



Inhibitory Activity of HEp-2 Cells by Honey from Indonesia

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Abstract

Indonesian local honey contains active compounds that have the potential as an antioxidant and anticancer, primarily as a laryngeal anticancer through the inhibition of HEp-2 cells. This study aims to determine the anticancer activity of several types of honey in Indonesia through the inhibition of HEp-2 cells. Samples used in the form of Trigona, Longan, Rambutan, and Kaliandra honey obtained from honey farmers in Sulawesi and Java, Indonesia. Honey samples extracted by using methanol, then liquids partition was carried out consecutively using n-hexane and ethyl acetate. The cytotoxicity test for HEp-2 cells was carried out using the MTT method (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide). The results showed that all honey samples were active against preventing HEp-2 cells with the highest inhibition activity from longan honey with ethyl acetate extract at 65.18% at 100 ppm. Longan honey has decreased HEp-2 cell inhibitory activity after fractionation. Indonesian local honey, namely trigona honey, kaliandra honey, rambutan honey, and longan honey, can be used as a supplementary supplement for patients with laryngeal cancer.

1. Introduction

Cancer or neoplasm is a disease that is notoriously dangerous and is one of the leading causes of death worldwide. In 2018 GLOBOCAN (Global Cancer Burden) [1] estimated that there were around 18.1 million cases of cancer worldwide, with 9.6 million cancer cases were causing death worldwide. One type of cancer commonly suffered throughout the world is head and neck cancer, which ranks fourth in the top ten fierce cancers in men and women. Also, laryngeal cancer is ranked second as the most aggressive cancer that attacks the epithelial cells in the head and neck [2]. The overall estimated time of a patient able to survive from laryngeal cancer for five years is around 61% [3]. Honey from Indonesia studied shows the presence of various secondary metabolites such as β carotene and vitamin C [4], vitamins E [5], and has high flavonoid and phenolic content around 2000–4400 ppm which consist of chlorogenic acid, caffeic acid, p -coumaric acid, ferulic acid, pinobanksin, quercetin, luteolin, pinocembrin, and chrysenes [6]. Flavonoids and

phenolic have been known to have high antioxidant activity and can be an inhibitor of cancer cells [7]. Several types of honey in Indonesia has also been studied for its various activities, such as being an antibacterial, antioxidant [8], and potential as anticancer and antioxidant with LC_{50} values of 1.50 ppm and IC_{50} values of 5453.75 ppm [9].

The efficacy of honey as an anticancer has also been proven by Spilioti *et al.* [7], which states that the ethyl acetate extract of Greek honey successfully inhibits MCF-7 cells (breast cancer) and PC-3 cells (prostate cancer) with concentrations around 200–500 μ g/mL. Tualang honey from Malaysia also has the ability to inhibit MCF-7 cells (breast cancer) with an IC_{50} value of 4% (w/v) [10]. Honey from Iraq also shows anti-proliferation activity and apoptotic effects on HEp-G2 and AMN-2 tumor cells in vitro [11].

The data on the activity of local honey produced in Indonesia against laryngeal cancer cells is still not yet available. One of the cells that are often used in-vitro to

determine the activity of substances as inhibitors of cancer cells is HEP-2 cells. HEP-2 cells are cancer cells that cause damage to laryngeal organs (laryngeal cancer). This cell has the same properties as its host so that it can be used for in-vitro anticancer activity research. The in-vitro process used the MTT method (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay with the target cell, which is HEP-2 cells.

The data of this research is hope to be able to provide information on the function of honey as alternative medicine or as a supplement to laryngeal cancer patients through inhibition of HEP-2 cells. Thus, the use of honey will be more diverse to help human health.

2. Methodology

2.1. Materials and Equipment

The samples used in this research were four different types of honey obtained from honey farmers in West Java (Bogor) and Central Java. The four types of honey were Kaliandra honey, Trigona honey, Rambutan honey, and also Longan honey. The chemical solvents used for the extraction method were (methanol pa (FULLTIME), n-hexane pa. (FULLTIME), ethyl acetate pa (SmartLab), and distilled water, TLC Silica gel 60 F254 (Merck), Na₂SO₄ anhydrous pa (Merck), Phosphate buffer saline (PBS) (Gibco, USA), trypsin (Gibco, USA), Dimethyl Sulfoxide (DMSO) (Sigma USA), ethanol (Merck, Germany), and MTT (Sigma, USA) HEP-2 cells (ATCC CCL 23) were obtained and cultured in the Microbiology and Immunology Laboratory, PSSP IPB-Bogor. Cell growth media consisted of Dulbecco's Modified Eagle Medium (D-MEM) (Gibco, USA), Fetal Bovine Serum (FBS) (Hyclone, USA), and penicillin-streptomycin (Invitrogen, USA).

