



Effect of Honey in Improving Breast Cancer Treatment and Gene Expression Modulation of MMPs and TIMPs in Triple-Negative Breast Cancer Cells

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ABSTRACT

Breast cancer treatment by chemotherapy or radiotherapy is widely used but has serious side-effects. Thus, honey which is a natural substance has been extensively studied for its anticancer properties. However, its mechanism of action remains unclear. The present study evaluated the effect of two varieties of natural honey (Sidr and Wild) on a human breast adenocarcinoma (MDA-MB-231) cell line. The MDA-MB-231 cell lines were treated with Sidr honey (H1) and Wild honey (H2) for 6, 24, or 48 h followed by cell morphology evaluation. Cell viability was examined by MTT assay and gene expression of three tissue inhibitors of metalloproteinase (TIMPs) and two matrix metalloproteinase (MMPs) were measured by real-time PCR. All subgroups exhibited altered morphology with accelerated detachment compared to untreated cells. The MTT assay after 48 h revealed that treatment with H1 and H2, reduced cell viability by 48% and 91% respectively, compared to that of untreated cells. These results suggest that anticancer effect of Sidr and Wild honey corresponds to their ability to modulate gene expression of MMPs and TIMPs in MDA-MB-231 cell lines. Further investigations are required to understand the molecular mechanism of honey as an anticancer agent.

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Authors' Contribution

RA conceived and designed the project and wrote the article. AA and SA performed the experimental work. ZH analysed the data. SA and AS co-drafted the manuscript.

Key words

Sidr honey, Wild honey, MMPs, TIMPs, Triple-negative breast cancer, Tissue inhibitors of metalloproteinase, Matrix metalloproteinase.

INTRODUCTION

Breast cancer (BC) is a serious health problem worldwide, occurring in both in developed and less developed countries (Jemal *et al.*, 2011). In 2012, it was estimated that more than 1.7 million new cases of breast cancer were diagnosed. Deaths from cancer are projected to increase to over 14.1 million in 2030 (ACS, 2016). There are five groups in breast cancer based on their molecular profiles, including triple-negative breast cancer (TNBC) (Irvin and Carey, 2008), which accounts for approximately 15% of breast tumors (Lara-Medina *et al.*, 2011). TNBC

is considered an aggressive but heterogeneous clinical disease for which there is no well-identified mechanism of therapy (Vaz-Luis *et al.*, 2014). However, the standard mechanism of treatment consists of radiation, surgery, and chemotherapy drugs with an anthracyclines basis (*i.e.*, gemcitabine, ixabepilone, vinorelbine, doxorubicin (Doxo), taxanes, and capecitabine). Although, chemotherapy increases the survival rate, it also causes side effects in various body systems (Yu and Jones, 2016).

Abbreviations

H1, Sidr honey; H2, Wild honey; TIMPs, tissue inhibitors of metalloproteinase; MMPs, Matrix metalloproteinase; PCR, polymerase chain reaction; MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); BC, breast cancer; TNBC, triple-negative breast cancer; Doxo, Doxorubicin.

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Natural products such as honey have been shown to exhibit potential anticancer effect (Ahmed and Othman, 2017). Honey is composed of various types of sugar, phenolic acids, amino acids, flavonoids, enzymes, and proteins, along with other compounds. Its composition changes according to the flower source and geographical origin (Gheldof *et al.*, 2002). The major factors responsible for the anticancer activity of honey are its phenolic acids (Abubakar *et al.*, 2012). Previous studies suggested several explanations for honey's anticancer effects, but the detailed mechanism remains unclear (Ahmed and Othman, 2013a). Honey from different floral sources may have different effects. The mechanisms suggested for anticancer effect of honey include interference with multiple cell-signaling pathways, such as antiproliferative (Pichichero *et al.*, 2010), inducing apoptosis (Jaganathan and Mandal, 2010), antimutagenic (Saxena *et al.*, 2012), and anti-inflammatory pathways (Hussein *et al.*, 2012).

