RESEARCH ARTICLE

Mechanism of Chemoprevention against Colon Cancer Cells Using Combined Gelam Honey and Ginger Extract via mTOR and Wnt/β-catenin Pathways

Lee Heng Wee[1], Noor Azian Morad[1], Goon Jo Aan[1], Suzana Makpol[1], Wan Zurinah Wan Ngah[1], Yasmin Anum Mohd Yusof[1]*

Abstract

The PI3K-Akt-mTOR, Wnt/β-catenin and apoptosis signaling pathways have been shown to be involved in genesis of colorectal cancer (CRC) . The aim of this study was to elucidate whether combination of Gelam honey and ginger might have chemopreventive properties in HT29 colon cancer cells by modulating the mTOR, Wnt/β-catenin and apoptosis signaling pathways. Treatment with Gelam honey and ginger reduced the viability of the HT29 cells dose dependently with IC_{50} values of 88 mg/ml and 2.15 mg/ml respectively, their while the combined treatment of 2 mg/ml of ginger with 31 mg/ml of Gelam honey inhibited growth of most HT29 cells. Gelam honey, ginger and combination induced apoptosis in a dose dependent manner with the combined treatment exhibiting the highest apoptosis rate. The combined treatment downregulated the gene expressions of Akt, mTOR, Raptor, Rictor, β-catenin, Gsk3β, Tcf4 and cyclin D1 while cytochrome C and caspase 3 genes were shown to be upregulated. In conclusion, the combination of Gelam honey and ginger may serve as a potential therapy in the treatment of colorectal cancer through inhibiton of mTOR, Wnt/β catenin signaling pathways and induction of apoptosis pathway.

Keywords: mTOR - Wnt/β catenin - apoptosis - combination - HT29 colon cancer cells

Asian Pac J Cancer Prev, **16 (15)**, 6549-6556

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer among males after lung and prostate cancer whereas for female, CRC is the second common cancer after breast (Jemal et al., 2011). In Malaysia, CRC is the second most common cancer in both females and males with a total of 2246 patients diagnosed with this fatal disease in the year 2007 (Zainal and Nor, 2007).

A multistep genetic model of colorectal carcinogenesis involving mutation of the APC gene, Kras, PI3K-Akt-mTOR, and Wnt have been suggested to play a major role in dysregulation of cell cycle progression, evasion of apoptosis, induction of genetic instability and enhanced invasiveness and metastasis (Moran et al., 2010; Wu et al., 2013). Activation of Wnt/β-catenin signalling pathway leading to transcription of Wnt target genes that regulate different cellular processes such as cyclin D1, c-myc, surviving, matrix metalloproteinase 7 and 14 (Herbst et al., 2014). mTOR is activated in CRC resulting from mutations in certain protein closely related to mTOR pathway; including amplification of PIK3CA gene, loss or inactivation of phosphatase and tensin homolog (PTEN), mutations of TSC1/2 complex and p53 (Guertin and Sabatini, 2007). Reduced rates of apoptosis in colonic mucosa are associated with an increased risk for colorectal adenomas (Wu et al., 2013). The antiapoptotic Bcl2 and Bcl-xl genes and proteins were found to be expressed in most of the colon cancer cell lines including HT29, HCT116, HCT15 and SW620 (Amundson et al., 2000).

The mTOR/Wnt/B-catenin pathways shared a same upstream target which is Akt. Activation of Akt by PI3K, mediates the activation and inhibition of several targets which include these three pathways: mTOR, GSK3/Akt and Wnt/Beta catenin, leading to increase in target genes, protein synthesis, and inhibition of apoptosis (Vara et al., 2004).

In recent years, the use of natural products as an alternative therapy in CRC treatment has become a growing interest among researchers. The protective effect of natural compounds is due to the presence of numerous phytochemicals such as resveratrol, quercetin, rutin, catechin, myricetin, kaempferol, EGCG which have been reported to modulate multiple key elements in signal transduction pathways affecting cellular proliferation, differentiation, apoptosis, inflammation, angiogenesis and

*[1]Department of Biochemistry, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Raja Muda Abdul Aziz, [2]Centre of Lipids and Engineering and Applied Research, Universiti Teknologi Malaysia, Jalan Semarak, Kuala Lumpur *For correspondence: anum@medic.ukm.my; rahmatyasmin@yahoo.com*

metastasis in CRC (Araujo et al., 2011).

