Tualang honey inhibits cell proliferation and promotes apoptosis of human lung adenocarcinoma cells via apoptosis signaling pathway

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ABSTRACT

Introduction: Tualang honey (TH) has been shown to have anticancer properties for breast, cervical, leukaemia, oral squamous, and bone cancers. This study was aimed at investigating the anti-proliferative and apoptosis effect of TH on human lung adenocarcinoma (ADC) cells.

Methods: MTT assay was used to detect the anti-proliferative effect of TH in ADC (H23 and A549) cells. Morphological changes of cells treated with TH were observed under phase-contrast and fluorescence microscopy. Flow cytometry was carried out to evaluate cell cycle progression. Annexin V-FITC assay was performed to investigate the effects of TH on the apoptosis of ADC cells. Apoptosis protein profile was detected using Human Apoptosis Antibody Array.

Results: MTT assay indicated that TH inhibited the proliferation of ADC cells in a dose- and time-dependent manner. The ADC cells treated with TH showed typical apoptotic features such as membrane blebbing, cell shrinkage, chromatin condensation and fragmented nucleus. The cell cycle analysis revealed the accumulation of TH treated ADC cells in sub-G1 and G2/M phases. Annexin V-FITC assay provided the evidence for apoptosis induction by TH. Apoptosis profiling array indicated that TH up-regulated the expression of caspases (caspase-3 and -8), pro-apoptotic (Bid, Bax, cytochrome c, SMAC), cell-surface death receptor (Fas), cell cycle regulator (p21) and tumor suppressor (p53), as well as down-regulating the expression of 2 anti-apoptotic proteins (Bcl-2 and Bcl-w) in ADC cells.

Conclusion: The present study suggests TH as a potential anti-cancer agent, following its capacity to promote the growth inhibition and apoptosis of ADC cells via apoptosis signaling pathways.

1. Introduction

Honey is the collection of nectar from many plants and processed by honey bees. Ancient civilizations and different cultures have consumed honey for its nutritional value and healing properties [1]. It is mainly composed of supersaturated solution of sugar, proteins, enzymes, amino acids, organic acids, lipids, vitamins, volatile chemicals, phenolic acids, flavonoids, carotenoids and minerals [2–7].

Tualang honey (TH) is a multiflora rainforest honey originating from Malaysia and is produced by the wild giant bees (Apis dorsata) that build their hives on the Tualang tree (Koompassia excelsa) [3,8]. The Tualang tree is one of the tallest trees in the world with a maximum height of 250 feet [9,10]. Interestingly, Koompassia excelsa trees and Apis dorsata can be found in tropical rainforest north-eastern region in the state of Kedah, Malaysia [10,11]. Each honeycomb in the beehive can span 2–3 meters in size with up to 450 kg of honey that can be collected from one tree [9]. TH has more phenolic and flavonoid content compared to other types of honey such as Gelam (Malaysia), Manuka, Pineapple and Indiana forest honey [12,13]. TH has been shown to exhibit anti-proliferative or antitumor activity besides capable of inducing programmed cell death (apoptosis) in several types of cancer cells such as oral squamous cell carcinoma, human osteosarcoma cell lines, human breast cancer cells (MCF-7) and cervical cancer cell lines [1,14,15].

Lung cancer is a major cause of mortality and morbidity among men and women worldwide, including in many Asian countries [16,17]. Lung adenocarcinoma (ADC) is the most common subtype of this lethal disease that accounts for 40–45% of total cases. Therapeutic
approaches to lung cancer such as chemotherapy, radiotherapy and surgery, besides incurring prohibitive costs, often offering limited survival benefits due to severe toxicity and development of resistance towards chemotherapy drug particularly ADC [18].

To date, there are currently no reports showing the anti-cancer effect of TH on ADC. Thus, the present study attempts to investigate the effects of TH on cell proliferation and their involvement in molecular mechanisms of cell death in the human ADC cells.