There are very complex mechanisms and destructive features in cancer, primarily metastasis (Martin *et al.*, 2013). Studies of honey's effects on cancer cells properties are limited. Many metastasis modifiers that may be affected by honey effect have not been examined. Matrix metalloproteinases (MMPs), extracellular-degrading proteases, contribute to metastatic and invasive cancer cells properties; their expression (particularly MMP-9 and MMP-2) is increased in carcinomas (Stamenkovic, 2000) MMPs activity can be specifically inhibited by a group of structurally related, endogenous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) (Al-Olayan *et al.*, 2014). However, some conflicting reports demonstrated that inhibitors or individual proteinases were correlated with the result of surgery in breast cancer patients (Brummer *et al.*, 1999). The effect of honey on MMPs expression and activity has not been widely examined (Porcza *et al.*, 2016). This study examined the role of sidr and wild honey as antiproliferative and cytotoxic agents. Additionally, the mechanisms of its action at the cellular level were examined by evaluated the effect of honey on the expression of MMPs and TIMPs.

MATERIALS AND METHODS

Cell culture

Human breast adenocarcinoma (MDA-MB-231) cell lines used in this study were obtained from the Genome Research Chair, King Saud University. Cells were seeded under sterile conditions into RPMI medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum and 1% penicillin-streptomycin (UFC Biotech, Riyadh, Saudi Arabia). Cells were cultured in a special flask and

the flasks were placed in a 5% CO₂ incubator with 37°C humidity. The cells were checked routinely under an inverted microscope to avoid contamination. Every 2-3 days, the media was changed until the cells reached a confluent state.

Honey preparation

Crude honey (both honeys were purchased from a local market in Riyadh, Saudi Arabia) was initially transfer by sterilized micropipette under sterilized condition in the biological safety cabinet class II then dissolved in culture medium [(RPMI) medium 1640 from (Gibco[®], UK) supplemented with 1% fetal bovine serum (FBS) (Gibco[®], UK) and 1% of 10000 unit/ml penicillin-10000 µg/ml streptomycin (UFC Biotech, KSA)] at a final concentration of 1% (v/v); that means for each 1 ml of medium which needed to coverage the cells of density 3×10^5 cells / well (in 6 wells culture plate) 10 µl of honey was add and for the density of 9×10^5 cells/25 cm² culture flask 30 µl of honey dissolved in 3 ml of culture medium then added directly on the cells after washing of the cells by PBS Phosphate-Buffered Saline (Gibco[®], UK).

Treatment

Cells cultured in 6-wells plates at 3×10^5 cells/well were divided into four groups: Group 1: untreated cells as a negative control, Group 2: cells treated with at 4% (v/v) concentration of doxorubicin (Doxo) as a positive control, Group 3: cells treated with Sidr honey (H1) at 1% (v/v) concentration, and Group 4: cells treated with Wild honey (H2) at 1% (v/v) concentration. Each group was further divided into three subgroups and cultures were maintained for 6, 24, or 48 h.

Morphological changes

An inverted microscope (Nikon, Tokyo, Japan) was utilized to monitor the morphological alterations of cells.

Cell viability

Cell viability in the culture was verified by subjecting confluent cells to the methyl thiazolyl tetrazolium (MTT) assay as described elsewhere (Klettner *et al.*, 2009) with some modifications. Briefly, 100 µL of MTT (5 mg/mL) was added to each well and the plate was incubated for 3 h at 37 °C with 5% CO₂ and in a humid atmosphere. Next, the flask was opened slightly for cell aspiration. The tetrazolium crystals were dissolved by viable cells after adding 500 µL of isopropanol-HCl (0.04 N). A plate reader was used to measure absorbance (X Mark Microplate Spectrophotometer, Bio-Rad, Hercules, CA, USA) at a wavelength of 550 nm.