Honey, is a natural product widely used since ancient times and most recently it has attracted attention of researchers due to its variable pharmacological effects mainly due to the vast amount of phenolic compounds found in it such as gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, quercetin, hesperetin and chrysin (Hussein et al., 2012). Teh et al. (2012) showed that local Gelam honey was able to inhibit proliferation of HT29 and HCT116 colon cancer cells by inducing apoptosis and inhibiting inflammation through modulation of the NFκb pathway. Ginger (Zingiber officinalis) has been used for thousands of years as food condiments and medicines (Shukla and Singh, 2007). It contains biological active phytochemicals such as gingerols, shagoals, paradols and zingerone (Ali et al., 2008). Abdullah et al. (2010) elucidated that ginger extract inhibited proliferation and induced apoptosis of HT29 and HCT116 colon cancer cells by arresting at G0G1 cell cycle.

The combination of food high in polyphenols has been shown to have a synergistic effect in chemoprevention rather than a single compound (Soerjomataram et al., 2010). A combination of EGCG and polyphenol E showed a synergistic effect by inhibiting cell growth, and inducing apoptosis in several colorectal cancer cell lines (Shimuzu et al., 2005). To our knowledge, the chemoprevention effect of the combination between ginger and honey has not been explored as the possible treatment for cancer especially CRC. A preliminary study in our laboratory showed that combined ginger and Gelam honey has antitumor effect against HT29 colon cancer cells by modulating the PI3K and NFκB pathway (Tahir, 2015). Interestingly, we have also shown previously ginger extract and honey were found to enhance the cytotoxic effect of 5-Fluorouracil, a chemotherapeutic drug, in colon cancer cells HCT116 (Hakim et al., 2014). We aimed to investigate the possible synergistic effect of Gelam honey and ginger extract in inhibiting the growth of on HT29 colon cancer cells at molecular level via modulation of the mTOR, Wnt/β-catenin and apoptosis pathways.

Materials and Methods

Gelam honey & ginger (zingiber officinale) extract

Gelam honey was obtained from the National Apiary, Department of Agriculture, Parit Botak, Johor, Malaysia. For sterilization purpose, the honey was sent to SINAGAMA, Malaysian Nuclear Agency for gamma radiation. The ginger ethanolic extract was obtained from the Center for Lipids Engineering Applied Research (CLEAR), Universiti Teknologi Malaysia (UTM).

Cell lines and culture

The human adenocarcinoma cell lines HT29 was purchased from American Type Culture Collection, ATCC (Rockville, USA). The monolayer cell lines were grown in T-25 flask containing McCoy's 5A Medium Modified supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Austria), 100 U/ml of penicillin and streptomycin (Flowlab, Australia) and 100 U/ml Amphotericin B (PAA laboratories GmbH, Austria) at 37°C in a humidified atmosphere of 5% CO2.

MTS Cell Viability Assay

Cell viability was determined by using MTS assay(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H-tetrazolium). The cells at a concentration of 2 x 10^4/ 100ul complete medium was seeded in 96 well plates and incubated for 24 hours at 37°C in 5% CO_2 incubator. The complete medium was changed and cells were treated with honey (12.5-400 mg/ml) and ginger extract (0.0625-4.0 mg/ml) dissolved in 0.01% DMSO as well as combination of both ginger and Gelam honey at various concentrations. Three replicates were performed for each treatment. The plate was further incubated for another 24 hours. After the incubation period, the culture medium was changed with MTS solution and incubated at 37°C, 5% CO_2 for another 2 hours. The reduction of the MTS solution was determined spectrophotometrically at 490 nm using ELISA microplate reader (VERSAmax, USA). The cells viability was calculated as a percentage for each treatment over the untreated control.

Combination Index Analysis

Combination index (CI) analysis was used to assess the interaction of combination of Gelam honey and ginger whether it is synergistic, additive or antagonistic. The concentrations required to produce 50% effect level (IC50) was determined for Gelam honey and ginger. CI was calculated using the equation: CI = (dH/DH) + (dG/DG) where dH and dG represent the concentration of Gelam honey and ginger in combination treatment and DH and DG represent the concentrations of Gelam honey and ginger in single treatment respectively. A combination index value below, equal or above 1 is indicated as synergism, additive or antagonistic effects respectively.

