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Research paper

In-vitro cytotoxicity of *Trigona itama* honey against human lung adenocarcinoma epithelial cell line (A549)

Siti Norfitrah Mohd Salim[a], Logaraj Ramakreshnan[a], Chng Saun Fong[a], Ridhwan Abdul Wahab[b], Mohammad Syaiful Bahari Abdull Rasad[b,*]

[a] *Centre for Occupational and Environmental Health, Department of Social and Preventive Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

[b] *Department of Biomedical Science, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang Darul Makmur, Malaysia*

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ABSTRACT

Introduction: Many efforts have been made to identify natural alternatives to reduce the side effects of cytotoxic drugs in cancer treatment. With this in mind, the current study aimed to investigate the cytotoxicity effects of one of the multifloral Malaysian honey, Kelulut honey (*Trigona itama*), as a potential natural anticancer agent in stimulating apoptosis and cell cycle arrest to a human lung adenocarcinoma epithelial cell line (A549).

Methods: The cells were treated with various concentrations of *T. itama* honey for 24, 48 and 72 h. The cytotoxicity and cell viability were determined using trypan blue exclusion assay (TBEA) and flow cytometric analysis.

Results: The moisture content in the analysed honey was 14.3 ± 0.8%, which was within the accepted international standard. The pH, electrical conductivity and proline content were 3.17 ± 0.02, 0.47 mS/cm - 0.55 mS/cm and 19.1 mg/kg 20.2 mg/kg respectively. The findings demonstrated a significant dose and time-dependent inhibitory effect of *T. itama* honey with the maximum cytotoxic effects observed at 72 h with 20% concentration of *T. itama* honey, indicating 100% growth inhibition. Meanwhile, IC_{50} of *T. itama* honey treatment for A549 cells was determined as 0.62% v/v. Moreover, *T. itama* honey had a promising cytotoxic effect and proven capable of inducing cell cycle arrest at G2/M phase at 72 h of exposure with IC_{50} concentration.

Conclusion: This study provided prefatory evidence on *T. itama* honey's significant anticancer activity against human lung cancer cell lines.

1. Introduction

Cancer is considered as one of the deadly health threats profoundly affecting most populations worldwide [1]. According to World Health Organization (WHO), there will be more than 15 million new cases of cancer by 2020 which exemplifies it as a substantial global issue [2]. In particular, lung cancer is regarded as the leading malignancy in terms of incidence and mortality compared to other types of cancers [3,4]. The majority of cancers are due to genetic mutations as well as environmental and lifestyle factors, but some are also due to inherited genes [5]. Surgery, chemotherapy, radiation therapy, immunotherapy and hormone therapy are some of the contemporary treatment approaches in curing cancer [6,7]. Notwithstanding their efficiency towards a significant improvement of cancer survival rates, these treatment approaches resulted in a greater variety of treatment related complications and side-effects. Of note, these approaches are also ineffective towards late stage cancer diagnosis [7]. Specifically, chemotherapy has proven to be detrimental to the neighbouring normal cells in terms of gene mutation, DNA methylation and histone modification which also induce resistance to certain chemotherapeutic agents [8,9]. In addition, most of the drugs used as chemotherapeutic agents may lose their efficacy due to the development of drug resistance [10,11].

In respect of this, there has been several efforts of finding potential natural product sources that could function as alternative anti-cancer agents which are responsible in suppressing, delaying and reversing carcinogenesis [12,13]. Natural honey, as one of the natural products that has been extensively used in traditional medicine, demonstrated

* Corresponding author.
E-mail addresses: fitrah91@gmail.com (S.N.M. Salim), logarajramakreshnan@gmail.com (L. Ramakreshnan), fongcs92@gmail.com (C.S. Fong), ridhwan@iium.edu.my (R.A. Wahab), syaiful@iium.edu.my (M.S.B.A. Rasad).

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anti-tumour activity by inhibiting proliferation and enhancing the early death of various cancer cells [14]. Natural honey contains various compounds such as phenols, vitamins and antioxidants that induce apoptosis in various types of cancer cells via depolarization of mitochondrial membrane [15]. In a critical review of five clinical trials, Charalambous et al. discussed that the anti-inflammatory, antimicrobial and healing properties of honey enhance the positive effective management of oral mucositis for head and neck cancer patients undergoing radiotherapy [16]. Though limited, a number of studies substantially evaluated the anti-cancer potential of the natural multifloral honey in human cells [11,17]. For instance, Ghashm et al. [18] and Fauzi et al. [11] reported that Tualang honey (*Apis dorsata*) exhibits a significant anticancer activity against human oral, breast and cervical cancer cell lines. Even though these studies demonstrated the potential of Tualang honey towards anti-cancer activities, more empirical evidences are still needed to explore the potential and mechanism of anti-cancer activity of other types of multifloral honey in Malaysia.

With such consideration, this study was designed to explore the role of Kelulut honey (*T. itama*) as a potential natural anti-cancer agent. The general objective of this study was to investigate the cytotoxicity effects *of T. itama* honey as a potential natural anti-cancer agent in delivering stimulus on apoptosis and cell cycle arrest to human lung adenocarcinoma epithelial cell line (A549). To achieve this, the cell viability at different concentrations of *T. itama* honey was examined at 24, 48 and 72 h respectively in this study. Besides, this study also determined the inhibitory concentration dose (IC_{50}) of *T. itama* honey treatment which effectively inhibit 50% cells proliferation on A549 cells. At the same time, this study also determined the effect of *T. itama* honey treatment on apoptosis induction and cell cycle arrest via flow cytometer.