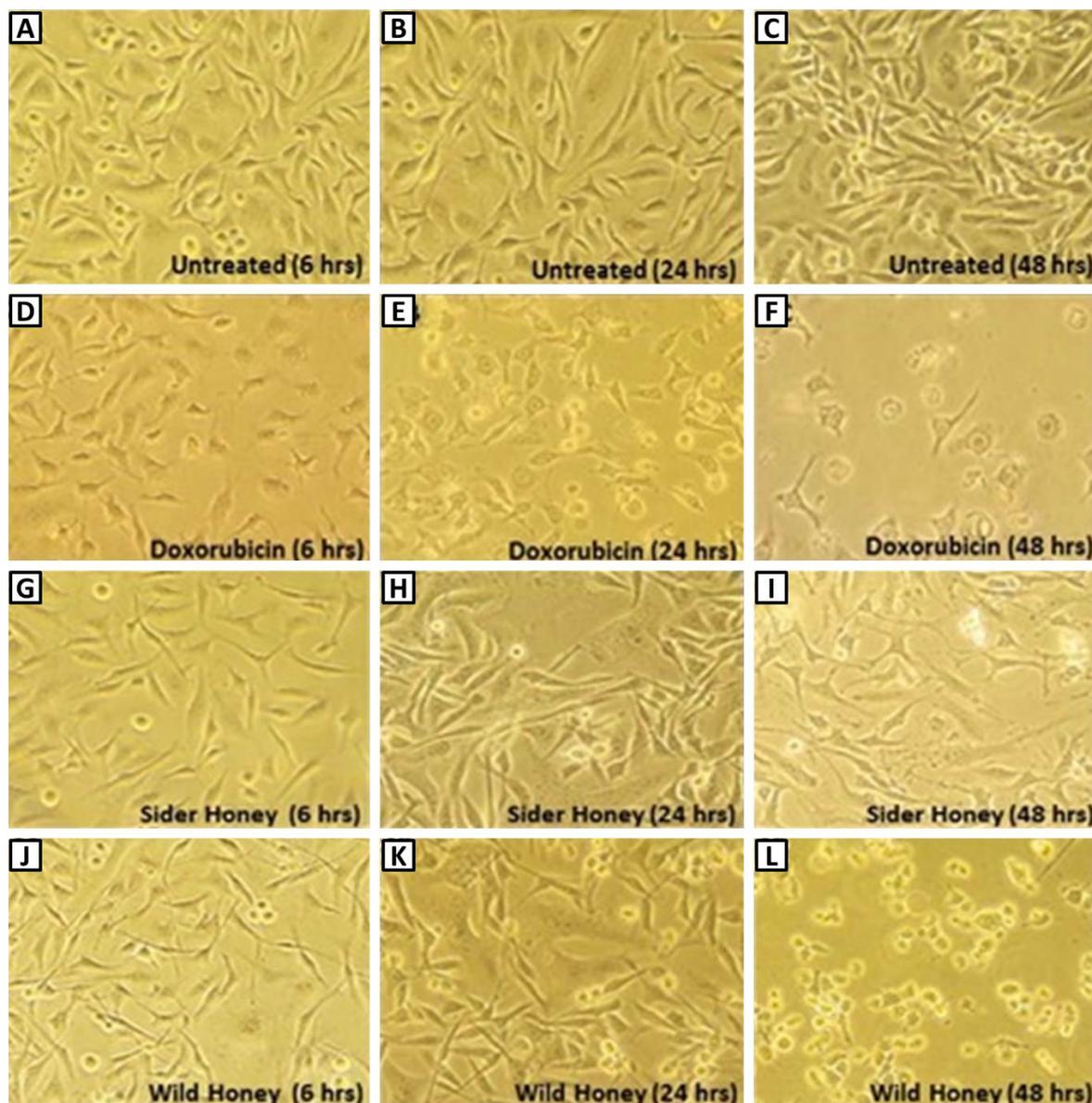


Fig. 1. Cell morphology at 6, 24, and 48 h of untreated cells (A-C), post-treatment with doxorubicin 4% (v/v) (D-F), sidr honey 1% (v/v) (G-I) and wild honey 1% (v/v) (J-L).

Table I.- Sequences of primers used in the experiment.