2. Materials and methods

2.1. Honey sample

TH was purchased from the Federal Agricultural Marketing Authority (FAMA), under the Ministry of Agriculture and Agro-Based Industry, Malaysia (product code number: 110013). TH was stored in the dark at room temperature. Upon treatment, TH was freshly prepared by dissolving it in culture medium and filter-sterilized with a 0.2 μm syringe filter unit (Sartorius Stedim, Germany).

2.2. Cell culture

ADC cell lines (H23 and A549) and human epithelial lung cell line (NL20) were purchased from American Type Culture Collection (ATCC, USA). H23, A549 and NL20 cells were propagated in RPMI-1640 (Biowest, Nuaille, France). Culture media were supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Biowest, Nuaille, France). Culture incubation was carried out at 37°C with 5% CO2 and 95% humidity. NL20 cells were used as control. In all experiments, the cells without TH were represented as the untreated cells.

2.3. MTT assay

ADC cell lines were treated with 100 μL freshly prepared and filter-sterilized TH (1.25–10% v/v in serum-free culture medium) for 24 and 48 h, respectively. Cell proliferation was determined by MTT assay. IC50 was defined as the inhibitory concentration at which 50% of the cells showed an inhibition of growth. The IC50 values were calculated from curves constructed by plotting the percentage of cell viability (%) versus concentration of TH. IC50 value was used for subsequent experiments.

2.4. Morphological analysis of TH treated cells under light and fluorescence microscopy

ADC cells were seeded and treated with various concentrations for 24 and 48 h. For light microscopy analysis, the cells were observed using an inverted phase contrast microscope (ECLIPSE TI-S, Nikon) at 200X magnification. For fluorescence microscopy analysis, 500 μL of Hoechst 33342 (Calbiochem, Germany) solution (10 μg/mL) and propidium iodide (PI) solution (2.5 μg/mL) (Sigma-Aldrich, USA) were added. The plates were kept in the dark for 15 min at RT and morphological changes were inspected using ECLIPSE TI-S (Nikon) at 100X magnification.

2.5. Molecular mechanisms of TH on ADC cell lines

2.5.1. Cell cycle analysis

Fluorescence-activated cell sorting analysis was performed on ethanol-fixed cells stained with PI/RNase (BD Pharmingen, San Jose, USA) using ACCURI C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA). A total of 20,000 events in each sample were acquired. Using ModFit LT software (version 4.0), the percentages of cells at different phases of the cell cycle were determined.

2.5.2. Annexin V-FITC flow cytometry assay

Annexin V-FITC Apoptosis Detection Kit (eBioscience, San Diego, USA) was used to evaluate the apoptosis-inducing activity of TH in cancer cells according to manufacturer’s protocol. The cells were analyzed using BD FACsCANTO II system (Becton Dickinson, USA).

2.5.3. Profiling of apoptosis-related proteins

The expression level of apoptosis-related proteins was determined using Human Apoptosis Antibody Array G-Series (RayBiotech, Inc) according to manufacturer’s instruction. Cancer cells treated with IC50 dose of TH and 500 μg/mL of protein from each cell were incubated with the human apoptosis array. The array slide was scanned using Agilent G2565CA Microarray Scanner System in the Cy3 channel. Spot signal intensity was analyzed using Image Studio® Lite software (ver. 5.2). Background corrections and normalization were carried out using the RayBio® Analysis Tool.

2.6. Statistical analysis

All statistical analyses were carried out using SPSS, version 24.0. All data were expressed as means ± SD. The significance of the data was evaluated using the Student’s t-test. The observation is considered statistically significant if the p-value is less than 0.05 (p < 0.05).

3. Results

3.1. MTT cell proliferation assay

MTT assay was utilized to evaluate the anti-proliferative effect of TH against ADC cell lines (H23 and A549). Results were expressed as 50% inhibitory concentration for 24 and 48 h treatments (Table 1). It was observed that TH had significantly inhibited (p < 0.05) the proliferation of H23 and A549 cells in a dose- and time-dependent manner (Fig. 1).

