RESEARCH COMMUNICATION

Gelam and Nenas Honeys Inhibit Proliferation of HT 29 Colon Cancer Cells by Inducing DNA Damage and Apoptosis while Suppressing Inflammation

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Abstract

Gelam and Nenas monofloral honeys were investigated in this study for their chemopreventive effects against HT 29 colon cancer cells. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolim) assays showed more effective inhibition of colon cancer cells proliferation by Gelam honey with IC_{50} values of 39.0 mg/ml and 85.5 mg/ml respectively after 24 hours of treatment. Alkali comet assays revealed both honeys increased DNA damage significantly in a dose dependent manner. In addition, annexin V-FITC/PI flow cytometry demonstrated that at IC_{50} concentrations and above, both Gelam and Nenas honeys induced apoptosis significantlyat values higher than for necrosis ($p<0.05$). Measurement of prostaglandin E_2 (PGE_2) confirmed that Gelam and Nenas honeys reduced its production in H_2O_2 inflammation-induced colon cancer cells. In conclusion, our study indicated and confirmed that both Gelam and Nenas honeys are capable of suppressing the growth of HT 29 colon cancer cells by inducing apoptosis and suppressing inflammation.

Keywords: Gelam honey - nenas honey, HT29 cells - antitumor - anti-inflammatory - apoptosis - DNA damage

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Introduction

Honey is a natural sweet substance produced by honey bees from floral nectar and has been used to treat several diseases since ancient times (Wollgast & Anklam, 2000; Molan, 2006). It is rich in antioxidants such as phenolic compounds (Cherchi et al., 1994), vitamins B, C & E (White & Crane, 1975), and other essential nutrients such as amino acids and enzymes (USDA National Nutrient Database for Standard Reference, 2011).

Research in the past few years has claimed that honey has several health beneficial effects which include protection against cardiovascular disease (Khalil & Sulaiman, 2010), anti-inflammatory and wound healing (Jaganathan & Mandal 2009[a]), anti-aging (Klatz & Glodman, 2003), antibacterial (Chambers, 2006; Molan, 2009) and antitumor properties (Jaganathan & Mandal 2009[a], Russo et al., 2004). Our Malaysian local honeys were also reported for their significant effects on treatment of diseases: Tualang honey was found to exert antitumor effects on MCF-7 and MDA-MB-231 breast cancer cell lines, HeLa cervical cancer cell lines (Fauzi et al., 2011), osteosarcoma and oral cancer (Ghashm et al., 2010); Gelam honey has been reported to exhibit anti-inflammatory effect in RAW 264.7 macrophage cells (Kassim et al., 2010[b]) and anti microbial properties against the growth of pathogenic bacteria Methicilin Resistant *Staphylococcus aureus* (MRSA) (Aljadi & Kamaruddin, 2002).

Colorectal cancer is the third leading causes of cancer-related deaths in the USA (American Cancer Society, 2011). In Malaysia, colorectal cancer is the second most common type of cancer affecting the population with male's frequency being higher than females. Furthermore, Chinese are the most prevalent ethnic for colon cancer compared to other ethnics such as Malays and Indians (National Cancer Registry, 2006). Treatment for colon cancer is crucial by intervention of new drugs or foods that can treat or prevent its dramatic increase of occurrence (Jaganathan & Mandal 2009b).

Apoptosis is a type of cell death that involves activation of caspase cascade pathway (Zoltán, 2008). Loss of regulation in apoptosis will cause uncontrollable cancer cells proliferation and therefore compounds that activate apoptosis are targeted to be a novel chemoprevention therapy for cancer (Sun et al., 2004). Inflammation is an immunological and patho-physiological response of tissues to infectious organisms, cancer, autoimmune disease, toxic chemical substances or physical injury (Vodovotz et al., 2009). There are many mediators of inflammation such as cytokines, nitric oxide (NO) and prostaglandin (PG). Excessive generation of PG is known to be responsible for pathologic effects in inflammation disorder, cardiovascular disease, hypertension as well as

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cancer (Renoa et al., 2004).