The instruments used in this research consisted of commonly used glassware, separating funnel, magnetic stirrer, Buchner funnel, rotary evaporator (Heidolph), vacuum pump, Whatman filter paper, microplate reader (Imax, BioRad, US), biosafety Cabinet Level- 2 (Nuair, USA), CO₂ incubator (Binder, Germany), centrifuge (Tommy, Japan), inverted microscope (Nikon, Japan), T25 flask (Corning, USA), haemocytometer (Improved Neubauer), 96-wells tissue culture plate (Corning, USA), 12-wells tissue culture plate (Corning, USA), aid pipettes, volumetric pipettes and media bottles.

2.2. Preparation and Phytochemical Screening

Samples of Trigona honey, Kaliandra honey, Rambutan honey, and Longan honey were obtained from honey farmers in West Java and Central Java. The four types of honey prior to extraction was carried out in some preliminary tests, which included (i) proximate analysis (water content, ash content and hydroxyl methyl furfural (HMF), (ii) diastase enzyme activity test to determine the quality and grade of honey according to SNI 3545: 2013 and (iii) phytochemical tests (alkaloid test, flavonoid test, terpenoid and steroid test, tannin/polyphenol test, saponin test) to determine the compounds contained in each sample qualitatively.

One hundred grams of honey was extracted in 300 mL methanol (pro analysis) for 24 hours so that it was homogenous. Furthermore, the mixture was concentrated using a rotary evaporator at a temperature of 64°C to obtain a concentrated extract of honey methanol [12]. Then it proceeded with liquids partition [12], so that water extract (polar) and ethyl acetate (semipolar) extracts were obtained from the four types of honey. Honey extracts from the liquids partition were tested for their proliferative inhibitory activity against HEP-2 cells using the MTT assay method. Extract samples with the highest activity were assessed by Thin Layer Chromatography (TLC) and fractionated [13]. The results of the fractionation were analyzed again for the inhibitory properties of the proliferation of HEP-2 cells using the MTT assay method.

2.3. Diastase Enzyme Activity According to SNI 3545:2013

Five grams of sample was put into a 20 mL beaker, and add 10-15 mL of distilled water and 2.5 mL of acetate in cold condition, the solution was stirred until the sample was completely dissolved. After that, the sample solution was put into a 25 mL volumetric flask containing 1.5 mL NaCl solution and adjusted to the limit of the flask with distilled water. The determination of absorbance was conducted by putting 5 mL of starch solution with a pipet into the sidearm of the test tube and 10 mL of sample solution to the bottom of the test tube (avoid mixing of both solutions). Furthermore, the tube was incubated in a water bath (temperature 40°C ± 0.2°C) for 15 minutes, then mixed its contents by moving the test tube back and forth in a tilted position while running the stopwatch. Precisely in 5 minutes, 1 mL of the mixed solution was quickly poured into 10 mL of dilute iodine in a 100 mL erlenmeyer and mixed thoroughly. After that, the solution was diluted to the same volume as the previous volume. Then the absorbance value was determined using a UV-Vis spectrophotometer. The reading was conducted at a wavelength of 860 nm or 600 nm with one cm cell. The reaction time was counted when the starch was mixed with the honey until the addition of liquid iodine. The solution was taken in an interval of 5 or 10 minutes until an absorbance value of <0.235 was obtained. The absorbance value was calculated by plotting the result against the reaction time. Thus, it can be determined the amount of time needed to reach the absorbance value of 0.235. When the time value has reached, the absorbance value of 0.235 had been obtained. The time value was used to divide the number 300 in the SNI 3545: 2013 formulation, the results indicated the diastase enzyme or *diastase number* activity.

2.4. Cytotoxicity test [14]

2.4.1. Honey Extract Preparation

One mg (milligram) of extract from each honey was then weighed, then dissolved in 1000 µL 99.5% DMSO, then centrifuged until homogeneous. This solution was used as the base solution of the honey extract with a concentration of 1000 ppm. The mother liquor was then

diluted with 99.5% DMSO to make a series of test solutions with concentrations of 50 ppm, 100 ppm, 200 ppm, 400 ppm, and 800 ppm.