3.2. Effects of TH on cancer cells morphology

The apoptogenic effect of TH was also investigated by observing morphological changes induced by TH on H23 and A549 cells. After 24 and 48 h treatments, the untreated cells exhibited normal morphology. Most of the cells were adhering to the wells even after 48 h of treatment. In contrast, the ADC cells treated with TH revealed apoptotic morphological features such as cell shrinkage, membrane blebbing, nuclear condensation, apoptotic bodies, blisters and echinoid spikes (Fig. 2). These changes appeared as early as 24 h and became progressively more pronounced after 48 h. Increased concentrations of TH contributed to a significant decrease in number of the viable cells. At high TH concentrations (7.5% and 10% v/v), the number of viable cells was noted to decrease in a time-dependent manner.

Fluorescence microscopy analysis was carried out using Hoechst 33342 and PI to discriminate apoptotic and necrotic cells. Results showed that untreated cancer cells (24 and 48 h) maintained intact cell morphology; the cells were oval-shaped with intact pale blue chromatin, indicating that the cells were alive (Fig. 3). In contrast, H23 and A549 cells revealed apoptosis (Fig. 4).

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incubation time</th>
<th>IC50 (% v/v)</th>
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<tbody>
<tr>
<td>H23</td>
<td>24 h</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>3.7 ± 0.2*</td>
</tr>
<tr>
<td>A549</td>
<td>24 h</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>2.2 ± 0.0*</td>
</tr>
</tbody>
</table>

The results are shown as mean of three independent experiments. *p < 0.05 compared with 24 h treatment.
A549 cells treated with TH for 24 and 48h showed dose- and time-dependent detrimental morphological changes. The TH-treated cells exhibited apoptotic features (nuclear shrinkage, nuclear fragmentation, apoptotic body formation and decrement of cell number) and emitted a bright blue fluorescence, which can be characterized as live cells with apoptotic nuclei. At higher concentrations of TH, a great number of cancer cells with pink chromatin was observed. The TH-treated cancer cells with normally shaped nuclei but emitted pink fluorescent signal were either necrotic cells, or they were at the late stage of apoptosis. Meanwhile, the cells that emitted bright pink fluorescence signal with fragmented and shrunken nuclei were dead cells with apoptotic nuclei.

3.3. Molecular mechanisms of TH on lung cancer cells

3.3.1. Cell cycle analysis

Flow cytometry assay was employed to examine the impact of TH on cell cycle progression. H23 and A549 cells treated with TH showed significant increase in percentage of apoptotic cells at sub-G1 phase, which were 35.52 ± 4.07 % and 35.96 ± 2.74 %, respectively (Fig. 4A). H23 cells treated with TH showed a significant reduction in percentage of cells at G0/G1 and S phase, which consequently led to a significant increase in percentage of cells at G2/M phase (Fig. 4B). Meanwhile, A549 cells treated with TH showed a significant reduction in the percentage of cells in G0/G1 phase (untreated; 55.26 ± 0.47 % and TH; 45.19 ± 0.47 %), while the percentage of cells in S and G2/M phases significantly (p < 0.05) increased compared to untreated cells (Fig. 4C).

3.3.2. Apoptosis-inducing activity of TH in H23 and A549 cells

Apoptosis-inducing activity was determined using flow cytometry assay (Annexin V-FITC). As shown in Fig. 5, untreated H23 and A549 cells exhibited the highest percentage of viable cells, which were 99.7 % and 99.9 %, respectively. Late and early apoptotic cells were almost absent in the untreated cells. In contrast, both cancer cells treated with TH demonstrated early and late apoptosis. In H23 and A549 cells treated with TH, 10.5 ± 4.6 % and 6.9 ± 3.4 % of the cells were in early apoptotic phase, respectively. H23 and A549 cells treated with TH also showed high percentages of late apoptotic cells, which were 28.4 ± 10.0 % and 15.8 ± 12.2 %, respectively.