Lately, phytochemical from natural sources are exploited to seek for high efficacy with minimal adverse side effects of anti-inflammation and anticancer reactions for cancer treatment. Not many studies have been conducted to evaluate the antitumor and anti inflammation effects of Malaysian honey on colon cancer cells. Thus our study was aimed to determine the chemopreventive properties of two Malaysian honeys which are Gelam and Nenas against colon cancer HT29 cells.

Materials and Methods

Materials

Fresh monofloral Malaysian honey (Gelam and Nenas honey were collected from *Apis mellifera*) was obtained from the National Apiary, Department of Agriculture, Parit Botak, Johor; Malaysia. It is stored in dark at 4°C. This pure unfractionated honey was diluted with RPMI 1640 medium of different concentration (weight/volume) for *in vitro* studies. All chemicals and reagents used were of analytical grade.

Cell culture and treatment

Colon carcinoma cell line HT 29 was obtained from the American Type Culture Collection (Rockville, MD USA) and was cultured in T-25 flasks containing RPMI 1640 medium (Flowlab, Australia) supplemented with 10% Fetal Calf Serum (FCS) (PAA Laboratories GmbH, Austria), 100 U/ml of penicillin and streptomycin (Flowlab, Australia) at 37°C in 5% CO_2. Cell proliferation, apoptosis and prostaglandin (PGE_2) secretion were assessed when cells reached 70% confluence.

HPLC analysis

Twenty microliters of standard mixtures of gallic acid, chlorogenic acid, caffeic acid, ellagic acid, ferulic acid, *p*-coumaric acid, rutin, hesperetin, qurecetin and chrysin (100 µg/mL for each) and phenolic extracts were injected into the HPLC machine (10A Shimadzu, Japan). The phenolic compounds were detected using UV absorption spectra and monitored at 290 nm and 340 nm; the majority of the honey flavonoids and phenolic acids showed maximum UV absorption at these two wavelengths (Martos et al., 1997). The column used was a reversed phase C18 column, ACE (4.6 × 250 mm, particle size 5 µM, USA). The mobile phases were 0.25% formic acid and 2% methanol in water (solvent A) and methanol (solvent B), at constant solvent flow rate of 1 mL/min. The following gradient was used, according to the previously mentioned method (Martos et al., 1997), except for minor modifications: 10% methanol (B) was flowed through the column isocratically with 90% solvent (A) for 15 min which was then increased to 40% methanol (B) for 20 min, to 45% methanol (B) for 30 min, to 60% methanol (B) for 50 min, to 80% methanol (B) for 52 min, to 90% methanol (B) for 60 min, and then followed by isocratic elution with 90% methanol (B) for 65 min. Finally, the gradient was changed to 10% methanol for 68 min, and this composition was held until 73 min. The phenolic and flavonoid compounds were identified by comparing the chromatographic retention time with those authentic standards. A calibration curve of caffeic acid at 290 nm was used to calculate phenolic acids concentrations, whereas calibration curve of quercetin at 340 nm was used for flavonoids. This is because the different phenolic compounds are absorbed better at these wavelengths (Martos et al., 1997). The calibration curves of the standards were used to determine the concentrations of the phenolic compounds in the extracts.

Cell viability assay

Cell viability was assessed with MTS colorimetric assay as described by Mossman, 1983 with some modifications. For cell viability assay, 2 x 10^4 cells HT 29 were plated in 100 μl RPMI 1640 media to each well of 96-well plates. Cells were incubated overnight at 37°C in humidified atmosphere of 5% CO_2 for cells attachment. Gelam and Nenas honeys were added at various concentrations ranging from 0 to 150 mg/ml respectively to appropriate wells. After 24-hour treatment with honey, culture medium was replaced with 20 μl MTS/PMS solution (Promega, Madison, WI, USA) diluted in 100 μl RPMI 1640 medium in each well and the plate was placed at 37°C in the incubator for 2 hours. The resulting MTS products were determined spectrophotometrically at the absorbance of 490 ηm with ELISA microplate reader (VERSAmax, USA).

