

ACACIA HONEY INDUCES APOPTOSIS IN HUMAN BREAST ADENOCARCINOMA CELL LINES (MCF-7)

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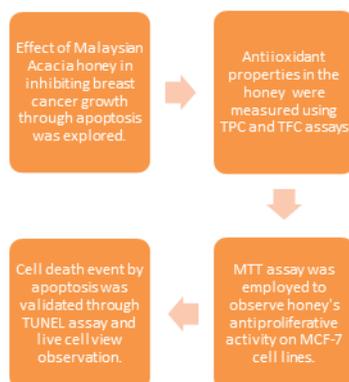
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Graphical abstract



Abstract

Apoptosis is one of markers considered in drug design including in treating cancers. Conventional treatments for cancer cause various side effects. One of possible alternatives is honey, an antioxidant driven by its phenolic and flavonoids content. This study aims to observe the effects of Malaysian Acacia honey in inhibiting breast cancer cell growth through apoptosis. Antioxidant properties of the honey were measured using total phenolic content (TPC) and total flavonoid content (TFC). Then, antiproliferative activity of the honey were observed using MTT assay. The honey exhibited IC_{50} at concentration of 5.5% (v/v). Further studies using TUNEL and live cell view revealed that Acacia honey induced apoptosis after 6 hours of treatment. Cell shrinkage, which is one of the apoptotic features, was observed as early as 2 hours, followed by the formation of apoptotic bodies within 6 hours of the honey treatment. Details of mechanism and actual compounds involved in displaying the results are being intensively studied.

Keywords: Acacia honey, antioxidant, apoptosis, TUNEL, live cell view

Abstrak

Apoptosis ialah salah satu penanda yang digunakan untuk mencipta sesuatu ubatan termasuk bagi rawatan kanser. Rawatan kanser yang sedia ada mempunyai beberapa kesan sampingan. Madu yang diketahui bersifat antioksidan dengan kehadiran fenol dan flavonoid antara bahan alternatif yang berpotensi. Penyelidikan ini dijalankan bagi memerhati kesan madu Akasia Malaysia dalam merencat pertumbuhan sel kanser payudara melalui apoptosis. Kandungan antioksidan madu diukur menerusi jumlah kandungan fenol (TPC) dan flavonoid (TFC). Menerusi asai MTT, madu Akasia diperhatikan mampu merencat pertumbuhan jujukan sel MCF-7 pada kepekatan IC_{50} 5.5% (v/v). Kajian lanjut menerusi asai TUNEL dan paparan sel secara langsung menunjukkan bahawa madu Akasia memulakan proses apoptosis seawal 6 jam selepas rawatan dimulakan, malah beberapa ciri awal seperti pengecutan sel dan pembetulan jasad apoptosis diperhatikan seawal 2 jam. Kajian lanjut sedang giat dijalankan bagi memahami mekanisme dan komponen penting yang meransang tindak balas ini.

Keywords: Madu Akasia, antioksidan, apoptosis, TUNEL, paparan sel secara langsung

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1.0 INTRODUCTION

Apoptosis is a programmed cell death, essential in controlling cell growth and damaged cells. Besides apoptosis, necrosis is another cell death mechanism. However, necrosis results in cell burst with the released of intracellular contents which will affect the surrounding cells [1]. In contrast, apoptosis occurs in our body to eliminate unnecessary cells with unnoticeable and impairment effects [1]. Basis of apoptosis is applied in many drug designs to maximize the effect (for example to kill cancer cells) but with low or none adverse effects commonly appeared due to necrosis [1-3].

Apoptosis is among foundation principle in drug design for treating cancer. Cancer is an abnormal cell growth and mainly caused by genetic disorders, be specifically by oncogenes and related to down regulation of apoptosis. Current cancer drugs for instance tamoxifen for breast cancer and 5-fluorouracil (5-FU) for colon cancer induce various side effects including steatosis, steatohepatitis, and hepatic sinusoidal injury [4]. Thus, one of the approaches to treat the cancer is by investigating alternative treatment using natural product including honey.

Honey is one of bee products and known as a natural sweetener with high antioxidant properties and capacity with other medical potencies [5]. There are many types of honey based on species of bees, floral sources, geography, climates, and technologies. In Malaysia, various types of honey are available such as Tualang, Gelam, pineapple, Acacia and honey harvested from different species of native stingless bees [6]. In this study, Acacia honey was selected to observe their effects on breast cancer, the highest incidence and prevalence cancer among Malaysian women.