Gene	Forward primer sequence	Reverse Primer sequence
<i>TIMP1</i>	5'-ACAACCGCAGCGAGGAGT-3'	5'-AGGTGACGGGACTGGAAGC-3'
<i>TIMP2</i>	5'-TTGACCCAGAGTGGAACG-3'	5'-ACCAAAGACGGGAGACGA-3'
<i>TIMP4</i>	5'-TACCAGGCTCAGCATTAT-3'	5'-CCACTTGGCACTTCTTATT-3'
<i>GAPDH</i>	5'-AAGGATAATGGCTTACAAC-3'	5'-TCACTTAGGGCTTCTCAC-3'
<i>MMP-2</i>	5'-TTTCCATTCCGCTTCCAGGGCAC-3'	5'-TCGCACACCACATCTTTCCGTCACT-3'
<i>MMP-9</i>	5'-CCTGCCAGTTTCCATTCATC-3'	5'-GCCATTCACGTCGTCCTTAT-3'

Gene expression

As described previously (Semlali *et al.*, 2017), RNA was extracted from harvested cells with a Qiagen kit and RNA purity was measured with a NanoDrop 2000 spectrophotometer (Wilmington, DE, USA). A QuantiTect® Reverse Transcription kit from Qiagen (Hilden, Germany) was used to reverse-transcribe total RNA (1 µg of each sample) into cDNA. Gene expression of MMPs and TIMPs gene was quantified by reverse transcription PCR. All experiments were conducted in triplicate. Expression levels were determined for *MMP-2*, *MMP-9*, *TIMP-2*, *TIMP-1*, and *TIMP-4* with an endogenous *GAPDH* as an internal control using a 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA) and universal iTaq SYBR green PCR master mix (Bio-Rad). Experiments were performed in triplicate. Primer sequences are listed in Table I. The 25µl RT-PCR reaction components comprising of SYBR Green (2x), 12.5µl (1x), Primers; (F+R) (10µM) 0.5µl (10pmole), H₂O 7µl, CDA 5µl were mixed in 96-well plates. The thermal cycling conditions comprised DNA polymerase activation at 95°C for 5 min followed by 40 cycles each of denaturation at 95°C for 30 seconds, Annealing at 55-60°C for 45 seconds and extension at 72°C for 30 min. After the reactions, the amplification plots were viewed. Baseline and threshold values were adjusted to determine the threshold cycle (CT) for the amplification curves. The results were analyzed using the $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) relative expression method.

Statistical analysis

The study involved three different experiments and each experiment was conducted in triplicate, with the results shown as the means ± SD. The statistical significance of the difference between the control (untreated cell) and test (cell treated by H1, H2, or doxorubicin) values were

determined by one-way analysis of variance. An unpaired two-way *t*-test and with a *P* value < 0.05 was used to determine significant differences between groups.

RESULTS

Cell morphology

MDA-MB-231 cell morphology was examined under an inverted microscope to investigate changes in the cells after treatment with Doxo or honey (H1 and H2) for 6, 24, or 48 h. As shown in Figure 1D-L, the morphology of breast cancer cells treated differed from that of untreated cells. The density of untreated cells (Fig. 1A-C) remained confluent throughout the experimental period. After treatment with Doxo (Fig. 1D-F), most cells showed reduced viability at all three time-points, while treatment with H1 (Fig. 1G-I) resulted in reduced cell density after 48 h of treatment. A few floating cells were also observed at 48 h. Treatment with H2 (Fig. 1J-L) also reduced cell density after 48 h of treatment. Many cells were detached from the surface and appeared round at this time point.

MTT assay

The cytotoxicity of various treatments in MDA-MB-231 cells was examined after 6, 24, and 48 h and compared to that in untreated cells (Fig. 2). Treatment with Doxo significantly reduced cell viability after 24 and 48 h of treatment (viable cells were 11.7% and 5.9%, respectively). Treatment with H1 and H2 significantly increased cell viability at 6 (viable cells were 106.7% and 112.6%, respectively) and 24 h (viable cells were 57.5% and 115.8%, respectively); while they reduced cell viability after 48 h (viable cells were 52.6% and 8.1%, respectively). Thus, Doxo was cytotoxic at 24 and 48 h treatments, while H1 and H2 were cytotoxic only after 48 h treatment.