2. Materials and methods

2.1. Honey and cell samples

T. itama honey sample was acquired from the hives that had been cultivated at Integrated Centre of Research an Animal Care and Use (ICRACU) in International Islamic University Malaysia, Kuantan Campus from March to June 2015. Human lung adenocarcinoma epithelial cell line (A549, ATCC No. CCL-2) was obtained from American Type Cell Culture (ATCC). The cell line was of a monolayer cell type.

2.2. Chemicals and solvents

All chemicals used in this study were from Sigma–Aldrich (Chemie, Steinheim, Germany) and Merck (Darmstadt, Germany). All solvents used were either of analytical or chromatographic grade.

2.3. Physicochemical analysis

All of the physicochemical parameters of honey were determined in triplicate according to the Harmonized Methods of the International Honey Commission - IHC (2002) and the Association of Official Analytical Chemists – AOAC (1990). The major criteria of interest to define the physicochemical quality of *T. itama* honey in this study were moisture content, Hydroxymethyl furfural (HMF), electrical conductivity, pH value and proline content.

2.3.1. Moisture content of T.itama honey

The moisture content of *T. itama* honey was measured according to the modified Karl' Fischer titration method proposed by Fischer [19]. It was ensured that the moisture content in all the replicated honey samples was constantly less than 20% to commensurate with the recommended international standard for honey moisture content [20].

2.3.2. Hydroxymethyl furfural (HMF)

Hydroxymethyl furfural (HMF) was measured using the spectrophotometric method of White with slight modifications [21]. Five grams of honey was dissolved in 25 mL of water, transferred quantitatively into a 50 mL volumetric flask, added with 0.5 mL of Carrez solution I and 0.5 mL of Carrez solution II and the mixture was made up to 50 mL with water. The solution was filtered through a filter paper rejecting the first 10 mL of the filtrate. Aliquots of 5 mL was placed in two test tubes in which 5 mL of distilled water was added to one of the test tubes and 5 mL of 0.2% sodium bisulphite solution was added to the other one.

2.3.3. Electrical conductivity

Electrical conductivity of 20% (w/v) honey solution on a dry matter basis was measured using a conductivity meter (SevenMulti™ S47, Mettler Toledo, Switzerland), following Harmonised Methods of the International Honey Commission. Results were expressed as milliSiemens per centimetre (mS/cm).

2.3.4. pH value

pH is an important indicator during honey extraction and storage as it influences its texture, stability and shelf life. A pH meter (HI 98127, Hanna instruments, Mauritius) was used to measure the pH of a 10% (w/v) solution of honey prepared in milli-Q water (Millipore Corporation, Billerica, Massachusetts, USA).

2.3.5. Proline content

The proline content of a honey was measured using ninhydrin. When proline reacts with ninhydrin, it forms a colored complex which is then measured spectrometrically at 520 nm. Proline is the main free amino acid present in honeys produced by stingless bees, making it useful for the characterization of the botanical source as it is related to the floral source and the amount of pollen present in the honey.

2.4. Preparation of T. itama honey dilutions

T. itama honey samples were prepared using a serial dilution method. The honey was initially dissolved in a Complete Growth Medium (CGM) at concentrations of 0.62% (v/v), 1.25% (v/v), 2.5% (v/v), 5% (v/v), 10% (v/v), and 20% (v/v) respectively. Then, the samples were kept in tightly packed and sterilized containers to avoid contamination. Of note, the honey samples were prepared 24 h before adding to the cell cultures and stored at 4 °C.

2.5. Cell culture

Cell culture was performed under sterile conditions in Biosafety Cabinet Class II. A549 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) in cell culture flask and supplemented with 10% (v/v) fetal bovine serum (FBS). The medium was supplemented with 1% antibiotics (100 µg/ml streptomycin/penicillin) (Gibco, US). The cell culture was maintained at 37 °C in 5% carbon dioxide (CO_2) humidified atmosphere.

2.5.1. Subculture of cells

The DMEM medium was discarded and the confluence cells in 25 mL culture flask were washed with 5 ml sterile phosphate-buffered saline (PBS) to remove the dead cells and excessive medium. 1 mL of trypsin (TrypLE, Invitrogen) was added into monolayer cells and incubated in CO_2 incubator for 10–15 minutes at 37 °C. 2 mL of CGM was added to neutralize the trypsin and the suspension was transferred to a sterile 15 mL falcon tube. Then, the suspension was centrifuged at 1500 rpm for 5 min at the room temperature. The supernatant was discarded. Cell pellet was re-suspended in 3 mL pre-warmed CGM. A haemacytometer (Neubauer, Germany) and trypan blue exclusion assay (TBEA) were used for cell counting. An appropriate amount of medium was added and the cell suspension was transferred to a new flask.