DNA damage analysis by alkaline Comet assay

The cell DNA fragmentation was assessed using alkaline Comet assay according to Singh et al., 1988. All the samples were prepared at room temperature under dark condition to prevent DNA damage. Sixty μl of cell suspension was mixed with 90 μl of 0.6% low melting point agarose gel (Sigma, USA) and added to fully frosted slides (StatLab Medical Product Anapath®, USA) that had been covered with a layer of 0.6% normal melting point agarose gel (ICN Biomedical Inc.). Subsequently, the slides were immersed in lysing solution (2.5 M NaCl, 1% Triton X-100; Ajax Chemical, Australia, 100 mM $Na_2EDTA.2H_2O$, sodium sarconate; ICN Biomedical Inc., 10 mM Tris-HCl, and dimethylsufoxide; Sigma, USA) for 1 hour at 4°C followed by alkaline electrophoresis buffer (10 M NaOH; Ajax Chemical, Australia and 1 mM EDTA; Sigma, USA) for 20 minutes to unwind cell DNA and electrophoresed for 20 minutes using 25 V with current adjusted to 300 mA. Finally, slides were added with neutralizing buffer (0.4 M Tris base pH 7.5; Sigma. USA) to neutralize residual alkali and were stained with 50 μl ethidium bromide (Ajax Chemical, Australia). Images of 150 randomly selected non-overlapping comets on each slide were visually analyzed using fluorescence microscope (Zeiss, German) as shown in figure 3. Images generated form Comet assay were scored numerically from 0-4. Score 0 = cell without DNA damage, score 1 and 2 = cell with mild DNA damage, score 3 = cell with moderate DNA damage and score 4 = cell with severe DNA damage.

Apoptosis assessed with annexin V-FITC/PI flow cytometry

Apoptosis was detected with annexin V-FITC kit

(Beckton-Dickinson, Canada). All adhering and floating cells were harvested after incubation for 24 hours with 39.0 and 60.0 mg/ml Gelam honey and 85.5 and 112.5 mg/ml Nenas honey. Cells were collected, washed with ice-cold PBS and centrifuged. The cells pellet were resuspended in ice-cold binding buffer (100 mM HEPES/ NaOH, pH 7.5 containing 1.4 M NaCl and 25 mM $CaCl_2$) at a density of 1 x 10^6 cells per ml. Five hundred μl of this cell suspension was transferred to a 5 ml culture tube, to which 2.5 μl of annexin V-FITC conjugate were added, gently vortexed and incubated for 10 min followed by addition of 10 μl of propidium iodide which is then gently vortexed and incubated for 3 min at room temperature in the dark. The fluorescence of the cells was immediately determined by flow cytometry (FACS, Becton-Dickinson, USA).

Measurement of Prostaglandin E_2 (PGE_2)

Production of PGE_2 by HT 29 cells after treatment was measured by PGE2-Monoclonal Enzyme-Immuno Assay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA). Cell culture supernatants were collected and kept on ice after 24-hour incubation with 36.0 mg/ml Gelam honey, 63.0 mg/ml Nenas honey and 600 μM indomethacin as positive control in cells with or without stimulation of inflammation by hydrogen peroxide (H_2O_2) (50 μM). Then, the supernatants were centrifuged at 4°C to remove cell debris and PGE_2 analysis was performed according to the manufacturers' guidelines.

Statistical analysis

The experiments were repeated at least 3 times and the results were expressed as mean ± S.E.M. Statistical evaluation was done using the analysis of variance, ANOVA (SPSS 17.0) where P<0.05 was considered significant.