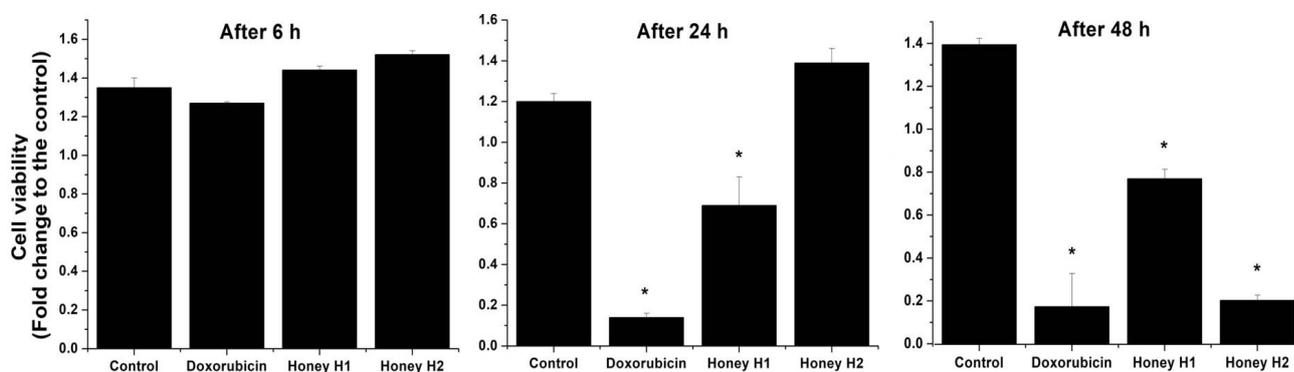


Fig. 2. Cell viability in different subgroups (6, 24, and 48 h) as estimated by MTT assay. The proliferation of MDA-231 cells was detected by MTT assay after treatment with H1, H2, Doxo and compared to untreated cells. **p*-value is significant at < 0.05.

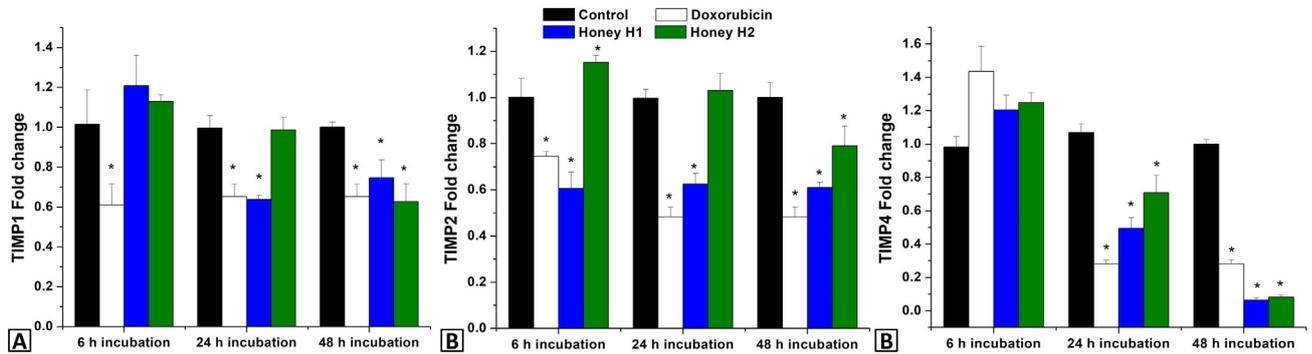


Fig. 3. Expression of *TIMPs* in (H1, H2 and doxo) treated and untreated breast cancer MDA-231 cells. A, *TIMP-1*; B, *TIMP-2*; C, *TIMP-4*. Columns, mean ($n = 3$); SD. * $P \leq 0.05$, significant compared to control (untreated cells).