Apoptosis evaluation - flow cytometry

Apoptosis of cells was evaluated using annexin V-FITC/PI kit (Beckton-Dickinson, Canada) according to the manufacturer's protocol. Briefly, 1 x 10^6 cells were seeded in 60 mm petri dish and incubated at 37°C in 5% CO_2 incubator for 24 hours. After incubation period, the cells were treated with single treatment of Gelam honey, ginger and combination and re-incubated for another 24 hours. The floating cells in the medium were collected while the adherent cells were detached with Accutase (GE Healthcare). The collected cells were centrifuged for 5 minutes at 2000 rpm. The supernatant was removed and the cells pellet were washed with ice cold PBS. The cells were centrifuged again at 2000 rpm for 5 minutes. The cells pellet were resuspended in 100 μl ice-cold 1X binding buffer (0.1M HEPES/NaOH, pH 7.5, 1.4 M NaCl and 25 mM CaCl2) followed by staining with 5 μl FITC Annexin V and propidium iodide each, gently vortexed and incubated for 15 minutes at room temperature in the dark. Another 400 μl 1X binding buffer was added and the cells were analyzed by flow cytometry (FACS, Becton-Dickson, USA) within 1 hour.

RNA extraction and cDNA Synthesis

HT29 cells (1 X 106) were incubated for 24 hours at 37°C in 5% CO_2 after they were seeded in 60mm culture dish. The cells were harvested after 24 hours treatment with different doses of Gelam honey, ginger and combination of both. Total RNA was extracted with TRIzol reagent (Molecular Research Centre, USA) and subsequently purified by ethanol precipitation. Concentration and purity of extracted RNA was determined by optical density at wavelength 260 nm and 280 nm using Nanodrop ND-1000 (Thermo Fisher Scientific, USA). Revere transcription was performed using 1 μg of total RNA in a final reaction volume of 20 μl using iScriptTM Reverse Transcription Supermix (BIORAD, USA), according to the manufacturer's instructions.

Primer Design & Quantitative Real-time PCR (qRT-PCR)

All primers were designed by using Primer 3 software (http://frodo.wi.mit.edu/primer3), with reference of Genbank (http://www/ncbi.nlm.nih.gov) database. Specific set of primers used are shown in Table 1. The mRNA level was quantified by SYBR-green detection in an iQ5 Real Time Cycler machine (Bio-Rad, USA). The PCR amplification condition was as follows: initial denaturation at 95°C for 180 seconds, followed by 40 cycles at 95°C for 10 seconds (denaturation) and 56°C for 30 seconds (annealing). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference gene that acts as an internal reference to normalize the mRNA expression.

Statistical analysis

The experiments were conducted in triplicates and the results are expressed as mean ± standard deviation. Statistical evaluation was analysed using SPSS software version 22. The normality of each test was performed using the Shapiro-Wilk test. Normal distribution data was then tested with one-way ANOVA and Dunnett T3 was selected as post-hoc test. Non parametric test, Mann-Whitney U was used if the data values were not normally distributed. The results are considered significant if $p<0.05$.

Results

Effect of Gelam honey and ginger extract on proliferation of HT29 cells was determined using MTS assay (Figure 1). Data showed that the HT29 cells viability decreased significantly in dose dependent manner for both treatments with ginger being more potent compared to Gelam honey. The IC_{50s} obtained were 88 mg/ml and 2.15 mg/ml for Gelam honey and ginger treatment respectively.

Figure 2 showed the combination effect of Gelam

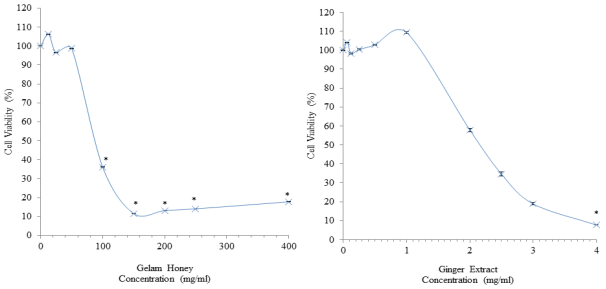

Figure 1. Inhibition of HT29 Cell Viability After Treatment with Gelam Honey and Ginger Extract. Data represents as mean ± SD of triplicate assays. *p<0.05 when compared to control (without treatment)

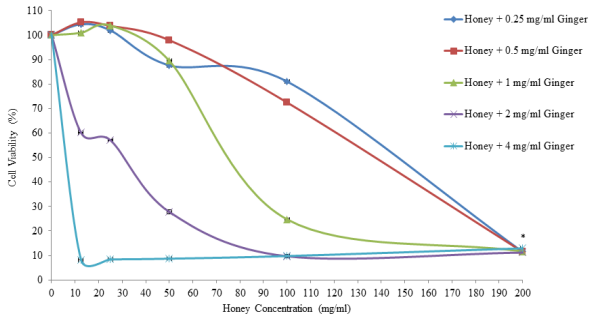

Figure 2. Inhibition of HT29 Cells Viability with Different doses of Combination Treatment of Gelam Honey and Ginger Extract. Data represent as mean ± SD of triplicate assays. * p<0.05 compared to control