2.4.2. Monolayer HEp-2 Cell Preparation

The confluent living HEp-2 cells were then sub-cultured. Cell media were removed, and then 10 mL of PBS was added to clean the flask from the rest of the media, then PBS was drained. Five milliliters of 0.125% Trypsin was added to the flask and incubated at 37°C for 5 minutes. Cells that have been released from the substrate were put into a 15 mL test-tube and then centrifuged 500 x g for 5 minutes, and the supernatant was removed. Cell counting was conducted using a hemocytometer, and then cells were prepared by the purposes of the test. Cells were re-incubated in a CO2 incubator with a concentration of 5%.

2.4.3. Cell Calculation

A precise amount of 50 µL cell solution was added to 50 µL *trypan blue* and then transferred into the hemocytometer, then the living cell was observed and counted (which not absorbing color) from 2 large boxes. The results obtained were calculated using the formula:

$$Cell\ per\ ml = average\ cell\ counted \times dilution\ factor \times 10^4$$

2.4.4. MTT Assay

Sustainable cells that have been grown in T25 flask were sub-cultured, then the cells were grown on 96 wells tissue culture plates with 5000 cells/well and were incubated for 24 hours in a growth medium at 37°C and 5% CO₂. An amount of 100 µL/well Ethyl acetate extract and honey water in each concentration were added, cells without treatment were also included as a control cell, and then re-incubated for 48 hours. The 3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 4 hours at 37°C and at 5% CO₂. The supernatant cell was then removed, and crystal formazan that formed were then dissolved in 70% ethanol. Optical density (OD) reading was carried out using a microplate reader at a wavelength of 565 nm.

The counting of viability counting percentage:

$$\% \text{ viability} = \frac{(1 - (OD \text{ control cell} - OD \text{ treatment of cell}))}{OD \text{ control cell}} \times 100\%$$

The counting of inhibition percentage:

$$\% \text{ inhibition} = \frac{OD \text{ control cell} - OD \text{ treatment of cell}}{OD \text{ control cell}} \times 100\%$$

3. Result and Discussion

3.1. Honey Proximate Analysis Result

Proximate analysis was conducted on the four honey samples (Trigona honey, Kaliandra honey, Rambutan honey, and Longan honey) included water content, ash content, hydroxymethylfurfural (HMF), and diastase enzyme activity, then compared with SNI 3545: 2013 standards (Table 1).

Table 1. The proximate analysis result of honey samples

Test Parameter	SNI Limits	Trigona Honey	Kaliandra Honey	Rambutan Honey	Kelengkeng Honey
Water Content (% b/b)	Max. 22	<i>22.88 ± 0.001</i>	16.88 ± 0.003	16.75 ± 0.003	15.41 ± 0.002
Ash Content (% b/b)	Max. 0,5	<i>5.01 ± 0.028</i>	0.11 ± 0.005	0.10 ± 0.003	0.05 ± 0.001
HMF (mg/kg)	Max. 50	14.6706 ± 0.060	23.1536 ± 0.091	3.38322 ± 0.030	11.1277 ± 0.017
Diastase Enzyme Activity (DN)	Min. 3	<i>1.27</i>	<i>1.53</i>	8.63	4.16

*Notes: numbers in italics and underscores indicate the test results do not meet SNI 3545: 2013 standards.

The results of the analysis showed that not all honey samples showed conformity with SNI standards, including the parameters of water content, ash content, and Diastase Number (DN) for Trigona and Kaliandra honey samples.

The yield (% w/w) of each liquids partition extraction process demonstrated that ethyl acetate and water extracts displayed a separate extract in ethyl acetate and water extracts. The yield data for each sample were Trigona honey (ethyl acetate: water = 23.98: 68.83), Kaliandra honey (ethyl acetate: water = 22.61: 65.08), Rambutan honey (ethyl acetate: water = 20, 42: 63.72), Logan Honey (ethyl acetate: water = 28.33: 60.55). While the n-hexane extract showed a 0% yield, so the next measurement only involved ethyl acetate and water extracts.

3.2. Honey Phytochemical Test Result

The phytochemical test aimed to determine the class of secondary metabolite compounds found in the four honey samples. Phytochemical test results showed that the four honey samples positive in containing secondary compounds i.e., saponin, flavonoid, and steroid compounds. While tannins and alkaloids were only detected in Trigona honey (Atr) and Kaliandra honey (Bkld); hence, both types of honey contained fairly complete secondary metabolite compounds (Table 2).