Acacia honey is the most commercially available honey in Malaysia and was reported to contain high antioxidant properties [7, 8]. Interestingly, the antioxidants were reported to inhibit several cancer cell growths [9,10]. However, effect of Acacia honey towards breast cancer is still unknown and yet to be discovered in this study.

Basically, antioxidant acts by neutralising and/or scavenging excess free radicals, thus inhibiting cancer development [11]. Honey is hypothesised to induce apoptosis in breast cancer cells and/by neutralising free radicals through its antioxidant properties. Mechanism of apoptosis can be further observed using microscopic techniques including terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and live cell view [1,12]. Both approaches were implemented in the study to observe effects of Acacia honey on breast cancer cell lines (MCF-7).

2.0 METHODOLOGY

2.1 Cell Culture

Human breast adenocarcinoma (HTB-22) was purchased from American Type Culture Collection (ATCC) and propagated in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and gentamicin. The cells were maintained at 37°C in 5% CO₂ humidified atmosphere [13].

2.2 Honey Preparation

Acacia honey was harvested from a bee farm in Batu Pahat, Johor, Malaysia. The honey was stored in the dark at 4°C. Upon treatment, the honey was freshly prepared by dissolving it in culture medium and filter-sterilised with a 0.2µm syringe filter.

2.3 Determination of Total Phenolic Content

Quantification of total phenolic content (TPC) was determined by Folin-Ciocalteu's phenol reagent as described by Ramli *et al.*, (2011) [14]. Briefly, 80µL of honey solution was added into 96-well microplate followed by 100µL of 15% Folin-Ciocalteu solution. Distilled water was added to adjust the volume to 200µL. The mixture was then incubated 5 minutes at room temperature before addition of 100µL of Na₂CO₃ aqueous (0.105g/mL). The absorbance was measured at 756nm after incubation at 30°C for 60 minutes. A series of concentration of gallic acid (0.03-1 mg/mL) were used to prepare the standard graph. The result obtained was measured as gallic acid equivalent/1mg gallic acid (GAE/1mg).

2.4 Determination of Total Flavonoid Content

Quantification of total flavonoid content (TFC) was determined according to the colorimetric assay phenol reagent as described by Khalil *et al.*, (2012) with slight modifications [15]. Briefly, 25 µL of honey solution was added into 96-well microplate, followed by distilled water to adjust the volume to 80µL. 30µL of NaNO₂ aqueous (1.25% w/v) was then added followed with 5 minutes incubation at room temperature. Then, 30µL of AlCl₃ aqueous (2.5% w/v) was added and incubated at room temperature for 6 minutes. Finally, 50µL of NaOH (1M) was added followed by 60µL of distilled water. The absorbance was measured at 510nm. A series concentration of catechin (0.03-1 mg/mL) were used to prepare the standard graph. The result was measured as catechin equivalent/1mg catechin (CE/1mg).

2.5 MTT Assay

Antiproliferative activity of the honey was assessed with non-radioactive cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA) assay. Briefly, cells were seeded in each 96-well plate and incubated for 24 hours. Then, the cells were treated with series of honey concentrations [3.125%, 6.25%, 12.5%, 25%, 50%, 100% (v/v)] for 24, 48, and 72 hours. 50 μ L MTT solution was then added and incubated for 4 hours. Produced formazan was then solubilised with dimethyl sulfoxide and determined by measuring the absorbance at 520nm with a micro titer plate reader (VersaMax, Molecular Device, USA).

2.6 TUNEL Assay

DNA fragmentation (an apoptotic feature) of treated cells with Acacia honey was analysed using TUNEL assay (Promega, USA). Briefly, samples were fixed with 4% formaldehyde, rinsed with phosphate buffered saline (PBS) twice, and permeabilised with 0.2% Triton-X. Samples were then rinsed again with PBS and equilibrated with 100 μ L equilibration buffer. 50 μ L of rTdT reaction mix was then added before incubation for 60 minutes in 37 °C humidified atmosphere. Reaction was stopped by immersing samples in 2X SSC buffer for 15 minutes followed by washing with PBS. Finally, samples were mounted and viewed under confocal laser scanning microscope (TSC SPE, Leica, Germany).