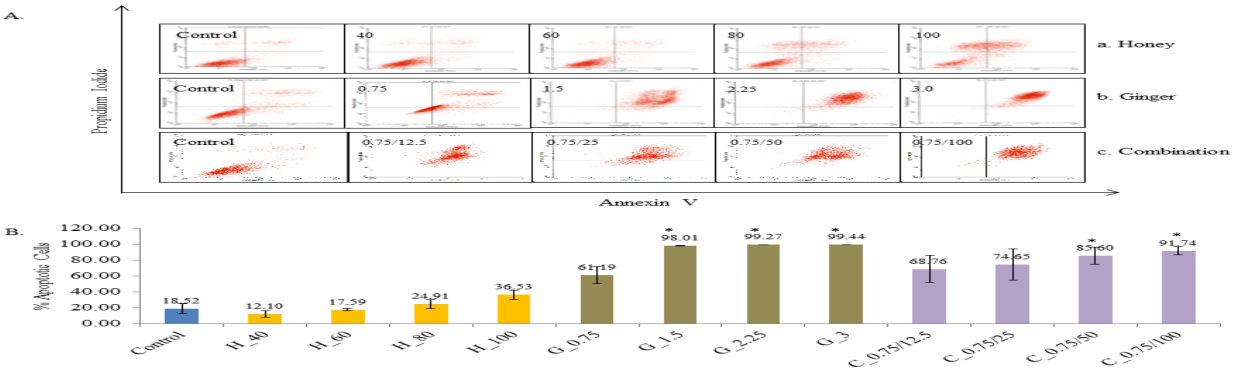

Figure 3. Effect of Gelam Honey, Ginger Extract and their Combination on Apoptosis of HT29 Cells by Flow Cytometry. (A). The cytogram of apoptosis analysis after treatment with a. Gelam honey, b. ginger and c. combination of Gelam honey and ginger. The value in the cytogram represents the concentration (mg/ml) used in the analysis. (B). The apoptosis rate (%) of HT29 cells after treatments. (H - gelam honey, G - ginger, C - combinations of Gelam honey and ginger). Data represent as mean ± SD of triplicate assays. * p<0.05 compared to control

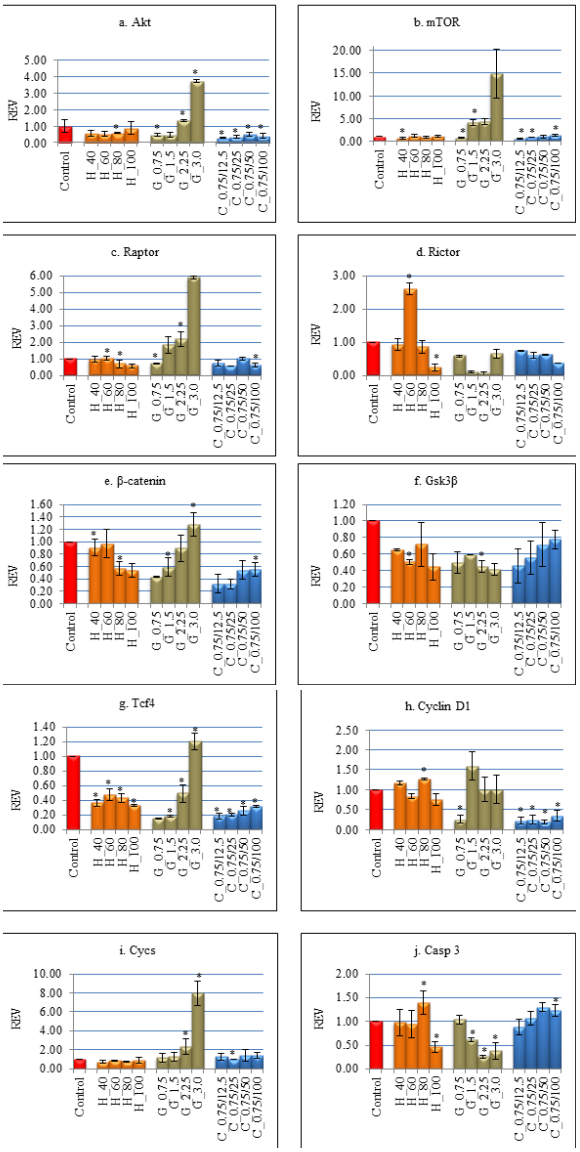

Figure 4. Expression of genes Involved in PI3k/Akt/mTOR, Wnt/β-catenin and Apoptosis Signaling Pathway After Treatment with Gelam Honey, Ginger and Combination of Both. Data represent as mean ± SD of triplicate assays. * p<0.05 compared to control (H - Gelam honey; G - Ginger; C - Combination of Gelam honey & ginger)