Table 2. Phytochemical analysis of honey from Trigona, Kaliandra, Rambutan, and Longan

Honey	Saponin Test	Tannin Test	Flavonoid Test	Uji Steroid	Alkaloid Test
Trigona	Positive, stable foam ± 10 minutes	Positive, black green	Positive, clear orange	Positive, red ring	Positive, brown sediments
Kaliandra	Positive, stable foam ± 10 minutes	Positive, black green	Positive, clear orange	Positive, red circle	Positive, brown deposits
Rambutan	Positive, stable foam ± 10 minutes	Negative	Positive, clear orange	Positive, red ring	Negative
Longan	Positive, stable foam ± 10 minutes	Negative	Positive, clear orange	Positive, red circle	Negative

Trigona honey contains five classes of secondary metabolite compounds; it was assumed as Trigona honey is multiflora honey produced by Trigona bees from various flower nectar. Hence, the content of secondary

metabolites was complete than other types of honey. Alves *et al.* [15] reported that inside the honeycomb of *Trigona.sp*, it can be found phenolic, flavonoids, and tannins compounds.

The presence of tannins and alkaloids compounds were only detected in *Trigona* honey and *Kaliandra* honey, while the other two types of honey, which are *Rambutan* honey and *Longan* honey, give negative results. In general, almost all flowering plants can feed honey bees in the form of nectar and pollen [16]. Phytochemical testing of local *Trigona* honey by Sumarlin *et al.* [9] showed that local *Trigona* honey has phenolic and flavonoid content. Also, beehives contain many phenolic, flavonoids, and tannins compounds [17] so that each honey should contain alkaloids and tannins derived from plants nectar. Still, there is no trace of alkaloids and tannins in both *Rambutan* honey and *Kaliandra* honey; the cause is alleged because there is only a small quantity of the secondary compounds of alkaloid and tannin. Therefore, it does not give positive results when the phytochemical test is performed.

3.3. Cytotoxicity Test Result of Honey Extract

The results of MTT water extract assay on four types of honey showed inhibitory activity against HEP-2 cells with different concentrations ranging from 50 ppm to 400 ppm. The inhibitory ability was seen in HEP-2 cell growth by the sample extracts, which were lower than controls (without the addition of honey extract) (Figure 1).

The results show that each honey water extract is active and has various inhibitory activity at specific concentrations. It indicates that the active compounds found in each honey can be polar and soluble in water. The inhibitory activity of HEP-2 cell, the polar extract of the four types of honey ranged from 18% - 46%. Inhibition of water extracts against hep-2 cells has also been carried out by Al-Asady *et al.* [18] through *Capparis spinosa* extract. Data on the inhibitory activity of ethyl acetate extract showed that all honey ethyl acetate extracts were active and able to inhibit the growth of HEP-2 cancer cells ranging between 12% - 65% (Figure 2). It was also seen that each honey had the highest anticancer activity at different concentrations. The smallest inhibition activity was found in *Rambutan* honey, which only reached 12.02% at a concentration of 200 ppm while the highest inhibition activity was observed in ethyl acetate extract of *Longan* honey, which reached 65.18% at a concentration of 100 ppm (Figure 2).

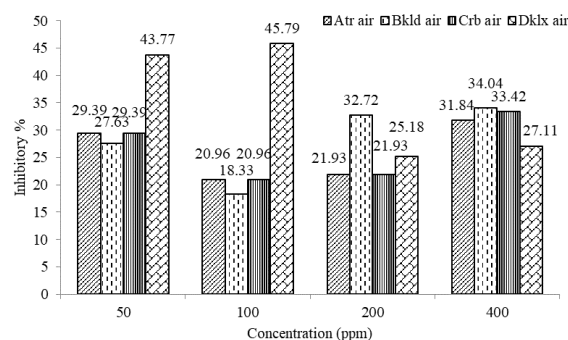


Figure 1. MTT assay results of *Trigona* (Atr), *Kaliandra* (Bkld), *Rambutan* (Crb) and *Longan* (Dklx) honey water extracts against HEP-2 cells