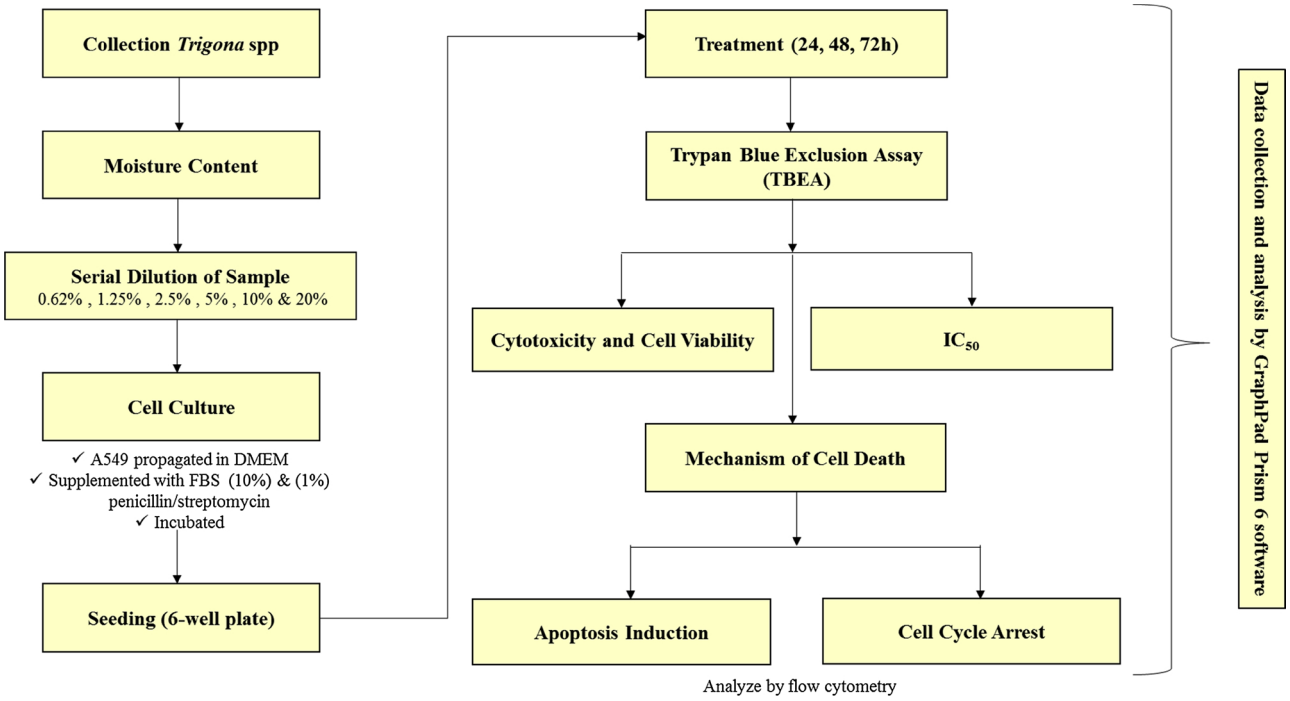

Fig. 1. The flowchart of the study.

2.5.2. Cryopreservation

Generally, the cells can be cryopreserved when they are in log phase of growth for a long-term storage. Freezing medium was prepared using dimethyl sulfoxide (DMSO) and DMEM at the ratios of 1:9. Cell pellet in the centrifuge tube was mixed with this freezing medium, and the suspension was homogenized using a vortex. Then, the suspension was transferred into the cryopreservation cryogenic vials and labelled with the essential information. Eventually, the cryogenic vial was placed into a Mr. Frosty Freezing Container with isoprophyl to prevent shock to the cells when stored at lower temperatures (− 80 °C).

2.6. Measuring cytotoxicity and cell viability

2.6.1. Trypan blue exclusion assay (TBEA)

A549 cells in the exponential growth phase were plated at 1 × 105 cells in a 6-wells plate. After 24 h of growth, various concentrations of *T. itama* honey treatment were applied to the cell wells and incubated at 37 °C with 5% carbon dioxide (CO_2) humidified atmosphere. After incubating for 24 h, 48 h and 72 h, cell viability of A549 was determined by TBEA method. TBEA is used to assess time dependent cytotoxicity as well as direct counting of living and dead cells. Dilution of the cell suspension in trypan blue (1:1) was prepared by mixing 20 µL of 0.4% (w/v) trypan blue dye. Cells were incubated for 5 min. Then, 10 µL of diluted suspension was placed at the edge of haemocytometer chamber and filled by capillary action. The haemocytometer filled with diluted cell suspension was observed and counted under an inverted microscope (Nikon) at 100X magnification. Only viable cells, which were not stained within square of the grid, were counted and all the blue stained cells (dead cells) were excluded. IC_{50} values (concentration that inhibited cell growth by 50% compared to the untreated control) was determined.

2.6.2. Flow cytometric analysis of apoptosis

A549 cells in the exponential growth phase were plated at 1 × 105 cells in a 6-wells plate. After 24 h growth, the inhibitory concentration dose (IC_{50}) of *T. itama* honey treatment that effectively inhibit 50% cells proliferation on A549 cells were applied into the culture wells and incubated at 37 °C with 5% CO_2 humidified atmosphere. The apoptosis analysis of A549 for 72 h with *T. itama* honey treatment was performed. Analysis was carried out using the Guava instrument flow cytometer coupled with InCyte 3.1 software. Cells were harvested, washed twice with cold PBS and centrifuged at 1200 rpm for 5 min. The supernatant was removed. Then, the cells were re-suspended and 100 µL of cells (1 × 105 cells/mL) were transferred into 5 mL flow cytometry tubes. Then 5 µL of Annexin V-PE and 5 µL of 7-AAD were added. The cells were gently vortexed and incubated for 15 min in the dark. Finally, 400 µL of Guava Nexin Reagent was added. Then, cells were analysed within 1 h.