honey and ginger on the viability of HT29 cells. The IC_{50s} of single treatment for Gelam honey can be reduced with several combination formula of ginger extract. In combination with 4, 2, 1, 0.5 and 0.25 mg/ml, IC_{50} of honey were 6, 31, 75, 137 and 147 respectively. However combination index analysis showed antagonistic effect (Table 2).

Figure 3 showed that Gelam honey, ginger extract and their combination induced apoptosis of HT29 cells in dose dependent manner with most of the cells were mainly in late apoptosis stage. As seen from figure 3b, Gelam honey was less effective in inducing apoptosis of HT29 cells compared to ginger. Synergistic apoptosis effect of the combination treatment on HT29 cells was seen although the combination index analysis for viability of cells was shown earlier to be antagonistic. For example, combination formula of 100 mg/ml Gelam honey and 0.75 mg/ml ginger induced 91.74±5.27% HT29 cells to undergo apoptosis which is significantly higher than single treatment alone for the same concentration.

Figure 4 showed the gene expression of the PI3K/Akt/mTOR, Wnt/β-catenin and apoptosis pathways after treatment with Gelam honey, ginger and combinations of both. Akt gene expression group was down regulated almost to half as compared to control when treated with Gelam honey. In contrast, the expression of Akt was up regulated by ginger treatment ($p<0.05$) at 2.25 and 3.0 mg/ml. The combination treatment however, downregulated Akt gene expression ($p<0.05$) at all concentrations (Figure 4a). A similar pattern of expressions was observed with mTOR and Raptor genes (Figure 4b,c). Rictor gene was down regulated in almost all the treatment group with an exception of 60 mg/ml of honey treatment, whereby an up regulation of this gene was observed (Figure 4d).

The β-catenin and Tcf4 gene expressions were downregulated when treated with Gelam honey and its combination but were upregulated with ginger treatment (Figure 4e & f). Again, the combined treatment was far more effective in down regulation of the genes. The negative regulator of this signaling pathway, Gsk3β was seen to be downregulated with all treatments (Figure 4g). The expression of cyclin D1 was seen to be upregulated and down regulated with Gelam honey at 80 mg/ml and

Table 1. Primers Sequence for qRT-PCR

Gene	Primer Sequence	
	Forward	Reverse
AKT	GTCGCCTGCCCTTCTACAAC	CACACGATACCGGCAAAGAA
MTOR	AGTGGACCAGTGGAAACAGG	CAGTTCAGACCAGCAGGACA
RPTOR	GGACCTGGCTGTTGACATCT	TGCACTTGGCTTAACAGCAC
RICTOR	CGGTTGTAGGTTGCCAGTTT	CATGAGGGTGGCAAGAAAGT
CTNNB1	GGAAGGTCTCCTTGGGACTC	TGATGTCTTCCCTGTCACCA
GSK3B*	ATTTCCAGGGGATAGTGGTGT	TCCTGACGAATCCTTAGTCCAAG
TCF4**	CATGCCGCAGCTGAACGGCGGT	TCATTCCGCCTCGGAATCGGAGGAG
CCND1	CCCTCGGTGTCCTACTTCAA	GGGGATGGTCTCCTTCATCT
CYCS	AAGTGTTCCCAGTGCCACA	GTTCTTATTGGCGGCTGTGT
CASP3	CAGATGTCGATGCAGCAAAC	TGGCTCAGAAGCACACAAAC
GAPDH	TCCCTGAGCTGAACGGGAAG	GGAGGAGTGGGTGTCGCTGT

* - Zeng et al. (2014); ** - Mologni et al. (2012)

Table 2. CI value of Combination of Gelam Honey and Ginger Extract

Concentration (mg/ml)	Gelam Honey	147	137	75	31	6
	Ginger	0.25	0.5	1	2	4
Combination Index		1.77	1.79	1.32	1.28	1.93
Description		Antagonistic	Antagonistic	Moderate antagonistic	Moderate antagonistic	Antagonistic

ginger at 0.75 mg/ml respectively ($p<0.05$), and the combination showed down regulation of the gene in all treatment groups ($p<0.05$) (Figure 4h).