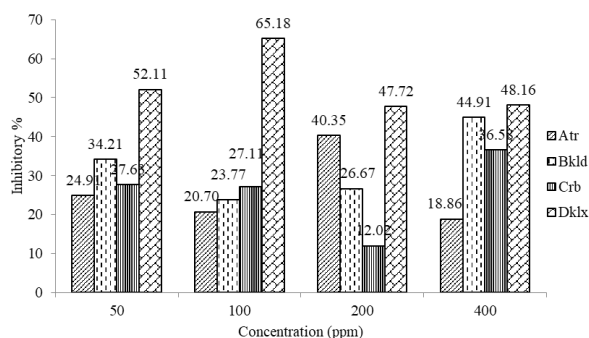


Figure 2. MTT assay results of *Trigona* (Atr), *Kaliandra* (Bkld), *Rambutan* (Crb), and *Longan* (Dklx) honey water extracts against HEP-2 cells.

Semipolar *Longan* honey ethyl acetate extract gives better activity than *Brassica oleracea* extract, which only has 50% inhibitory activity against HEP-2 cells at a concentration of 125 ppm [19]. Also, a semipolar extract of *T. foenum graecum*, which can inhibit HEP-2 cells by 31.22% at a concentration of 250 µg/µL [20]. It is suspected that ethyl acetate extract of *Longan* honey has a more active anticancer compound compared to *Brassica oleracea* extract, a semipolar extract of *Dewa* leaves, and semipolar extract of the three other types of honey. Hence *Longan* honey ethyl acetate has the highest inhibitory activity to HEP-2 cell.

The data above shows that all four types of honey, both polar and semipolar extracts are active and have the best inhibitory activity against HEP-2 cells ranging from 12% - 65% (Figure 2). Previous studies have shown that some types of honey were active and can inhibit the proliferation of cancer cells, such as Greek honey, can inhibit prostate and breast cancer cells [7]. Egyptian monofloral honey can inhibit colon, breast, and liver cancer cells [21], and honey from India impede the growth of prostate cancer cells (PC-3) [22].

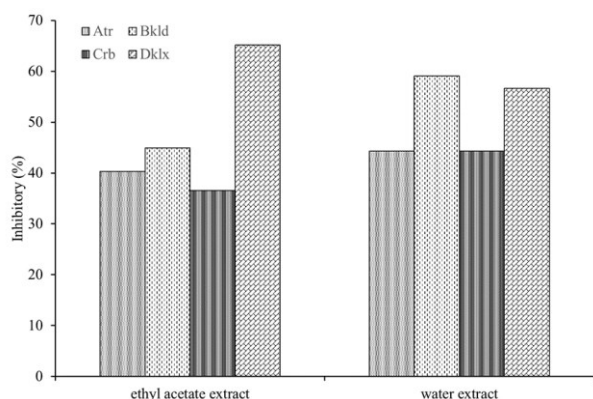


Figure 3. The highest inhibitory activity (%) of each honey extract.

Multiflora honey (Atr) has lower inhibitory activity compared to the three types of monoflora honey (Bkld, Crb, and Dklx) (Figure 3). Although based on phytochemical tests, Atr honey contains complete secondary metabolite compounds but does not guarantee that Atr honey provides the best inhibition. This result provides preliminary information that the inhibitory ability of Hep-2 cells is also influenced by more specific compounds not yet identified in this study.

The observations of the anti-proliferation effect of Hep-2 cells on the addition of water extracts and ethyl acetate of honey after incubation for 48 hours were observed under an inverted microscope (Figure 4). The morphology of the Hep-2 cells is oval-shaped, with both ends taper like a spindle and stick to the bottom of the plate, forming a monolayer layer. The decrease in Hep-2 cell growth can be seen from the cell density that decreases after adding the honey extract. The control density of Hep-2 cells almost covered the entire surface, and the decrease is due to the addition of the honey

extract, which is characterized by an empty zone that is not overgrown with Hep-2 cells.

Water extracts and ethyl acetate of the Trigona honey showed a decrease in the density of Hep-2 cell growth at concentrations of 400 ppm and 200 ppm, respectively (Figure 4a). While the addition of water extracts and ethyl acetate of Kaliandra honey were able to inhibit Hep-2 cells with the highest inhibition at concentrations of 200 ppm and 400 ppm (Figure 4b), the water extract and ethyl acetate of Rambutan honey can provide the highest inhibition at the same concentration of 400 ppm (Figure 4c). It is clearly seen that the addition of ethyl acetate extract of Longan honey with a concentration of 100 ppm will reduce the number of Hep-2 cells marked by the largest empty zone, and Hep-2 cells are rarely overgrown when compared to the addition of other honey extracts (Figure 4d).