2.6.3. Flow cytometric analysis of the cell cycle

A549 cells in the exponential growth phase were plated at 1 × 105 cells in a 6-wells plate. After 24 h growth, the inhibitory concentration dose (IC_{50}) of *T. itama* treatment that effectively inhibit 50% cells proliferation on A549 cells were applied into the culture wells and incubated at 37 °C with 5% CO_2 humidified atmosphere. The apoptosis analysis of A549 for 72 h with *T. itama* treatment was performed. Analysis was carried out using the Guava instrument flow cytometer coupled with InCyte 3.1 software. Cells were detached using 0.01% (w/v) trypsin (TrypLE, Invitrogen) solution for 5 min, transferred to centrifuge tube and centrifuged for 5 min. The supernatant was removed. Then, 5 × 105 cells/mL cells were suspended into 250 µL propidium iodide (PI), mixed well and incubated for 10 min at the room temperature. Finally, 200 µL Cell Cycle reagent (light sensitive) was added, mixed well and incubated for 10 min at the room temperature on ice. Then cells were analysed within 3 h.

2.7. Data analysis

Data were obtained from at least three independent experiments of cell viability. The data were analysed by two-way ANOVA using GraphPad Prism 6 Package (USA). Mean values were calculated and $p < 0.05$ was considered as statistically significant. However, apoptosis and cell cycle analysis were only performed once due to some limitations.

The overall flow of the study was illustrated in Fig. 1.

3. Results

3.1. Physicochemical analysis of T. itama honey

Moisture content in the *T. itama* honey samples ranged from 13.5% to 15.1% (mean: 14.3 ± 0.8%) which was within the limit (≤ 20%) recommended by the international quality regulations [20]. HMF was not detected in the honey samples, indicating the freshness and high quality of the honey. Of note, HMF is a widely known indicator of honey freshness due to their high content during the processing and aging of the products. *T. itama* honey was acidic in nature with the pH value of 3.17 ± 0.02. The electrical conductivity ranged from 0.47 mS/cm to 0.55 mS/cm. Furthermore, the proline content ranged from 19.1 mg/kg to 20.2 mg/kg. Based on these qualities, it can be elucidated that the *T. itama* honey samples used in this study were of good quality.

3.2. Cytotoxicity effect of T. itama honey treatment on A549

T. itama honey was screened for potential in-vitro cytotoxic activity against A549 at different concentrations of 0.62%, 1.25%, 2.5%, 5%, 10% and 20% (v/v) for 24, 48 and 72 h of exposure. Untreated A549 cells served as cell control exhibiting 100% cell viability. The results of cell viability at different concentrations of honey samples were illustrated in Figs. 2–4 and Table 1. In brief, it is eminent that the exposure of A549 cells to *T. itama* honey treatment significantly inhibited the growth of cancer cells in a dose and time-dependent manner. Moreover, the maximum percentage of cell death was observed at 72 h of exposure, particularly at 20% v/v of honey treatment as indicated in Fig. 4.

3.3. Evaluation of IC_{50} concentration of T. itama honey treatment on A549

Based on the Figs. 2 and 3, the inhibitory concentration (IC_{50}) of *T. itama* honey samples in effectively inhibiting cell viability of A549 cells occurred at 1.25% v/v. Hence, *T. itama* honey concentration of 1.25% v/v was chosen as the IC_{50} for 24 and 48 h of exposure. Conversely, much lower honey concentration (0.62% v/v) was determined as IC_{50} for 72 h of exposure as illustrated in Fig. 4. Based on these results, it can be deduced that the lowest concentration of *T. itama* honey that effectively inhibits the cancer cell's viability was 0.62% v/v at 72 h of exposure.

3.4. Apoptosis induction effects of T. itama honey treatment at IC_{50} on A549

The results of apoptosis analysis at IC_{50} on A549 cells after 72 h of exposure were illustrated in Fig. 5. Quantitative analysis using the Annexin V/7-AAD assay showed that the proportion of early stage (Q4) apoptotic cells (Annexin V+ / 7-AAD-) increased from 1.3% to 1.6% and the proportion of late stage (Q2) apoptotic cells (Annexin V+ / 7-AAD +) increased from 8.2% to 9.6% respectively. Nonetheless, there was no difference in the percentage of the live cells (Q3) (Annexin V- / 7-AAD -) between the controlled and treated cells. Percentage of dead cells (Annexin V+ / 7-AAD-) was higher in the controlled cells compared to the treated cells, with the values 2.2% and 0.7% respectively. Based on the findings, it can be suggested that *T. itama* honey treatment does not induce apoptosis in the A549 cells due to a slight difference in the early and late apoptosis values when compared between the treated and controlled cells. The flow cytometer results showed that inhibition of A549 cell growth after *T. itama* honey treatment at IC_{50} concentration was not mediated by apoptosis induction after 72 h of exposure. Since there was no induction of apoptosis in the early stage, it can be assumed that *T. itama* honey treatment exerted cell death via other possible pathways that needs further research.

3.5. Cell cycle arrest effects of Trigona spp honey treatment at IC_{50} on A549

The findings of cell cycle analysis at IC_{50} after 72 h of exposure were presented in Fig. 6. The results demonstrated that the A549 cells treated with *Trigona* spp honey induced 16.72% accumulation of cells compared to the untreated cells, which was about 12.92% in G2/M phases. The cells in G0/G1 phase after 72 h of exposure were reduced in the treated cells compared to the untreated cells. Such observation explains