3.3.3. Profiling of apoptosis-related proteins expression

The antibody array used in the present measured the expression of apoptosis-related proteins in H23 and A549 cells after TH treatment. The array is capable of detecting the expression of 11 apoptosis-related proteins (Fig. 6). TH up-regulated the expression of caspases (caspase-3 and -8), pro-apoptotic (Bid, Bax, cytochrome c, SMAC), cell-surface death receptor (Fas), cyclin-dependent kinase inhibitor 1 (p21) and tumor suppressor (p53) in H23 cells. Meanwhile, TH-A549 treated cells showed the expression of caspase-3, p21, p53 and Fas were significantly (p < 0.05) up-regulated. In contrast, Bcl-2 and Bcl-w were significantly (p < 0.05) down-regulated in ADC treated cells.

4. Discussion

Honey is a natural product produced by honey bee and has been used as food as well as traditional medicine since ancient times [19]. In this study, TH was observed to exert cytotoxic effect on H23 and A549 cells in a time- and dose-dependent manner. This observation was similar with that of previous studies, which reported anti-proliferative activity of TH against other types of cancer cells including oral squamous cell carcinoma, human osteosarcoma, human breast cancer (MCF-7) and cervical cancer cell lines [1,14].

In this study, the cancer cells treated with TH demonstrated apoptotic morphological features, which include cell shrinkage, chromatin condensation, membrane blebbing and apoptotic bodies (Fig. 2). Moreover, the presence of apoptotic bodies and nuclear fragmentation in TH treated cells was detected (Fig. 3). The presence of cell shrinkage and chromatin condensation can be characterized as an early apoptosis [20,21]. Additionally, cells that are at late apoptotic stage demonstrate nuclear DNA fragmentation [22-24].

TH treatment in this study was observed to significantly increase the percentage of ADC cells in G2/M phase, which suggests its ability to induce G2/M arrest. This activity was also observed in TH treated MCF-7 cells [25]. The G2/M arrest could have been induced by DNA damage, which may have been triggered through p53-dependent and/or p53-independent pathways [26,27]. A report stated that an arrest in G2/M phase was associated with enhanced apoptosis and cytotoxicity [28]. In the prostate cancer cell line DU145, silibinin was reported to enhance doxorubicin cytotoxicity by increasing the percentage of cancer cells arrested at G2/M phase and apoptotic cell death. For this reason, Tyagi et al. suggested that the G2/M phase arrest can be an effective target point of controlling cancer cells proliferation [29]. Besides, TH treatment displayed a significant increase in the percentage of sub-G1 phase population in cancer cells, which indicated that apoptosis induced-DNA fragmentation had taken place [30]. DNA fragmentation occurs in the later stage of apoptosis event [31]. Similarly, in this study, the induction of apoptosis by TH in the cancer cells was confirmed by Annexin-V FITC assay. The result of Annexin-V FITC assay showed that TH treatment increase the percentage of late apoptotic ADC cells.

The result of apoptosis antibody array showed that p21 and p53 proteins were up-regulated in TH-treated cancer cells. p21 (cyclin-dependent kinase inhibitor 1) is cell cycle regulator which is activated by tumor suppressor p53 protein. These two proteins played vital roles in preventing tumor development, by inhibiting cell proliferation and...
inducing cell cycle arrest at G2/M phase [32–34]. Furthermore, the up-regulation of p21 and p53 protein expression with G2/M phase arrest in breast cancer cells treated with TH has been reported by Fauzi and Yaacob [25]. Taken together, this study suggested that TH inhibits the proliferation of human ADC cells by inducing cell cycle arrest at G2/M phase via p53 and p21 signaling.

Disruption of apoptosis signaling pathway has implicated the tumor initiation, development and metastasis [35]. There are two major pathways of apoptosis, which include intrinsic (mitochondrial) and extrinsic (death receptor) pathways [31]. Activating the extrinsic pathway of apoptosis requires the binding of extracellular ligands to cell-surface death receptors (tumor necrosis factor-alpha (TNF-alpha)/TNFR1 and Fas-ligand (FasL)/Fas receptor). The binding of death ligand to its receptor facilitates the formation of a large protein complex at the cell membrane known as death-inducing surface complex (DISC), which recruits and activates caspase-8. Activated caspase-8 activates downstream effector caspases including caspase-3, -6 and -7, which in turn cleave several hundred caspase substrates and eventually result in apoptotic cell death [36–39].