Results

Phenolic compounds in Malaysian honey

Solid phase extraction (SPE), using C18 cartridges, were used to extract and recover phenolic compounds from honey. The recoveries were good for all standard phenolic compounds eluted from SPE, at 290 nm for phenol acids and 340 nm for flavonoids. The recoveries of phenolic acid standards were 71.5–98.8% while the flavonoid standards were 71.94-90.74%, indicating the suitability of this procedure for the recovery of phenolics in honey (Aljadi & Kamaruddin, 2003). The chromatograms of the extract samples from Malaysian honey showed a number of phenolic acids which absorb more strongly at 290 nm and flavonoids which absorb strongest at 340 nm (Kassim et al., 2010[a]) (Table 1). Caffeic acid, chlorogenic acid, *p*-coumaric acid, ellagic acid, quercetin and hesperetin were identified in both types of honey. On the other hand, gallic acid, ferulic acid and chrysin were identified in Gelam honey while rutin was identified only in Nenas honey. Generally, Gelam honey contains significantly higher quantity of phenolic compounds than Nenas honey as calculated from the peak areas.

Table 1. Concentrations of Phenolic Compounds Detected in Gelam and Nenas Honeys

Phenolic Compounds	Gelam Honey		Nenas Honey	
	Retention time (min)	µg/100 g honey at 290/340 nm	Retention time (min)	µg/100 g honey at 290/340 nm
Gallic acid	7.56	876.80±07.47	ND	ND
Chlorogenic acid	22.40	528.08±06.31	22.69	433.73±48.17
Caffeic acid	23.77	442.01±32.70	23.66	278.26±30.42
P- Coumaric caid	26.20	308.31±18.69	26.18	312.10±45.79
Ferulic caid	26.85	381.37±17.07	ND	ND
Rutin	ND	ND	28.50	1597.5±125.37
Ellagic acid	29.50	0575.67±17.66	29.71	339.61±44.41
Quercetin	37.35	1594.30±38.40	37.76	1700.90±93.97
Hesperetin	39.21	1477.78±01.91	39.20	1536.60±76.38
Chrysin	53.31	1504.60±03.20	ND	ND

* 'Data are expressed as the mean ± Standard Deviation from three independent experiments (n = 3). The honey extract were analyzed with HPLC with UV detector set as 290/340 nm. The concentrations of phenolic compounds in honey extract were derived by calculating the peak area from the calibration curves of the standards used. ND = Not Detected.

Antiproliferative effects of Gelam and Nenas honeys

Figure 1 showed Gelam and Nenas honeys inhibited proliferation of HT 29 cells significantly (p<0.05) dose dependently. Both honeys exhibited effective inhibition on cancer cells proliferation by suppressing about 80% of cells growth after 24 hours treatment with the highest concentration of honey used (150 mg/ml). The IC_{50} of Gelam honey was 39.0 mg/ml while Nenas honey was 85.5 mg/ml indicating that Gelam honey was more potent than Nenas honey in suppressing the growth of colon cancer cells.

DNA damage

The results demonstrated increased total DNA damage of cells with increasing dose of both types of honey (Figure 2). Both Gelam and Nenas honeys induced significant DNA damage at IC_{50} concentrations with elevated score 4 and reduced score 0 significantly (p<0.05) compared to respective scores of the control (Figure 3).

Apoptotic effects on HT 29 cells

With increasing concentrations of Gelam and Nenas honeys, the percentage of apoptotic cells was significantly

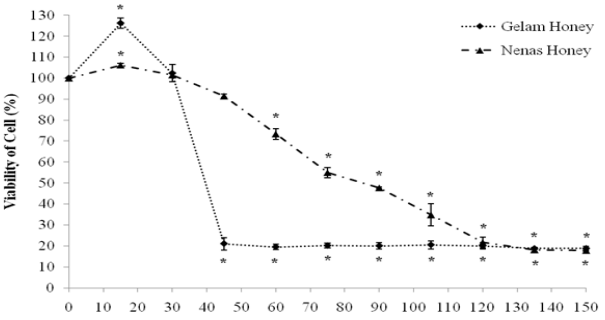

Figure 1. Effects of Gelam and Nenas Honeys on Proliferation of HT 29 Colon Cancer Cells. Cells viability was determined by MTS assay after 24 hour-treatment with increasing concentrations of both honeys. Data represents the mean ± S.E.M of 3 independent observations. *p<0.05 when compared to cells without honey treatment