2.7 Live Cell View

Live cell imaging was observed using confocal laser scanning microscope (TSC SPE, Leica, Germany). The treated cells were initially plated in a fluorodish and placed under a confocal microscope (37 °C, 5% CO₂). Then, images were acquired every 30 minutes for 72 hours.

2.8 Statistical Analysis

All parameters in this study, except TPC and TFC were measured in triplicate. Meanwhile, TPC and TFC assays were conducted duplicate. Results were expressed as mean \pm standard error mean (SEM). The differences between mean data value obtained in this study were determined using the Student's *t* test ($p < 0.05$).

3.0 RESULTS AND DISCUSSION

The values of TPC and TFC for Malaysian Acacia honey used in this study were 79.08mg GAE/1mg and 20.98mg CE/1mg respectively. TPC value was higher than TFC because phenolic acids have been

identified as the major component of antioxidant in honey [16]. The TPC value was higher compared to measurement made on Acacia honey originated from Slovenia [17]. In comparison to other Malaysian honey, the value was slightly lower compared to Tualang honey (83.96mg GAE/100g) but quite comparable with Gelam honey (74.12mg GAE/100g) [18].

In general, various types of honey contain approximately 56 to 500 mg/kg of total polyphenols as their major antioxidant properties [5]. However, the level detected in many Malaysian honeys was below than 100 mg/kg depending on seasons [19]. Thus, value of the antioxidant properties in Acacia honey in this study was above average when compared to other types of Malaysian honey.

Besides displaying potent antioxidant, polyphenols also have the capabilities to exert multiple biological activities such as proliferation and apoptosis [20]. For cancer cells, large but tolerable amount of reactive oxygen species (ROS) is often found. Intriguingly, polyphenols commonly known able to scavenging the excess ROS, suppress oxidative stress-responsive genes prior to inhibition of cell cancer proliferation [20].

A few studies demonstrated the similar behaviour of polyphenols found in honey. Aljadi *et al.*, (2004) revealed a direct correlation between antioxidant properties of Malaysian honey (Gelam and coconut honey) with scavenging effect [7]. Another study demonstrated a mild toxicity of phenolics extract of Gelam honey on murine fibrosarcoma cell lines [21].

Through MTT assay, Acacia honey inhibited MCF-7 cell line growth with low IC₅₀ level (Table 1). IC₅₀ is an inhibitory concentration some substances required to inhibit 50% of cell growth of their cell population [22]. Acacia honey displayed time-dependent inhibition effects, which cells treated for 72 hours showed the lowest IC₅₀ value (Table 1).

Table 1 IC₅₀ value of MCF-7 cell lines treated with Acacia honey

Time	IC ₅₀ Value. % (v/v)
24 hours	13.09 \pm 2.43
48 hours	7.06 \pm 1.70
72 hours	5.49 \pm 1.27

To further understand and confirm the inhibition effect either it is caused by apoptosis or necrosis, microscopic observation through TUNEL assay and live cell view imaging was conducted on the cell treated with the honey. The IC₅₀ value from MTT assay at 72 hours (5.5% v/v) was set as treatment concentration for both assay. The concentration is an optimal concentration since lower concentration would not exhibit satisfactory result and higher concentration would leads to artefactual positive result [23].

Only few apoptotic cells were observed in negative control (untreated MCF-7 cell lines) since it is also a physiological process. It was confirmed by red-stained colour in the assay; indicated viable cells (Figure 1). Meanwhile, cells treated with Acacia honey for 24, 48 and 72 hours appeared in the green-stained colour, indicating cells undergone apoptosis (Figure 2).

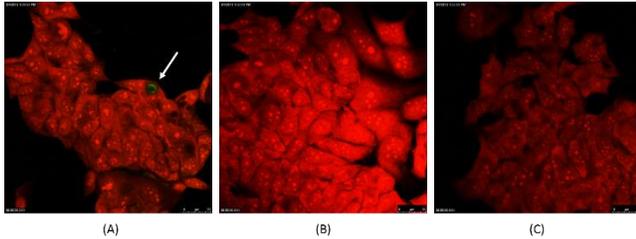


Figure 1 Negative control MCF-7 cell lines for 24 hours (A), 48 hours (B), and 72 hours (C) observation. Apoptosis (Arrow) was observed within 24 hours, however less than viable cells