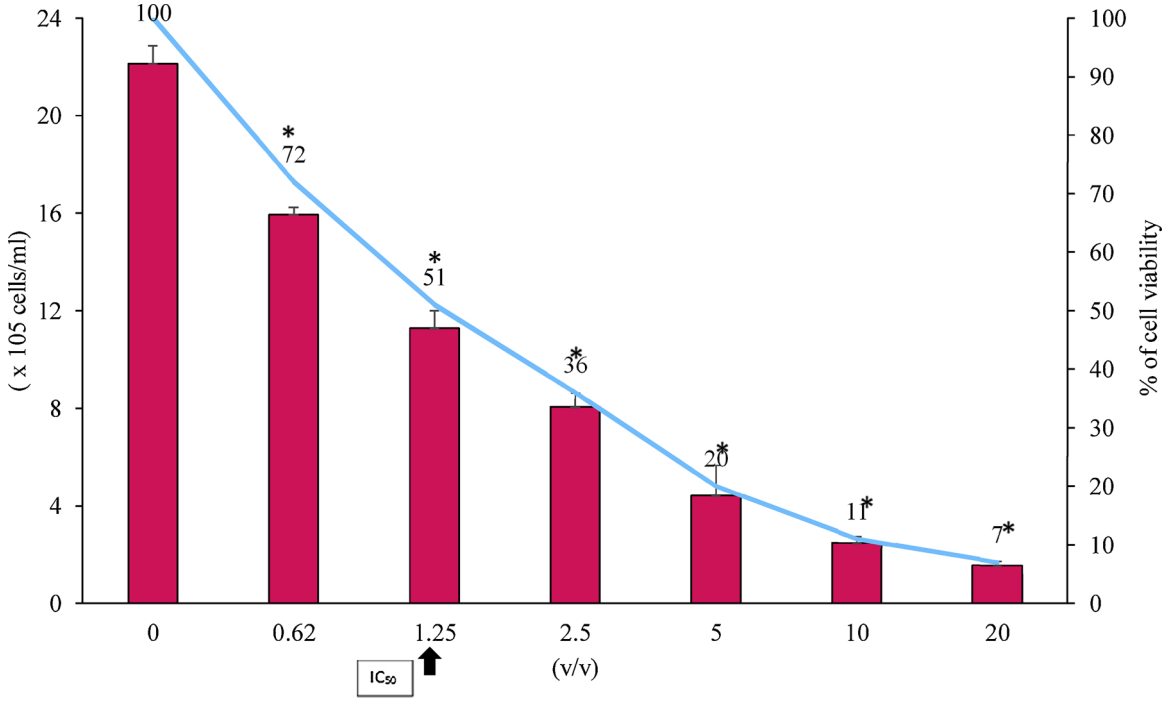

Fig. 2. Percentage of cell (A549) viability treated under different concentrations of *Trigona itama* honey for 24 h. IC_{50} concentration was indicated by the arrow. * indicated significance at $p < 0.05$ versus control (100% of cell viability).

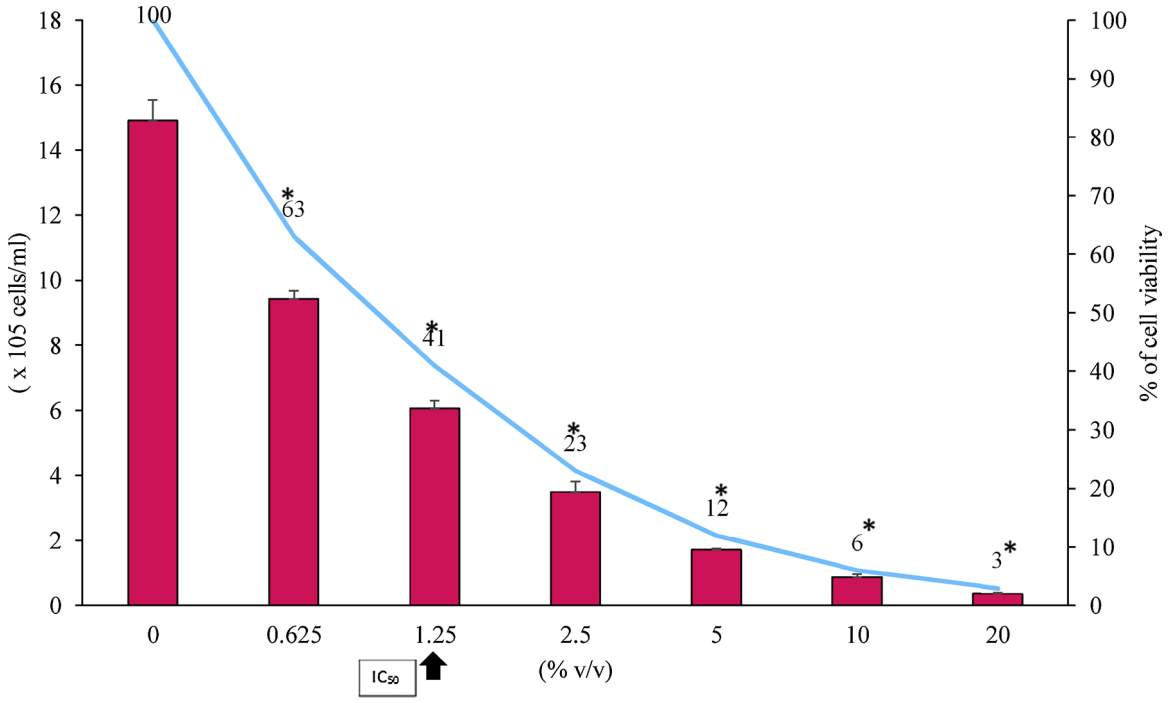

Fig. 3. Percentage of cell (A549) viability treated under different concentrations of *Trigona itama* honey for 48 h. IC_{50} concentration was indicated by the arrow. * indicated significance at $p < 0.05$ versus control (100% of cell viability).

that the compounds available in the honey arrest the cancer cells at this phase and stopping them from progressing into the next phase. The flow cytometer results suggested that inhibition of A549 cells growth after the treatment of *T. itama* honey treatment was mediated by cell cycle arrest pathway. In brief, it can be elucidated that *T. itama* honey at IC_{50} concentration has a promising cytotoxic effect and capable of inducing cell cycle arrest at G2/M phase after 72 h of exposure.