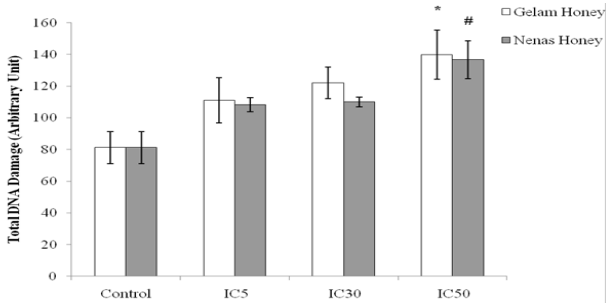

Figure 2. Effect of Gelam and Nenas Honeys on Total DNA Damage of HT 29 Colon Cancer Cells. Oxidative DNA damage of cells was assessed by alkaline Comet assay after treatment with IC_5, IC_{30} and IC_{50} concentrations of both honeys. Data are expressed as mean ± S.E.M of 3 independent observations. */ # represent p<0.05 compared to control

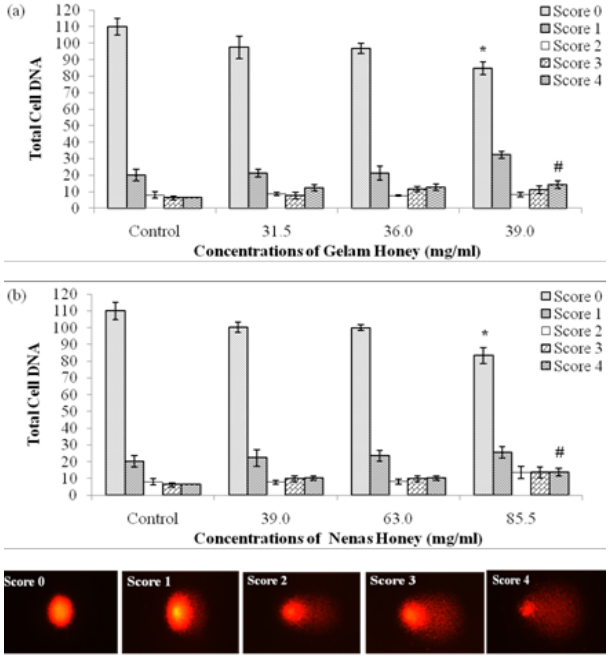

Figure 3. Effect of (a) Gelam and (b) Nenas Honeys on Individual DNA Damage Score of HT 29 Colon Cancer Cells. Alkaline Comet assays were performed after treatment with different concentrations of both honeys. Data shown are mean ± S.E.M of 3 independent experiments. *p<0.05 compared to score 1 of control; #p<0.05 compared to score 4 of control. Representative image of a comet generated in alkaline Comet assay. Score 0 = cell without DNA damage, score 1 and 2 = cell with mild DNA damage, score 3 = cell with moderate DNA damage and score 4 = cell with severe DNA damage

raised over control (p<0.05) with concomitant reduction of necrotic cells (Figure 4). The number of apoptotic cells was higher than necrotic cells (p<0.05).

PGE_2 production

Inflammation induced-HT 29 cells by H_2O_2 produced higher concentration of inflammatory marker, PGE_2 compared with control (p<0.05) (Figure 5). Interestingly, when inflammation-induced cells were treated with both types of honey and indomethacin (anti-inflammatory drug as positive control), PGE_2 levels were significantly reduced compared to H_2O_2 induced cells without treatment (p<0.05). Our results revealed that the PGE_2 secreted by inflammation-induced cells after treatment with both honeys and indomethacin were similar when compared