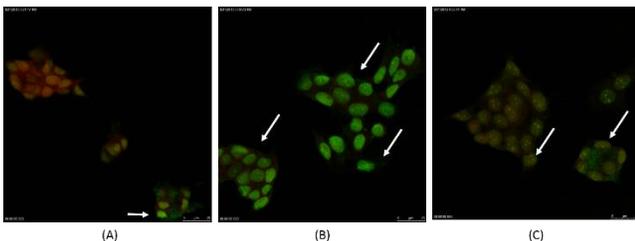


Figure 2 MCF-7 cell lines treated with Acacia honey (5.5%) for 24 hours (A), 48 hours (B) and 72 hours (C). apoptotic cells, green-stained (Arrow) color appeared and observed

The TUNEL results are in parallel with the MTT assay, which apoptotic cells are visually observed increased

over the time. TUNEL assay is directly tagged at fragmented DNA of cell lines at the 3' end of DNA strand. The fragmentation is among the properties of cells undergoing apoptosis [2]. Through the staining method, apoptotic and non-apoptotic cells can be directly distinguished via colour; apoptotic cells are stained green, while viable cells are stained red, as similar observed in this study.

To identify the actual time of apoptosis occurred in the breast cancer cell lines after additional of Acacia honey, live cell view was conducted using a confocal microscope. No apoptotic event was observed in the negative control. The cells observed actively propagate and growing from 0 to 24 hours of culture (Figure 3). In contrast, in the treated cells, apoptosis occurred after 6 hours of the treatment (Figure 4). The process was continued up to 24 hours onwards.

Since apoptosis involves several series of process, the results were predictable and consistent over the treatment period (Figure 4). After treated with 5.5 % v/v of Acacia honey, the breast cancer cell lines were start shrinking slowly as early as 2 hours [Figure 4(B)], dissymmetrical of cell membrane after 4 hours [Figure 4(C)], and formation of apoptotic bodies and condensed DNA after 6 hours and up to 72 hours [Figure 4 (D-I)]. The live cell view procedure was conducted up to 72 hours according to the cell cycle. After 72 hours, the cells were undergone to stationary phase [24]. The results were confirmed with the higher magnification of confocal microscope [Figure 5 (A-F)].