Gene expression profile

TIMP genes

As shown in Figure 3A, the expression of *TIMP-1* was significantly reduced (0.61-fold) after 6 h of treatment with Doxo ($p = 0.013$). After 24 h of incubation, *TIMP-1* gene expression was reduced both in H1-treated (0.63-fold) and Doxo-treated (0.65-fold) cells ($p = 0.0003$ and $p = 0.001$, respectively). After 48 h of treatment, *TIMP-1* expression was further reduced in H1-treated (0.748-fold) and H2-treated (0.628-fold) cells ($p = 0.001$).

Figure 3B shows *TIMP-2* expression levels in various subgroups. Compared to untreated cells, *TIMP-2* was significantly downregulated in both Doxo-treated (0.74-fold) and H1-treated (0.60-fold) cells after 6 h of incubation ($p = 0.003$ and $p = 0.002$, respectively). After 24 h of incubation, *TIMP-2* gene expression was further reduced both in H1-treated (0.48-fold) and Doxo-treated (0.62-fold) cells ($p < 0.001$). Incubation for 48 h downregulated *TIMP-2* expression both in H1-treated (0.61-fold, $p < 0.001$) and H2-treated (0.79-fold, $p = 0.013$) cells.

In contrast to the expression of *TIMP-1* and *TIMP-2*, the expression of *TIMP-4* was non-significantly ($p < 0.05$) changed (Fig. 3C) after 6 h treatment with H1, H2, and Doxo (1.20-, 1.24-, and 1.43-fold, respectively). However, 24 h of treatment with Doxo and H1 downregulated the expression of *TIMP-4* to 0.28- and 0.49-fold ($p = 0.001$ and $p = 0.019$), respectively. Incubation for 48 h downregulated *TIMP-4* expression both in H1-treated (0.064-fold, $p < 0.001$) and H2-treated (0.081-fold, $p < 0.001$) cells (Fig. 3C).

MMP genes

Treatment with H1 alone for 6 h significantly downregulated *MMP-9* gene expression levels (0.003-fold, $p < 0.001$). After 24 h of treatment with Doxo alone, *MMP-9* expression was reduced by 0.23-fold ($p = 0.01$). After 48 h of incubation, expression of *MMP-9* was

significantly reduced by 0.46- and 0.63-fold in response to H1 and H2 honey treatment ($p = 0.002$, $p = 0.013$), respectively (Fig. 4A).

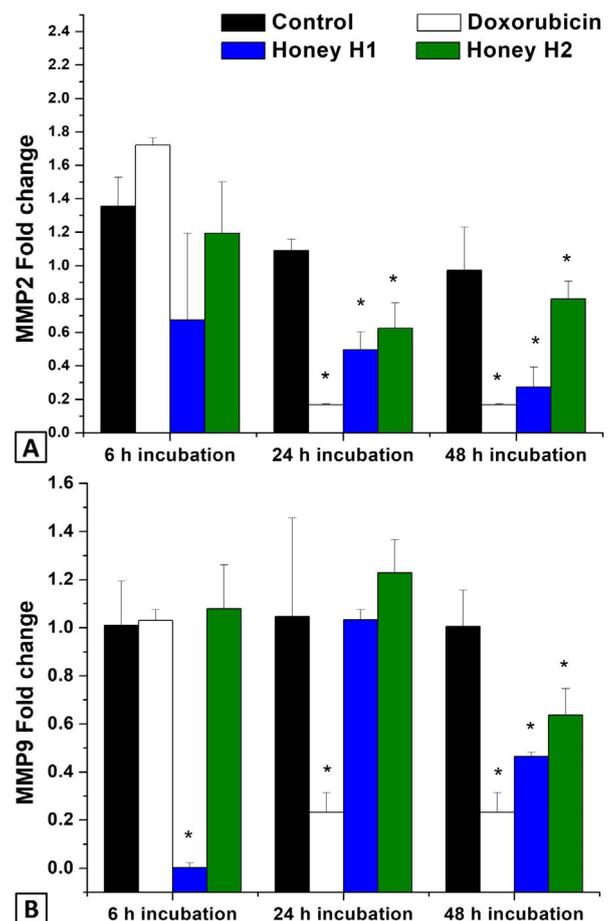


Fig. 4. Expression of *MMPs* in (H1, H2 and Doxo) treated and untreated breast cancer MDA-231 cells. A, *MMP-9*; B, *MMP-2* gene. Columns, mean ($n = 3$); SD. * $p \leq 0.05$, significantly compared to untreated control.