4. Discussion

Despite numerous use of multifloral honey as anti-oxidants, anti-inflammatory, anti-microbial and anti-tumour activities [22–24], little is known about the medicinal properties of multifloral honey specific to Malaysian region. Of note, there was no proper studies evaluated the cytotoxic effects of a multifloral honey, *T. itama*, on human lung cells.

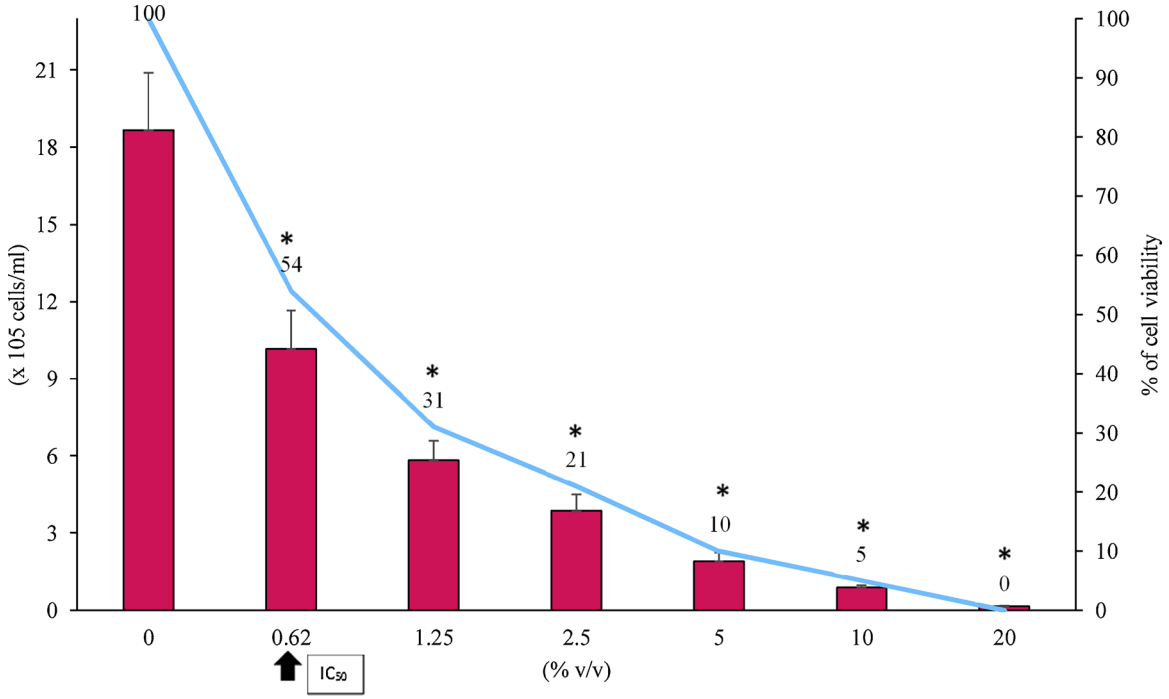

Fig. 4. Percentage of cell (A549) viability treated under different concentrations of *Trigona itama* honey for 72 h. IC_{50} concentration was indicated by the arrow. * indicated significance at $p < 0.05$ versus control (100% of cell viability).

Table 1
Summary of cell (A549) viability under different concentrations of *Trigona itama* honey treatment for 24, 48 and 72 h.

Concentration % (v/v)	Percentage of cell growth		
	24 h	48 h	72 h
0	100 ± 72.5	100 ± 62.2	100 ± 221.4
0.62	72 ± 30.4	63 ± 24.8	54 ± 148.3
1.25	51 ± 71.7	41 ± 23.0	31 ± 75.3
2.5	36 ± 56.9	23 ± 32.9	21 ± 64.1
5.0	20 ± 124.7	12 ± 4.4	10 ± 32.5
10.0	11 ± 26.2	6 ± 8.7	5 ± 7.7
20.0	7 ± 16.3	3 ± 4.7	0 ± 1.9

Thus, this study made an attempt to demonstrate the potential of *T. itama* honey as a natural anti-cancer agent in delivering stimulus on apoptosis and cell cycle arrest to A549 cells. As the results indicated, the moisture content in the *T. itama* honey (14.3 ± 0.8%) was within the accepted international standard [25] and can be treated as potential natural candidate of good quality for cancer treatment. Investigation on the cytotoxicity effect of *T. itama* honey revealed that it was able to induce significant inhibition of cancer cell growth after 24, 48 and 72 h in a dose and time-dependent manner under various concentrations. The findings also suggested that the higher the concentration of *T. itama* honey, the higher the percentage of cancer cells that would be effectively suppressed from proliferating. In addition, higher percentage of cell deaths was expected to occur when the cells were exposed to *T. itama* honey treatment for a longer period of time.