In apoptosis signaling, the low expression of cytochrome C was observed when treated with gelam honey but it was up regulated when treated with ginger and the combined treatment (Figure 4i). Ginger downregulated the expression of caspase 3 gene at almost all concentrations, while Gelam honey down regulated its expression at 100 mg/ml only ($p<0.05$). The expression of caspase 3 was up regulated with 80 mg/ml Gelam honey and the combination treatment at 0.75 mg/ml ginger with 50 and 100 mg/ml Gelam honey ($p<0.05$) (Figure 4j)

Discussion

Plant based compounds are becoming a popular alternative therapy for many chronic diseases including colorectal cancer. The miracle substance phytochemicals found in many plants and all parts of plants (flowers, fruits, barks, roots and seeds) were known to exhibit antioxidant effects by eliminating the reactive oxygen species (ROS) known to cause organ/tissue damage in the body leading to many chronic diseases (Palmer and Paulson, 1997; Mandal et al., 2009).

Ginger has been used as a cooking spice for decades and some of the medicinal properties associated with ginger are: antiemetic, anti-tumour, anti-inflammation, anti-oxidative activities and for treatment of, gingivitis, toothache, asthma, stroke, constipation and diabetes (Surh 2002; Shukla and Singh, 2007; Ali et al., 2008). Polyphenols in ginger extract were shown to exhibit very high antioxidant activity (Shukla and Singh, 2007; Ali et al., 2008). Gelam honey was also shown to contain many polyphenols with antioxidant activity such as gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, ellagic acid, quercetin, hesperetin and chrysin (Hussein et al., 2012; Teh et al., 2012). The polyphenols were shown to exert anti-inflammatory effect by inhibiting the production of nitric oxide and prostaglandin E2 while reducing edema and pain (nociceptive activity) in inflammatory tissues in rats induced with paw edema (Kassim et al., 2010). The anti-inflammatory property of Gelam honey was also reported to be mediated by NF-ϰB pathway in rats induced with paw edema (Hussein et al., 2013).

The synergistic effect of chemoprevention using combination food therapy has been a major interest lately. EGCG and curcumin showed synergistic effect via cell cycle arrest at the G1 and G2/M phase in oral cancer cells. The combination therapy also helps in reducing the dose used, of EGCG and curcumin to 4.4-8.5 and 2.2-2.8 fold respectively as indicated by the dose reduction index (DRI) compared to a single treatment (Khafif et al., 1998). Shimuzu et al. (2005) reported that combinations of 1μg/ml of epicatechin with 10 μg/ml of EGCG displayed synergistic effects on HT29 cells growth inhibition and induction of apoptosis. We have evaluated the combinations of Gelam honey and ginger in their ability to halt the proliferation of HT29 colon cancer cells and found that combination treatment inhibited growth of HT29 colon cancer cells better than single treatment with reduction of IC_{50} of Gelam honey from 88 mg/ml to 75 mg/ml when combined with 1 mg/ml of ginger. Surprisingly, the combination index analysis showed moderately antagonistic effect contrary to a previous finding which showed that when 3 mg/ml of aqueous ginger extract was combined with 27 mg/ml of Gelam honey a synergistic inhibition effect in killing HT29 cells was seen (Tahir, 2015). The different results obtained could be due to the water based extraction of ginger in the previous study compared to alcohol ginger extract used in the present study.

HT29 cells were induced to undergo apoptosis more potently by ginger extract compared to Gelam honey. Similarly, Abdullah et al. (2010) and Teh et al. (2012) reported that ginger was able to induce apoptosis of HCT116 and HT29 cells in a dose dependent manner by arresting cell cycle at G0/G1 phase. We noticed a synergistic effect on the rate of apoptosis in a dose dependent manner when low concentration of ginger was combined with various concentrations of Gelam honey, and the effect was far better than in single treatment. Majumdar et al. (2009) showed that curcumin and resveratrol combination induced colon cancer cells, HCT116 p53+/+ and p53-/- to undergo apoptosis more effectively compared to the single treatment and at the same time, inhibited the activation of EGFR accompanied by attenuation of NFϰB activity.

mTOR is one of the signalling pathways which is frequently associated with various cancers and it is a downstream target of PI3K/Akt (Khan et al., 2013). Mutations of genes such as PIK3CA and PTEN of PI3K-Akt-mTOR signalling pathway lead to cell proliferation and survival of the colorectal cancer cells (Gulhati et al., 2009, Wu et al., 2013).