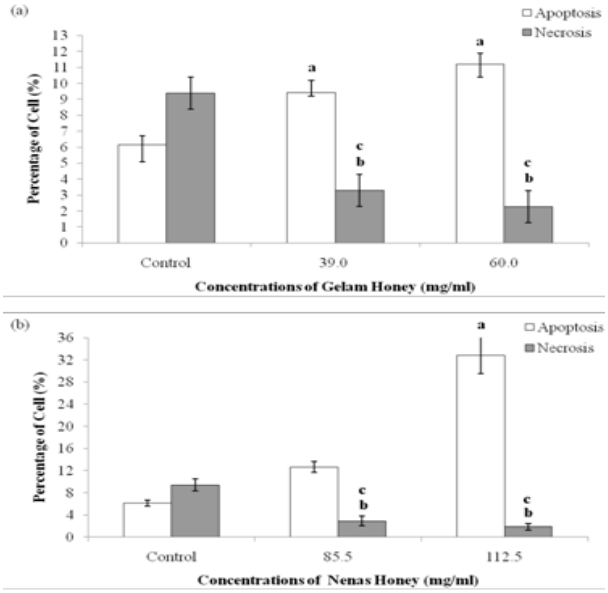

Figure 4. Effect of (a) Gelam and (b) Nenas Honeys on Mode of Cell Death of HT 29 Colon Cancer Cells. The apoptotic effect of both honeys was determined by flow cytometry annexin V-FITC and propidium iodide after 24-hour treatment with various concentrations of honey. All data are expressed in mean ± S.E.M of triplicate. a = significant (p<0.05) compared to control apoptosis; b = significant (p<0.05) compared to control necrosis and c = significant (p<0.05) compared to apoptosis for the same concentration of honey

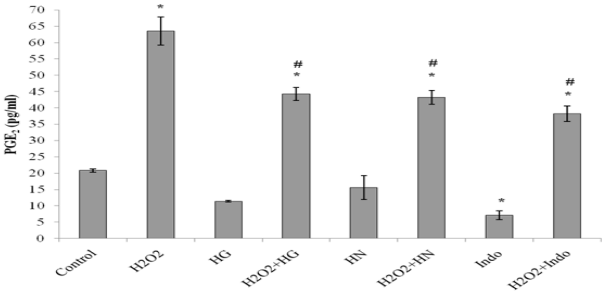

Figure 5. Effect of Gelam and Nenas honeys on PGE2 production in HT 29 colon cancer cells. Cells with or without inflammation induction by H_2O_2 were treated with Gelam Honey (HG), Nenas Honey (NH) and Indomethacin (Indo) as positive control for 24 hours. Cell culture supernatants were analyzed using PGE_2-Monoclonal Enzyme-Immuno Assay kit. Data shown are mean ± S.E.M. for triplicates. * = p<0.05 compared with control while # = p<0.05 when compared with H_2O_2 inflammation induced-cells without treatment

statistically (p>0.05).

Discussion

Honey is a universal food known to mankind since archaic time. Its benefit for human is mentioned in the Bible, Torah and the holy Quran. The medicinal benefit of honey is widely gaining attention all over the world based on promising scientific evidences. It has been reported to be effective in gastrointestinal disorders (Mobarok & Al Swaye, 2003), in the healing of wounds and burns (Subrahmanyam et al., 2001; Molan, 2006), and as an antimicrobial or antibacterial agent (Chambers, 2006; Kucuk et al., 2007; Molan, 2009).

Malaysia is known for its wide variety of honeys either

obtained from the deep forest such as Tualang honey or from plantations such as Nenas and Gelam. Our study provided results which showed significant inhibition of HT29 colon cancer cells by both Nenas and Gelam honeys, with the latter having higher potential as anti-tumor dietary agent. High polyphenols in honey has been attributed for its anti-tumor property (Russo et al., 2004; Jaganathan & Mandal, 2009[a]) The present and previous study showed that both Gelam and Nenas honeys have high levels of total phenolic compound which correlated significantly with their antioxidant activity (Hussein et al., 2010). Thus, it is highly probable that the high potency of Gelam honey in curbing the growth of HT29 colon adenocarcinoma cells is mainly due to high phenolic compounds it contained. Jaganathan and Mandal, 2009[a] reported similar findings whereby Indian honeys reduced the viability of colon cancer cells to about 10% after 48 hours of treatment.