It is noteworthy that IC_{50} concentration that causes 50% of inhibition in vitro is a prominent measure of antagonist drug potency in pharmacological research [26]. In this study, it was reported that IC_{50} concentration at 72 h to be the lowest concentration which represents the highest potency compared to the other dose-time responses. Although no independent test was performed to identify the phytochemical compounds of *T. itama* honey, these compounds were highly responsible in triggering the cytotoxicity activities on cancer cells. Previous studies supported such claims by reporting that vitamins, minerals and flavonoids that exist in *T. itama* honey possess many interesting biological properties such as anti-cancer and anti-oxidant potential [20,27,28]. Likewise, many other types of naturally-occurring honey were reported to contain abundance of phenolic and flavonoid content contributing to its impressive anti-oxidant activity and disease prevention [11,27,29]. By considering this, further analysis on phytochemical components of *T. itama* honey was essential to elucidate their roles in cancer prevention.

Besides, apoptosis and cell cycle have been evidenced as a critical mechanism for cancer chemoprevention and chemotherapy [30]. However, the present study indicated that inhibition of A549 cell's growth after the treatment of *T. itama* honey at IC_{50} concentration was not mediated by apoptosis induction after 72 h of exposure, as the results of both treated and untreated cells were almost the same. Since there was no induction of apoptosis in the early stage, it can be deduced that *T. itama* honey treatment exerted cell death via a different mechanism that warrants further research. In general, mutations in the cell cycles will cause the cell to divide uncontrollably, ignoring apoptotic signal and causing cancer to develop [31]. This study revealed that apoptosis induced by *T. itama* honey was also associated with the G2/M cell cycle arrest. This finding was in agreement with Toogood [32] and Yunlan et al. [33] who claimed that the anti-cancer agents usually cause cell cycle arrest at G1 or G2/M phases.

In summary, the study findings provided preliminary evidence that *T. itama* honey was capable of inducing cytotoxicity effect on human lung cancer cells in a dose and time-dependent manner. Thus, it could be viewed as a viable candidate of natural origin to treat cancer cells. The treatment of *T. itama* honey that selectively induce cell cycle arrest in cancer cells could be more effective in combination with the current drug such as cisplatin, doxorubicin and methotrexate to treat lung cancer and to overcome drug resistance.

4.1. Limitations of the study

It should be noted that the current study was also subjected to several limitations that need to be taken into consideration when interpreting its findings. The present study did not involve the use of normal human cells as the control of the study. This situation hindered the evaluation of the cytotoxic effect of honey on normal cells.

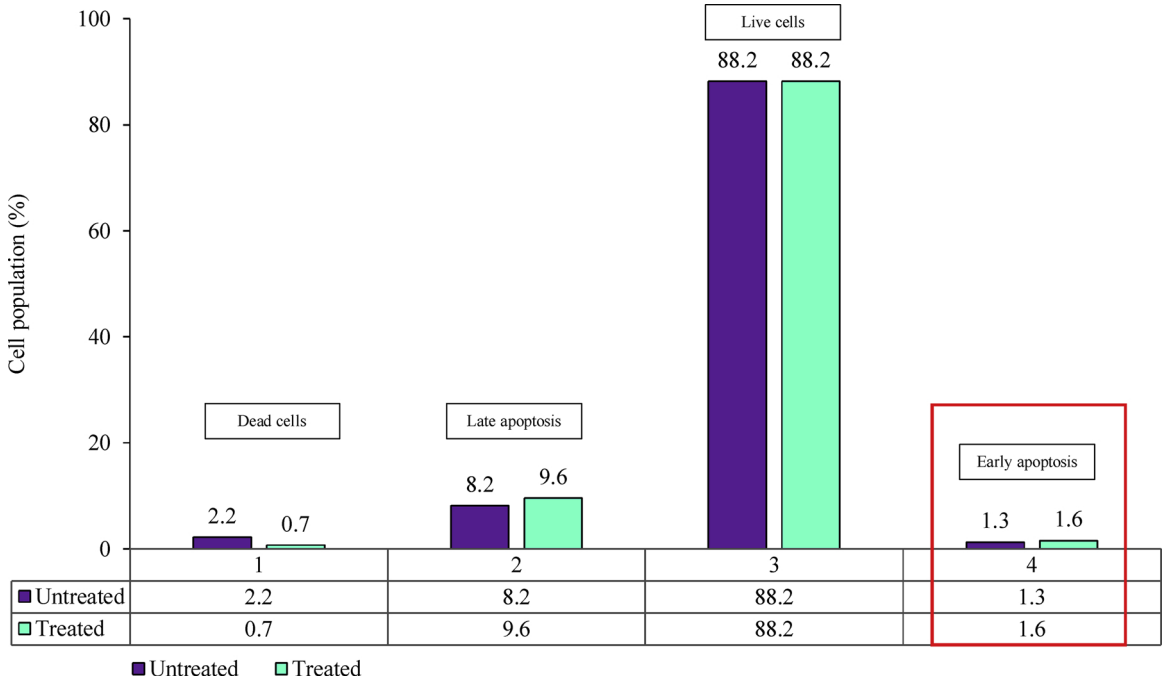

Fig. 5. Apoptosis induction of A549 cells upon treated to IC_{50} of *Trigona itama* honey for 72 h.