The highest inhibition of all four types of honey was shown by ethyl acetate (semipolar) extract of Longan honey, which was able to inhibit Hep-2 cells by 65% at a concentration of 100 ppm. While water extract (polar) from Longan honey was only able to inhibit 45.79% at a concentration of 100 ppm. The result shows that the semipolar extract of honey is more active compared to the polar extract of honey. So, it is estimated that the active compound which provides the best inhibition of Hep-2 cells is semipolar. The same thing was proven by El-Gendy [21] showed that some ethyl acetate extracts from honey originated from Egypt inhibited the proliferation of colon, breast, and liver cancer cells better than acetone, methanol, and chloroform extracts. The cytotoxic ability of the ethyl acetate fraction was also tested by Diba *et al.* [23] from lime parasites, which caused T47D cell death to be even stronger than the alcohol fraction.

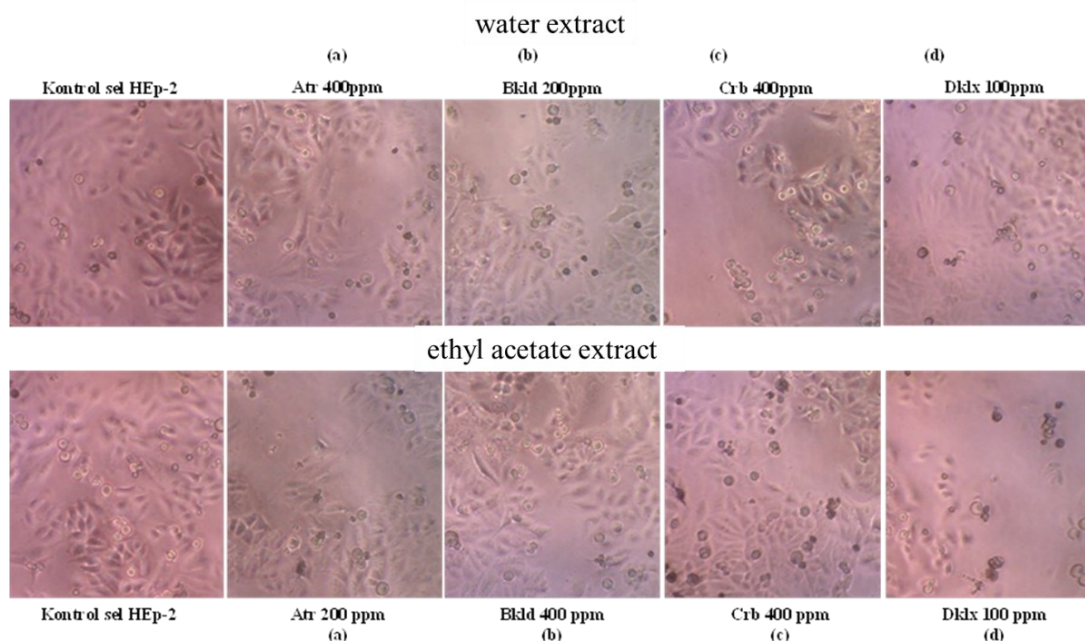


Figure 4. The best inhibitory effect of water extract and ethyl acetate of Trigona (Atr), Kaliandra (Bkld), Rambutan (Crb), and Longan (Dklx) honey samples on Hep-2 cell growth after 48 hours.

Spilioti *et al.* [7] reported that ethyl acetate extract from honey originated from Greece was rich in phenolic compounds such as protocatechuic acid, vanillin, caffein, fumaric acid and cinnamic acid (Figure 5). This extract had an anticancer activity of prostate cancer cells (PC-3) and breast cancer cells (PC-3) MCF-7) of 30–60% and 20–50%. Thus, it is suspected that semipolar extracts of honey contain active compounds, especially flavonoids and phenolic, which have the potential as anticancer agents. Some of the primary polyphenols presented in honey show anti-proliferative effects on various cancer cells such as caffeic acid, phenyl ester folic acid (CAPE), chrysene, galangin, quercetin, acacetin, kaempferol, pinocembrin, pinobanksin, and apigenin [24].

antioxidant, antimicrobial, and anti-carcinogenic properties, including those for HEP-2 cells [28].