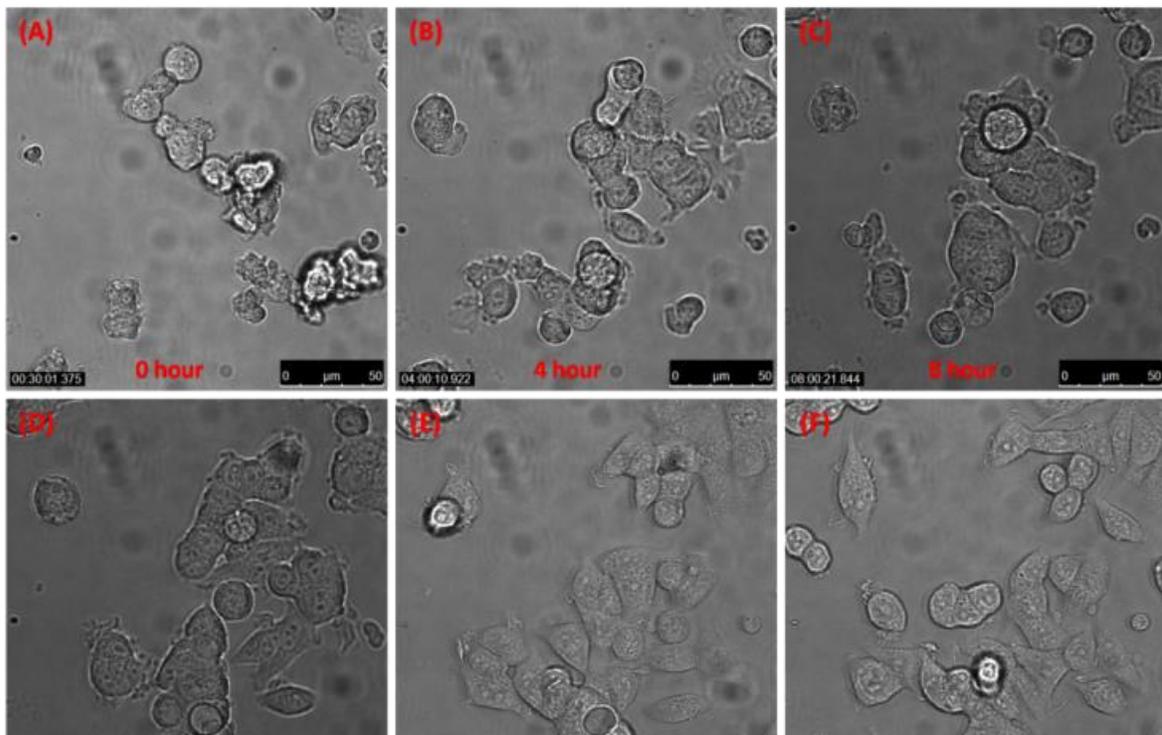


Figure 3 Live cell view observation (0 to 24 hours) on negative control (Untreated MCF-7 cell lines) using a confocal microscope. Negative control observed at 0 hours (A). The cells rapidly propagated started 4 hours (B) up to 24 hours (F)