Meanwhile, the intrinsic pathway involves mitochondria as the main site of apoptosis initiation. This pathway is initiated when there is mitochondrial outer membrane permeabilization (MOMP) [37,40]. The intrinsic pathway is modulated by Bcl-2 family of proteins comprising anti-apoptotic (Bcl-2, Bcl-xl, Bcl-w, A1 and Mcl-1) and pro-apoptotic (Bax, Bak, Bok, BID, Bad and BIM) members [37]. Pro-apoptotic Bcl-2 proteins induce MOMP causing the release of cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) and serine protease HtrA2/OMI (high-
temperature requirement protein A2) \[41,42\]. The cytochrome c released from mitochondria facilitates the formation of the apoptosome-containing adaptor Apaf-1 (apoptotic protease-activating factor 1) and activates caspase-9, which further activate caspase-3, -6 and -7 \[31,37,41,43\].

Collectively, the findings of this study indicated that TH treatment induced apoptosis in ADC cells by modulating the apoptosis-related proteins involved in intrinsic and extrinsic pathways. TH was observed to up-regulate the expression of Fas receptor and caspase-3 in ADC treated cells. The results were consistent with Fauzi et al., which reported that TH had induced apoptosis by activating caspase-3/7 activity in MCF-7 and MDA-MB-231 cells \[14\]. TH was observed to up-regulate the expression of caspase-8 in H23 cells. The activation of caspase-8 had also been reported in breast cancer cells treated with TH \[25\]. Besides, it was noted that TH treatment in H23 and A549 cells resulted in the up-regulation of four other apoptosis related proteins, which include Bax, BID, cytochrome c and SMAC. This is consistent with that of previous studies showing that the expression of cytochrome c and Bax protein were up-regulated in honey-treated cancer cells \[44–46\].

The apoptosis in cells can be inhibited by the intrinsic apoptosis regulator including Bcl-2 and Bcl-w \[47\]. The expression of Bcl-2 protein and Bcl-w was significantly down-regulated in ADC cells treated with TH. Meanwhile, oral supplementation of TH in a rat model of breast cancer was reported to induce down-regulation of Bcl-2 protein expression in the breast tissue of the animal model \[48,49\]. Down-regulation of Bcl-2 expression has been also reported in lung cancer cells NCI-H460 after its treatment with acacia honey, leading to cell growth inhibition and apoptosis induction \[50\].

Fig. 3. Morphological changes of H23 and A549 cells treated with TH and stained with Hoechst 33342/PI. Cells were treated with TH for 24 and 48 h and visualised under fluorescence microscopy at 100X magnification. Arrows indicate (1) viable cells with normal nuclei, (2) live cells with apoptotic nuclei, (3) dead cells with normal nuclei, (4) dead cells with apoptotic nuclei and (5) apoptotic bodies. (Scale = 50 μm).
This research, however, is subject to some limitations. The main limitation was related to the concentration of TH and time of treatment. This study was focused on the anticancer activity of TH at only one concentration (IC50) for 24h treatment. Further experiments should be performed to clarify the anticancer activity of TH on ADC at different concentrations and the time of exposure. Moreover, we examined the anticancer activity of TH on ADC by using whole TH itself as the rationale is that, this is how TH has been consumed traditionally, either eaten directly from the jar or mixed with water. Therefore, the active components of TH sample in this study should be characterized in future to investigate the possible component that responsible for its anticancer activity on ADC.

5. Conclusion

In conclusion, the present study suggested that TH as a promising potential anticancer agent, following its capability to promote the growth inhibition, cell cycle arrest and apoptosis of ADC cells. This was obtained via modulation of the apoptosis signaling pathway-related proteins.
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CRediT authorship contribution statement

Nazirah Amran: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. Wan Izlina Wan-Ibrahim: Writing - review & editing. Johari Mohd Ali: Writing - review & editing. Puteri Shafinaz Abdul-Rahman: Conceptualization, Methodology, Resources, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eujim.2020.101149.

References