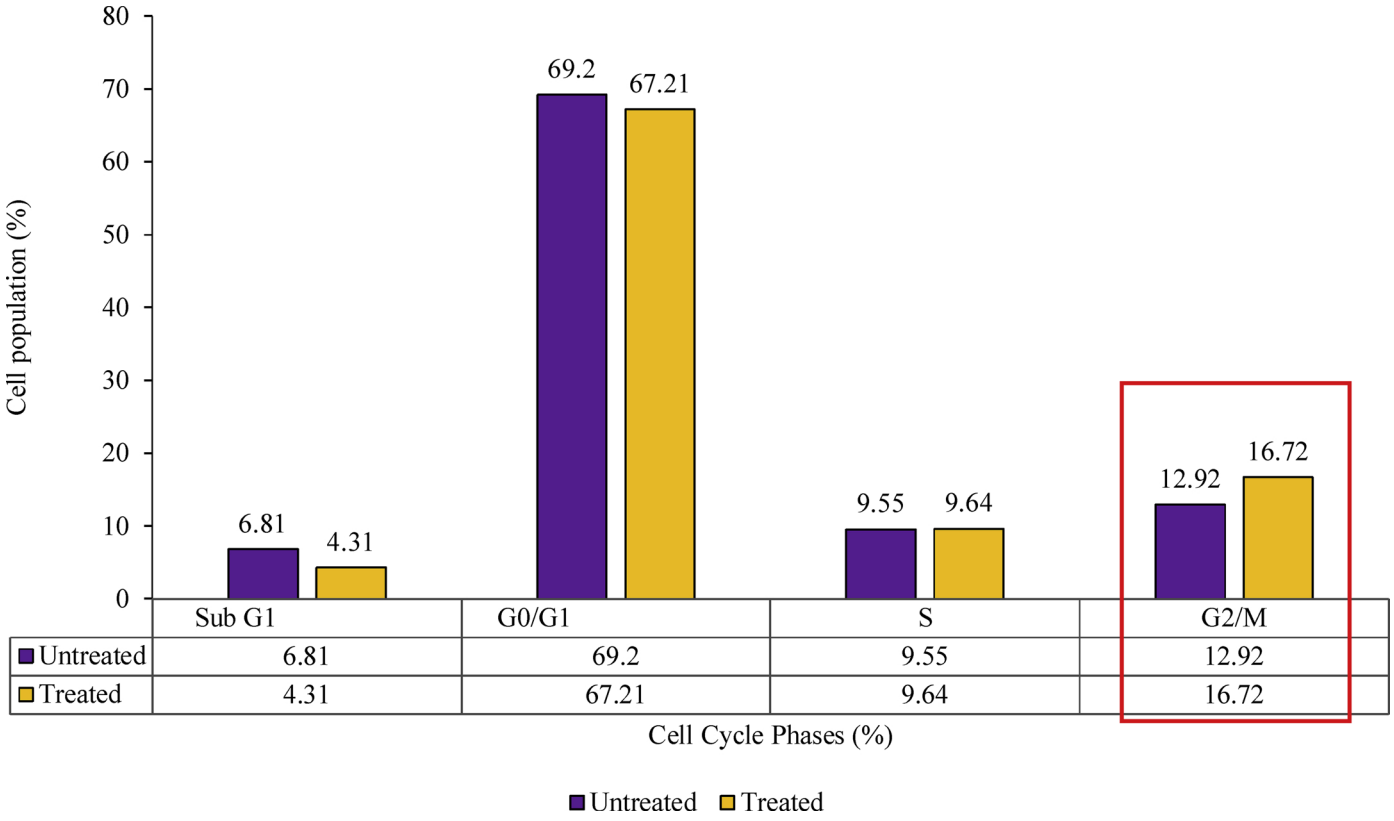

Fig. 6. Cell cycle arrest effects of *Trigona itama* honey treatment at IC_{50} for 72 h.

Furthermore, the present study did not profile the active compounds of *T. itama* honey as the concern of this study was to access the anti-cancer potential of the honey and therefore, the chemical characterization of the honey was not within the scope of this paper. Further investigation on the quantification of the phenolic compounds and flavonoids in the *T. itama* honey deemed necessary to evidence the involvement of these active compounds in preventing cancer cells. Despite these limitations, this study still provided valuable insights into the potential of the *T. itama* honey as a natural anti-cancer agent that can alleviate global cancer burden.

5. Conclusion and future directions

In short, the findings suggested that *T. itama* honey can act as a potential anti-cancer agent against A549 cells as it was capable of inhibiting the cells growth in a dose and time-dependent manner. The IC_{50} which effectively inhibit 50% cells proliferation was 1.25% v/v for both 24 and 48 h whereas 0.62% v/v for 72 h of exposure to honey treatment respectively. The results also demonstrated that *T. itama* honey was capable of inducing cell cycle arrest at G2/M phase. Even though this study provided valuable insights on the potential of *T. itama* honey as a promising treatment for cancer cells, further studies are still needed to verify the bioactivity of this honey on normal cells. If no cytotoxic effect of *T. itama* honey was identified on normal cells, it would provide an advantage over current anti-cancer agents that cause many cytotoxic and side effect on normal cells. Besides, further direction can be geared towards specific composition analysis and phytochemical content of *T. itama* honey to identify the role of each components towards cancer prevention. Furthermore, the mechanism of cell death caused by *T. itama* honey needs further investigation to determine the induction of apoptosis through specific signalling pathway, depolarization of the mitochondrial membrane or other molecular mechanisms.

Authors' contribution

Abdull Rasad, M.S.B. and Abdul Wahab, R. conceived and supervised the study. Mohd Salim, S. N. performed data collection and analysis. Mohd Salim, S. N. and Ramakreshnan, L. wrote the original version of the manuscript. Mohd Salim, S. N., Ramakreshnan, L. and Fong, C. S. revised the manuscript. All authors gave approval of the final version to be submitted for publication.

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Declaration of Competing Interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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