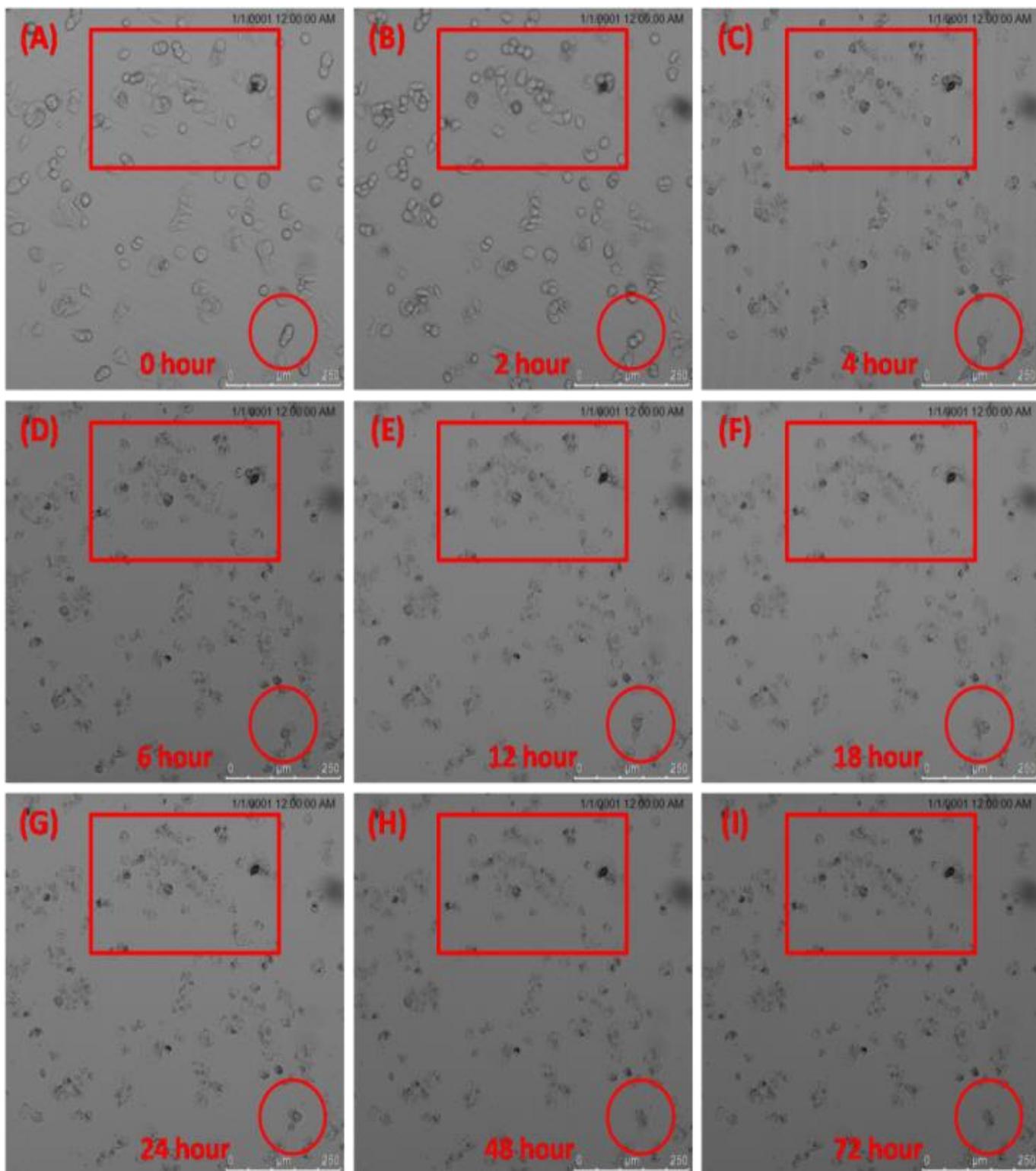


Figure 4 Low magnification observation on MCF-7 cell lines incubated with 5.5% (v/v) Acacia honey solution for 72 hours. The effect was observed in the square and circle parts as early as 2 hours when the cell lines started shrinking slowly (B), dissymmetrical of cell membrane observed after 4 hours (C) and formation of apoptotic bodies and condensed nucleus after 6 hours onwards