Down regulation of Akt gene expression was observed in HT29 cells treated with Gelam honey but not with ginger treatment in contrary to findings of Tahir et al., (2015) who had previously shown that the expression of Akt was down regulated after ginger treatment. Interestingly, we had shown that in combination treatment, Akt gene expression was down regulated and perhaps the action was attributed by the Gelam honey treatment. Down regulation of Akt gene expression was also reported by Nakamura et al. (2009) using combination

of indol-3-carbinol and genistein. They had also shown the combination treatment was able to induce apoptosis through inhibition of Akt activity and progression of the autophagic process mediated by the dephosphorylation of mTOR protein.

We observed upregulation of mTOR and raptor genes after treatment with ginger while downregulation of the genes were observed with Gelam honey and when combined with ginger extract. We speculated that the elevated expressions of Akt, mTOR and Raptor genes after ginger treatment may be the results of the resistance of HT29 cells to the ginger ethanolic extract or perhaps the ethanol itself acts as a stimulant of the respective genes. On the other hand, down regulation of the genes expression observed with honey and combination treatment may be explained due to the bioactive compounds or polyphenols found in Gelam honey.

The Cancer Genome Atlas (2012) reported that adenomatous polyposis coli (APC) mutation was detected in 93% of colorectal cancer tumor and 94% of the tumor was found with mutation of genes involved with Wnt/β-catenin signaling pathway. Previous studies found that natural bioactive compound was able to influence the β-catenin level and activity. The isoflavone genistein from soy bean decreased accumulation of nuclear β-catenin, decreased c-myc, cyclin D1, Wnt 5a, Sfrp1 Sfrp2 and Sfrp5 levels in azoxymethane (AOM)-induced rat colon cancer model (Zhang et al., 2013). Resveratol disrupted the formation of β-catenin/Tcf complex in the nucleus, supressed the Wnt target genes such as cyclin D1, conductin and inhibited the growth of Wnt-stimulated cells and Wnt-driven colorectal cancer cells (Chen et al., 2012). In this study, Gelam honey was shown to down regulate β-catenin gene expression in a dose-dependent manner. Unexpectedly, up regulation of Beta-catenin expression was observed with ginger treatment whereas the combinations treatment down regulated its expression.

Tcf4 was down regulated with Gelam honey and the combination treatment. A peculiar behavior was seen with ginger treatment, whereby, down regulation of Tcf4 gene expression was observed at the lower concentrations but it was upregulated at the highest concentration (3 mg/ml). This peculiar event may be due to the ethanol used in extraction method, that may serve as stimulant at higher concentration or the ginger itself may act as pro-oxidant at higher concentrations.

Cyclin D1 is a rate-limiting factor during cell cycle progression through G1 phase (Klein and Assoian, 2008). High level of cyclin D1 was found in many cancer cells (Klein and Assoian, 2008), thus, by targeting cyclin D1, it is one of the alternative targets in cancer therapy. However, we did not find changes in cyclin D1 gene after single treatment of Gelam honey and ginger but the combination treatment was effective in down regulating cyclin D1.

Gsk3β, which is the negative regulator of the Wnt/β-catenin was analysed and unexpectedly we found this gene to be down regulated in all treatment. In contrast, treatment with quercetin in SW480 colon cancer cells did not interfere the level of Gsk3β as reported by Park et al. (2005). On the other hand, treatment with sphingadienes was found to reduce the level of phospho-GSK3β in APCMin/+ mice as well as in HT29, SW480, DLD1 and HCT 116 colon cancer cell lines (Kumar et al., 2012).

Gene expressions of cytochrome C and caspase 3 which are involved in the intrinsic pathway were evaluated in this study. An intrinsic pathway is triggered by an internal stimuli such as DNA damage, hypoxia and severe oxidative stress causing mitochondria to release cytochrome C to the cytoplasm which then formed an apoptosome complex consisting of cytochrome C, pro-caspase 9 and Apaf1 and eventually triggers activation of pro-caspase 3 (Wong, 2011). We did not see an up regulation of cytochrome C gene with Gelam honey treatment but the combination treatment upregulated cythochrome C. As for caspase 3, an inconsistency of its expression was noted with single treatment of Gelam honey and ginger extract treatment. We found that caspase 3 gene expression was up regulated at 80 mg/ml of Gelam honey followed by its down regulation at 100 mg/ml. At all concentrations of ginger with the exception of 0.75 mg/ml, down regulation of caspase 3 expression was observed. Interestingly, up regulation of caspase 3 expression was observed in combination treatment.