MMP-2 gene expression levels (Fig. 4B) showed no significant difference at 6 h post-treatment for all samples. A significant reduction in gene expression was observed after 24 and 48 h incubation with H1, which reached the lowest expression level of 0.27-fold at 48 h incubation ($p = 0.007$). In H2-treated cells, *MMP-2* expression was significantly reduced to 0.62-fold after 24 h treatment ($p = 0.015$). Doxo reduced the expression of *MMP-2* to 0.168-fold after 24 h incubation ($p = 0.001$).

DISCUSSION

Cancer is one of the most common cause of death worldwide and its prevention and treatment are challenging. TNBCs are the most aggressive type of breast cancer. Unlike the positive hormone receptor breast cancer tumors or HER-2/Neu amplified breast cancer tumors, TNBC does not respond to hormonal therapy or therapies targeting HER2 receptors. The present treatments for TNBCs are surgery, chemotherapy, and radiotherapy, which cause adverse side effects in the patient's body (Chavez *et al.*, 2010).

Therefore, a large number of scientific studies have focused on natural food-derived products with minimum side effects for treating breast cancer patients. Previous studies showed that honey contains several bioactive compounds and exerts various anticancer effects via diverse mechanisms affecting different stages of cancer development and progression (Erejuwa *et al.*, 2014). The mechanisms suggested for the anticancer effects of honey include its antiproliferative, apoptotic, antioxidant, antitumor necrosis factor, estrogenic and immunomodulatory activities, and anti-inflammatory effects (Ahmed and Othman, 2013a). However, the full mechanism remains unclear (Roy *et al.*, 2009). The antioxidant and anti-inflammatory action of honey is mainly related to its phenolic constituents (Ahmed and Othman, 2013b). The highest phenolic contents have been found in manuka honey and tualang honey, while the lowest identified phenolic contents were in 2 types of honey, acacia and gelam. The phenolic content of honey is affected by numerous factors, including the type of flower utilized as the source of honey along with geographical origin (Sergiel *et al.*, 2014). Honey of various floral resources may have different results. Thus, further intensive studies are required to provide a more comprehensive understanding of the positive impact of using honey for treating various types of cancer. Traditionally, Sidr honey has been clinically used to treat infected wounds, as medication in traditional medicine, and as an antibacterial agent. Sidr honey is mono-floral and is produced by feeding of bees from Sidr tree (*Ziziphus*

spina-christi) only. A toxicity study of Sidr honey revealed that it is safe to consume and there is no threat of toxicity at high concentrations. This type of honey possesses potent anti-inflammatory, antipyretic, and analgesic properties. These properties may originate from the phytochemicals present in the honey (Mijanur *et al.*, 2014). Wild honey is polyclonal, but is unique in that it is not subjected to heat processing before it is sold, leaving most bio-active compounds intact.

The present study investigated the effect of two varieties of honey (Sidr and Wild honey) on an MDA-MB-231 cell line (considered as TNBCs) on the cell morphology, proliferation, and MMPs and TIMPs gene expression.

We confirmed that treatment with H1 and H2 changed the cell morphology. The changes in H2- and H1-treated cells were most clearly observed at 48 h post-treatment. The cells had detached from the surface, floated, and tended to be round. Because rounding, shrinkage, membrane blebbing, and losing contact with adjacent cells are typical apoptotic features (Elmore, 2007); both the varieties of honey selected in the current study have the potential to induce apoptosis in TNBC cells after 48 h of treatment.