Oxidative DNA damage with subsequent apoptosis has been associated with the probable mechanisms how cancer cells are killed by chemotherapy drugs and chemopreventive herbs. Chemotherapy agents such as antracycline, epipodophylotoxin and alkylating agents (Belousova, 1977; Burden & Osheroff, 1998) were shown to induce DNA fragmentation and damage that lead to suppression of cancer cells progression (Ploski & Aplan, 2001). Natural compound extracted from Ganoderma lucidum generally known as "Lingzhi" which is a novel anticancer agent was also reported to induce DNA damage in pre-cancerous human uro-epithelial cell (HUC-PC) tumorigenic model (Yuen & Gohel, 2008). Our results clearly showed that both types of honey were able to induce DNA damage of colon cancer cells indicating its potential as antitumor agent. To our knowledge no experimental evidences to date have shown the association of honey in inducing DNA damage in colon cancer cells. However, a close relative of honey, propolis extract which is rich in flavanoids, galangin and caffeic acid was also shown to cause dose-dependent DNA damage on Caco-2 colon adenocarcinoma cells after 72 hours of treatment (Russo et al., 2004).

DNA damage is a common event in life following which, repair mechanisms and apoptosis will be activated to maintain genome integrity. However in cancer cells, the induction of apoptosis is known to be an efficient strategy for cancer therapy. Both types of honey treatment induced early and late apoptosis of HT29 colon cancer cells. Jaganathan and Mandal, 2009[b] also demonstrated similar findings on Indian honeys. Tualang honey which consisted of high flavonoids, phenolic acid and hydroxymethylfurfural was also reported to cause apoptosis of breast and cervical cancer cells (Fauzi et al., 2011). A bioactive compound extracted from honey such as eugenol showed increased apoptosis of colon cancer cells by arresting cell cycle at sub-G1 phase and activation of p53 and caspase-3 (Jaganathan et al., 2010). Chrysin which is one of the polyphenols in honey also induced apoptosis on melanoma cells by activation of caspases cascade pathway (Pichichero et al., 2011). It is highly probable that the apoptotic effect of Gelam and Nenas honeys may be due to the presence of flavonoids and phenolic compounds which activated caspases signaling pathway.

PGE_2 is one of the inflammatory markers secreted by colon cancer cells that act as local chemical messenger for inflammation and metastasis process in cancer cells (Takahashi et al., 2004; Banu et al., 2007). H_2O_2 induced PGE_2 production by forming ROS that oxidizes phospholipids in the membrane leading to mobilization of arachidonic acid which promotes activation of COX enzyme. This enzyme was responsible for the production of prostaglandins such as PGE_2 (Poole et al., 2006) Both honeys showed significant anti-inflammatory effect on inflammation induced-HT29 cells by decreasing the level of PGE_2 of cells as effective as indomethacin. Indomethacin is a non-steroidal anti-inflammatory drug commonly used to reduce fever, pain, stiffness, and swelling. It works by inhibiting the production of prostaglandins which cause these symptoms. Interestingly, Gelam honey was also shown to depress production of PGE_2 and NO on exudates of rat's paw induced with carrageenan and lipopolysaccharide (Kassim et al., 2010[a]). Over stimulation of COX-2 enzyme and inducible nitric oxide (iNOS) were observed in colon cancer (Kawai et al., 2002) and indirectly led to increased of inflammatory mediators such as PGE_2 and antiapoptotic protein Bcl-2 that play important role in the promotion and progression of cancer cells (Surh et al., 2001; Issa et al., 2006). As a consequence, molecular mechanism of anti-inflammatory activities of polyphenols which existed in Gelam and Nenas honeys was postulated to down regulate COX-2 activity and PGE_2 levels that ultimately prevent the development of colon cancer.

In conclusion, our study indicated and confirmed that both Gelam and Nenas honeys are capable of suppressing HT29 colon cancer cells growth by inducing DNA damage and apoptosis as well as suppressing inflammation. As such, this study has revealed a novel nutritional value of honey in cancer treatment. Perhaps, this may open the door for more in-depth investigations on molecular mechanism of honey as an effective antitumor agent in preventing cancer development.

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