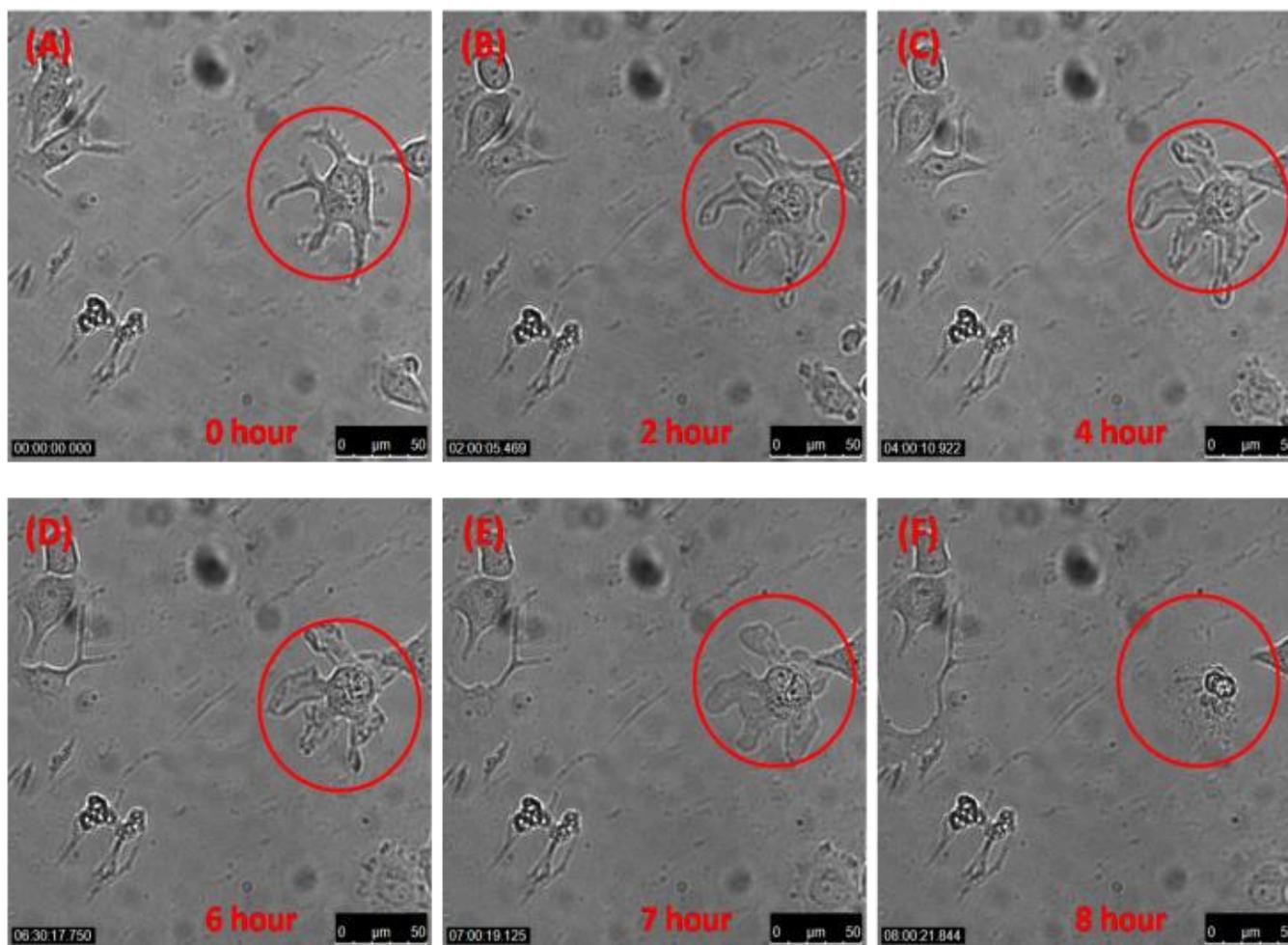


Figure 5 Higher magnification observation on MCF-7 cell lines incubated with 5.5% (v/v) Acacia honey solution for 8 hours. The effect was observed in the circle parts as early as 2 hours when the cell lines start shrinking slowly (B-E), and totally degraded at 8 hours

Above findings revealed that Acacia honey is able to kill breast cancer cells via inhibiting the cell growth. Through TUNEL and live cell view imaging, the mechanism appears through apoptosis rather than necrosis. The finding is parallel to other studies. Previous studies conducted on acacia honey Pakistan-origin by M. Aliyu *et al.*, (2012) on human prostate cancer cell lines (PC-3) with IC_{50} of 1.9% v/v also proved induction of apoptosis through modulation of $TNF-\alpha$, a key factor in cancer survivor [25]. The study also concluded cell cycle arrest was induced at G0/G1 phase [25]. The different IC_{50} indicated variety of honey quality based on geographical origin.

Honey also exhibits different dose based on cell lines, for example Tualang honey, a Malaysian honey was reported induced apoptotic effect on MCF-7 cancer cell lines through the disruption of mitochondrial membrane at IC_{50} concentration of 5% [9]. Same research group later reported apoptotic

effect on acute myeloid leukemia (K-562) and chronic myeloid leukemia (MV4-11) at IC_{50} concentration of 0.2% and 1.2% respectively [26].

Another extensively studied Malaysian honey, Gelam honey also confirmed the anti-proliferative effect by inducing DNA damage and apoptosis on colon cancer cells (HCT119), together with Pineapple honey [27]. Another study also on Gelam honey conveyed the ability of the honey to downregulated Kirsten rat sarcoma viral oncogene homolog (KRAS), extracellular signal-related kinase (ERK) and Akt genes [28] towards colorectal cancer cell lines (HT29) at 40-100mg/mL. The expression of caspase-9, a proapoptotic gene and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha ($I\kappa B\alpha$) gene, an inhibitor for nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) also upregulated [28].

In comparison to other foreign honey, Manuka honey induced apoptotic through activation of a

caspase-9-dependent apoptotic pathway at quite lower IC₅₀ concentration of 0.6% w/v in MCF-7, murine melanoma (B16.F1) and mouse colon carcinoma (CT26) [29]. Similarly, in P. Morales and A. Haza (2013) study, three types of Spanish honeys (heather, rosemary and polyfloral) induced apoptosis at concentration of 5% through a ROS-independent pathway on human peripheral blood promyelocytic leukemia cells (HL-60) [30]. Meanwhile, assessment of Astragalus honey towards human hepatic carcinoma cell lines (HepG2) and human bladder carcinoma (5637) reduced the expression of anti-apoptotic gene, Bcl-2 with IC₅₀ of 2.4% and 1.9% w/v respectively [31].

Major components in honey contribute to the effects were postulated through its antioxidant properties particularly polyphenol and flavonoids. Both the antioxidant properties were reported high in the honey [7, 8]. They act as a scavenger agent to neutralise free radical (normally high in the condition of cancer) and simultaneously induce apoptosis to the cancer cells [20]. The exact mechanism of the apoptosis, either through extrinsic or intrinsic pathway is required for further investigations.

4.0 CONCLUSION

Acacia honey inhibits MCF-7 cancer cell growth with low IC₅₀ value. Apoptosis appears to contribute to the cell inhibition as early as 6 hours after treated with Acacia honey (5.5% v/v). Results from TUNEL and live cell view assays are parallel to the results obtained from the MTT assay. Antioxidant properties in the honey may play the role. Details of the mechanism are projected to be studied.

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