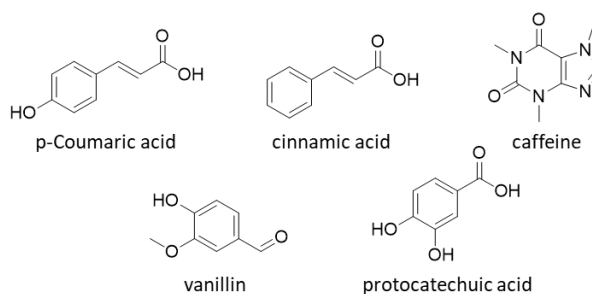


Figure 6. Phenolic compounds found in honey from Greece [7].

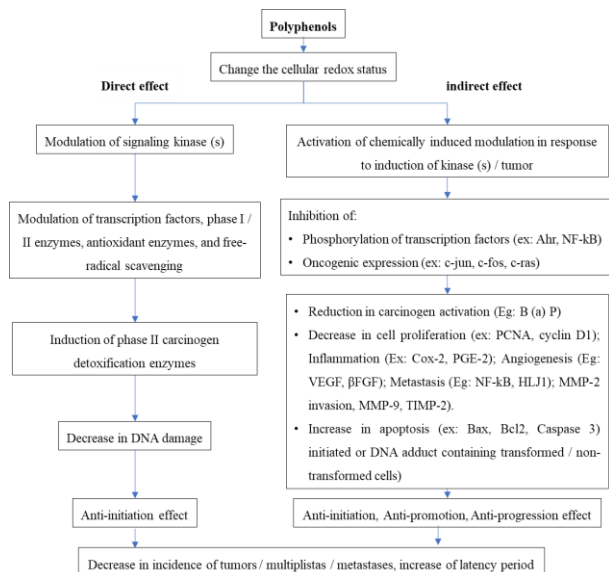


Figure 5. Summary of the in vivo chemopreventive effects of polyphenols and their underlying mechanisms [25].

Studies using in vivo mouse model experiments have shown that polyphenols play a role in modulating the incidence, multiplicity, latent periods of carcinogen-induced tumors at various organ sites and tumor angiogenesis, metastases and transplanted/xenografts tumor behavior. The anti-initiation, anti-promoting, and anti-progress or activities of polyphenols in chemical and genetic models involve modulation of kinase signaling, which ultimately leads to their effects on genes and cell signaling pathways at various levels. Overall, the mechanisms involved in polyphenol inhibition are diverse and include influences on several molecular pathways and genes (Figure 5).

Abd Elhamid *et al.* [26] found that various types of natural bee honey, such as eucalyptus honey, were cytotoxic and showed the highest anti-proliferation activity against HEP-2 cells and showed that the total phenolic content analyzed had a direct impact on honey's anticancer activity. Luteolin (flavone, a type of flavonoid) had the potential to treat lung cancer because of its ability to stimulate various responsible targets. These targets include apoptosis, cell cycle termination, decreased regulation of HNF4α expression, TAM-secreted CCL2, IL-4, and M2 related genes [27]. Additionally, phenolic extracts containing chlorogenic acid and also have

Fluctuating MTT assay results on all extracts were also reported by Tsiapara *et al.* [29], where some Greece honey extracts produced varying MTT assay data in inhibiting breast cancer cell proliferation (MCF-7), endometrial cancer cells (Ishikawa), and prostate cancer cells (PC-3) (Figure 6). This fluctuating result is thought to be due to the presence of carbohydrates and proteins in honey, which become nutrients for HEP-2 cells to carry out cell proliferation. Therefore, on one side, compounds that were contained in honey can inhibit the growth of HEP-2 cells, but on the other hand, the compounds in honey could become nutrients for the process of HEP cell proliferation -2.

Protein and carbohydrates become a source of energy for cells to carry out cell division, especially carbohydrates contained in honey, which acts as a substrate in the process of glycolysis, thereby triggering increased HEP-2 cell growth. When all the nutrients in the extract are used up by HEP-2 cells, the active compounds in honey are just beginning to play a role in inhibiting the growth of HEP-2 cells so that the number of HEP-2 cells decreases again. This result was confirmed by Oršolic [30], who stated honey might increase tumor growth because it contained large amounts of vitamins, minerals, amino acids, and glucose. Porcza *et al.* [31] also reported that glucose in honey could provide nutrition to breast cancer cells so that it can trigger the growth of cancer cells more quickly.

