *in vitro* antifungal activity of honey. *Journal of Medicinal Plants Research,* 6(18): 3512-3516.
69. Moussa, A., Noureddine, D., Saad, A., Abdelmelek, M. and Abdelkader, B. (2012). Antifungal activity of four honeys of different types from algeria against pathogenic yeast: *Candida albicans and Rhodotorula* Sp. *Asian Pacific Journal of Tropical Biomedicine,* 2(7): 554-557.
70. Shah, K. N., Pawar, P. and Wankhade, P. (2022). Honey and its properties: A comprehensive review. *International Journal of Food Science and Nutrition,* 7(1): 16-20.

71. Estevinho, L., Pereira, A. P., Moreira, L., Dias, L. G. and Pereira, E. (2008). Antioxidant and antimicrobial effects of phenolic compounds extracts of northeast portugal honey. *Food and Chemical Toxicology,* 46(12): 3774-3779.
72. Chavez, K. J., Garimella, S. V. and Lipkowitz, S. (2010). Triple negative breast cancer cell lines: One tool in the search for better treatment of triple negative breast cancer. *Breast Disease,* 32(1-2): 35-48.
73. Erejuwa, O. O., Sulaiman, S. A. and Wahab, M. S. (2014). Effects of honey and its mechanisms of action on the development and progression of cancer. *Molecules,* 19(2): 2497-2522.
74. Ahmed, S. and Othman, N. H. (2013). Honey as a potential natural anticancer agent: a review of its mechanisms. *Evidence-Based Complementary and Alternative Medicine,* 2013(1), 829070.
75. Martinello, M. and Mutinelli, F. (2021). Antioxidant activity in bee products: a review. *Antioxidants (Basel),* 10(1): 71.
76. Silva, L. R., Videira, R., Monteiro, A. P., Valentão, P. and Andrade, P. B. (2009). Honey from Luso region (Portugal): Physicochemical characteristics and mineral contents. *Microchemical Journal,* 93(1): 73-77.
77. Liu, J.-R., Ye, Y.-L., Lin, T.-Y., Wang, Y.-W. and Peng, C.-C. (2013). Effect of floral sources on the antioxidant, antimicrobial, and anti-inflammatory activities of honeys in Taiwan. *Food Chemistry,* 139(1-4): 938-943.
78. Portokalakis, I., Yusof, H. M., Ghanotakis, D., Nigam, P. S. and Owusu-Apenten, R. (2016). Manuka honey-induced cytotoxicity against mcf7 breast cancer cells is correlated to total phenol content and antioxidant power. *Journal of Advances in Biology and Biotechnology,* 8(2): 1-10.
79. Yaacob, N. S., Nengsih, A. and Norazmi, M. N. (2013). Tualang honey promotes apoptotic cell death induced by tamoxifen in breast cancer cell lines. *Evidence Based Complementary and Alternative Medicine,* 2013: 989841.
80. Seyhan, M. F., Timirci-Kahraman, Ö., Kısakesen, H. İ., Ünal, E., Eronat, A. P., Yılmaz, E., Öztürk, T., Saygılı, N., Gazioğlu, S. and Yılmaz-Aydoğan, H. (2015). Turkish honey is not only sweet, yet also anti carcinogenic: prominent effects of nonfloral honey with respect to floral honey on breast cancer cell lines. In: *IVEK 2nd International Convention of Pharmaceuticals and Pharmacies,* 2015: 1-10.
81. Celebioglu, H. U. (2020). Probiotic bacteria grown with chestnut honey enhance in vitro cytotoxicity on breast and colon cancer cells. *Archives Biological Sciences,* 72(3):329-338.
82. Almeer, R., Alqarni, A., Alqattan, S., Abdi, S., Alarifi, S., Hassan, Z. and Semlali, A. (2018). Effect of honey in improving breast cancer treatment and gene expression modulation of mmps and timps in triple-negative breast cancer cells. *Pakistan Journal of Zoology,* 50(6): 1999-2007.
83. van der Woude, H., Gliszczyńska-Swigło, A., Struijs, K., Smeets, A., Alink, G. M. and Rietjens, I. M. (2003). Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. *Cancer Letters,* 200(1): 41-47.