The results of the MTT assay emphasize the cytotoxic effect of H1 and H2 on the MDA-MB-231 cell line. Although less effective than Doxo in killing the cancer cells, both H1 and H2 showed considerable cytotoxicity after 48 h of treatment. Our results support those of previous studies showing a cytotoxicity effect against human breast cancer cell lines by Tualang (Fauzi *et al.*, 2011) and Thyme (Tsiapara *et al.*, 2009) honey. In contrast to these studies, Tsiapara *et al.* (2009) showed a stimulatory effect of fir honey on breast cancer cell line proliferation. This may be because different varieties of honey contain different nutrient (minerals, amino acids, and glucose), hydrogen peroxide, and phenolic constituent contents (van der Woude *et al.*, 2003). In the present study, H2 was found to be more cytotoxic than H1.

Another unclear aspect that may impact the results of *in vitro* studies is the effects of sugars on cell proliferation. Glucose is the favored nutrient of cancer cells. Sugar present in honey has been suggested to have mutagenic as well as antimutagenic effects. A review by Porcza *et al.* (2016) described the impact of honey on the viability of prostate, colon, breast, and various types of brain tumor cell lines. They demonstrated that only in some cell lines, the effect of honey could overcome the impact of sugars. This may be because of the phenolic content of honey and/or metabolism of sugars, as well as the number of glucose transporters expressed by cancer cells (Medina and Owen, 2002). In the present study, treatment with both H1 and H2 for 6-24 h increased the viability of MDA-MB-231

cells; however, prolonging the treatment to 48 h resulted in cytotoxic effects.

Matrix metalloproteinases are vital in the invasive and metastatic properties of cancer cells, and their expression (particularly MMP-2 and MMP-9) is promoted in a range of carcinomas (Deryugina and Quigley, 2006). MMP-9 is one of 70 genes in the Rosetta poor prognosis signature for breast cancer patients (van't Veer *et al.*, 2002). These statistics indicate that variances in MMPs expression can precede and increase the potential of developing breast cancer. The effect of honey on MMPs has not been studied extensively (Roy *et al.*, 2009). This is the first study to evaluate the effect of Sidr and Wild honey on gene expression of MMPs and TIMPs in MDA-MB-231 cell lines. We found that both varieties of honey could modulate the expression of *MMP-9* and *MMP-2*.

Caffeic acid (CA) is a common phenolic compound found in various natural sources such as honey, fruits, herbs, and vegetables. Chung *et al.* (2004) established the mechanism of the anti-cancer effects of CA. They found that both CA and its derivative CA phenethyl ester inhibit the enzymatic action of MMP9.

TIMPs are essential anticancer molecules (Shimoda *et al.*, 2014). TIMP2 has evolved to exhibit broad inhibition effects towards many MMPs (Radisky and Radisky, 2015). Reports that correlate individual proteinases or inhibitors with the results in patients with breast cancer have shown conflicting results. For example, *in situ* hybridization revealed a positive relation between *TIMP-1* and *TIMP-2* expression and tumor grade (Porcza *et al.*, 2016). Another study showed that *MMP-1* mRNA expression was mainly correlated with the phase of invasion of breast tumors (Benson *et al.*, 2013). In contrast, this relationship has not been reported in most studies (Baker *et al.*, 2002). In many cases, increased expression of MMPs activity and increased expression of one or more TIMPs occurred at the same time. This is likely a defensive mechanism in the body, that is eventually overcome by the tumor (Brown, 1998). In the present study, although *TIMP-1* and *TIMP-2* were downregulated by both H1 and H2, *TIMP-4* expression was upregulated after 6 h treatment. Increasing evidence shows that TIMPs can modulate crucial pathways independent of metalloproteinase inhibition (Sun, 2010). These findings suggest that the mechanism of anticancer activity possessed by honey may occur through modulation of gene expression of MMPs and TIMPs.

The results of the present study suggest that honey has anticancer effects and is promising as an adjunct to conventional cancer therapy with minimum side effects. The anticancer activity of honey may be related to its ability to modulate the expression of MMPs and TIMPs. Additional studies are required to test of the effects of

using honey at the molecular level and improve our understanding of the positive effects of honey in relation to cancer therapy.

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Statement of conflicts of interest

The authors declare that they have no conflict of interests.

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