3.4. Cytotoxicity Test Result of Longan Honey Ethyl Acetate Fraction

Ethyl acetate extract of Longan honey, which provides the most significant inhibitory activity, is separated again through the fractionation process, which produces six fractions (called ethyl acetate fraction with eluents in the form of a mixture of n-hexane, ethyl acetate, and acetone). Each fraction was re-tested for its ability to inhibit the growth of HEP-2 cells at a concentration of 50–400 ppm. The results showed that all ethyl acetate fractions of Longan honey were active against HEP-2 cells with inhibitory values ranging from 4.50% to 23.93% (Table 3). The highest inhibition of HEP-2 cell proliferation was owned by fraction 6, with a percentage of 23.93%. Only fractions 1, 3, and 5 stimulated HEP-2 cells at the highest concentration (400 ppm), whereas at lower concentrations (50 ppm), only

fractions 2 and 4 stimulated HEp-2 cell proliferation by 0.26% and 2.44%.

Stimulation of increased HEp-2 cell growth is thought to be caused not only by protein and amino acids that function as cell nutrients but also because of the content of phenolic compounds found in each fraction. This result is confirmed by the research of Tsiapara *et al.* [29], who reported that ethyl acetate from thyme honey had inhibitory activity. While the ethyl acetate extract from pine honey stimulated MCF-7 cell growth due to the total phenolic compounds contained in ethyl acetate extract of thyme honey (990 mg/100 g) was higher than ethyl extract acetate from pine honey (550 mg/100 g). However, phenolic compounds that trigger the growth of HEp-2 cells need to be studied even further. Moreover, the character of HEp-2 cells, which is quite resistant to active compounds, changes in temperature, nutrition, and environmental changes, is thought to make HEp-2 cells can continue to survive and multiply, even in conditions that are lacking in nutrients. So that honey samples have not able to completely inhibited the proliferation process. Devi and Thangam [19] reported that five semipolar fractions of the god's crown leaves were able to stimulate the growth of HEp-2 cells with an increase in cell number by 10.3%. Besides, 100 ppm ethanol extract of Sargassum sp is also able to stimulate HEp-2 cells [32].

Table 3. Comparison of inhibitory activity of ethyl acetate extract of longan honey with fractionation results.

Concentration (ppm)	Inhibitory (%)						
	EA Dktx	F1	F2	F3	F4	F5	F6
50	52.11 ±0.42	22.36 ±0.12	-0.26 ±0.04	16.26 ±0.06	-2.44 ±0.08	8.62 ±0.15	4.79 ±0.06
100	65.18 ±1.20	4.79 ±0.08	18.58 ±0.06	12.78 ±0.02	7.43 ±0.08	12.78 ±0.09	16.61 ±0.02
200	47.72 ±2.40	4.50 ±0.08	16.61 ±0.12	-0.15 ±0.04	12.60 ±0.09	9.61 ±0.02	23.93 ±0.17
400	48.16 ±12.23	-2.21 ±0.2	16.52 ±0.04	22.88 ±0.14	15.04 ±0.14	-6.21 ±0.08	18.67 ±0.06

When compared to before fractionation, it seems that the inhibitory activity of HEp-2 cells had decreased (Table 3). This result is thought to be due to the effect of the process during fractionation, which allows the loss or change of some active compounds that were initially present in the Logan honey sample. However, this research has proven that in vitro honey samples can reduce the growth of laryngeal cancer cells with different concentrations, especially Logan honey. Thus, honey has the potential to be used as a supplement for cancer patients, especially laryngeal cancer patients, to suppress the growth of cancer cells in conjunction with the use of chemotherapy drugs.

4. Conclusion

Water extracts and ethyl acetate of Trigona honey, Kaliandra honey, Rambutan honey, and Longan honey each have an inhibitory activity to HEp-2 cell growth with

different concentrations and inhibitory values. Honey ethyl acetate extract is better in inhibiting the growth of HEp-2 cells. The highest inhibition of ethyl acetate extract was retained by Longan honey, which was able to inhibit the growth of the HEp-2 cell for 65.18% at a concentration of 100 ppm. Longan honey extract can only inhibit the proliferation of the HEp-2 for 45.79% at a concentration of 100 ppm. The fractionation process of ethyl acetate extract of Longan honey can reduce the inhibitory activity of HEp-2 cells.

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