The erratic behavior of caspases could be explained by the fact that apoptosis occurs in relatively short time for about 2-3 hours, from its initiation to completion. The caspases are expressed transiently, thus, cultured cells undergoing apoptosis in vitro will undergo secondary necrosis (Elmore, 2007). From flow cytometry data, most of the cells were found to be at late apoptosis stage after 24 hours treatment, it is plausible that caspase 3 protein was further degraded after apoptosis occurrence. Similar findings were also reported by Fauzi et al. (2011) where Tualang honey induced late stage apoptosis after 24 hours treatment in MDA-MB-231 breast cancer cells.

Other phytochemicals shown to trigger apoptosis in HT29 colon cancer cells via intrinsic pathway were maslinic acid (Reyes-Zurita, 2011) and kaempferol (Lee et al., 2014). Combination treatment using quercetin and trans-resveratol was found to trigger MIA-PACA2 pancreatic carcinoma cells to apoptosis stage. Combination of these substances was found to be far more effective in inducing the release of cytochrome C and caspase 3 activation compared to single treatment respectively (Mouria et al., 2002).

In conclusion, we find that the combination treatment of Gelam honey and ginger extract were far more effective in halting or delaying the growth of HT29 colon cancer cells through induction of apoptosis and modulation of genes involved in PI3K/Akt/mTOR and Wnt/β-catenin pathways. Thus, the combination of dietary phytochemicals found in both Gelam honey and ginger extract may serve as an alternative or complimentary therapy for colon cancer in the future.

Acknowledgements

This study was funded by Universiti Kebangsaan Malaysia Grant no. FF-2013-442. The authors duly acknowledge the contributions from the staff of Biochemistry Department, Medicine Faculty, UKM.

References

Abdullah S, Abidin SAZ, Murad NA, et al (2010). Ginger extract (Zingiber officinale) triggers apoptosis and G0/G1 cells arrest in HCT 116 and HT 29 colon cancer cell lines. *Afric J Biochem Res*, **4**, 134-42.

Ali BH, Blunden G, Tanira MO, et al (2008). Some phytochemical, pharmacological and toxicological properties of ginger (Zingiber officinale Roscoe): A review of recent research. *Food Chem Toxicol*, **46**, 409-20.

Amundson SA, Myers TG, Scudiero D, et al (2000). An informatics approach identifying markers of chemosensitivity in human cancer cell lines. *Cancer Res*, **60**, 6101-10.

Araujo JR, Goncalves P, Martel F (2011). Chemopreventive effect of dietary polyphenols in colorectal cell lines. *Nutr Res*, **31**, 77-87.

Chen HJ, Hsu LS, Shia YT, et al (2012). The β-catenin/Tcf complex as a novel target of resveratrol in the Wnt/β-catenin signaling pathway. *Biochemical Pharmacol*, **84**, 1143-53.

Elmore S (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol*, **35**, 495-516.

Fauzi AN, Norazmi MN, Taacob NS (2011). Tualang honey induces apoptosis and disrupts the mitochondrial membrane potential of human breast and cervical cancer cell lines. *Food Chem Toxicol*, **49**, 871-8.

Guertin DA, Sabatini DM (2007). Defining the role of mTOR in cancer. *Cancer Cell*, **12**, 9-22.

Gulhati P, Cai QS, Li J, et al (2009). Targeted inhibition of mTOR signaling inhibits tumorigenesis of colorectal cancer. *Clin Cancer Res*, **15**, 7207-16.

Hakim L, Alias E, Makpol S, et al (2014). Gelam honey and ginger potentiate the anti cancer effect of 5-FU against HCT116 colorectal cancer cells. *Asia Pac J Cancer Prev*, **15**, 4651-7.

Herbst A, Jurinovic V, Krebs S, et al (2014). Comprehensive analysis of β-catenin target genes in colorectal carcinoma cell lines with deregulated Wnt/β-catenin signaling. BMC Genomics, 15, 74.

Hussein SZ, Yusoff KM, Makpol S, et al (2012). Gelam honey inhibits the production of proinflammatory, mediators NO, PGE2, TNF-α, and IL-6 in carrageenan-induced acute paw edema in rats. *Evid Based Compl Alt Med*, **2012**, 109636.

Hussein SZ, Yusoff KM, Makpol S, et al (2013). Gelam honey attenuates carrageenan-induced rat paw inflammation via NF-ϰB pathway. *PLoS ONE*, **8**, 72365.

Jemal A, Bray F, Center M, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69-90.

Kassim M, Achoui M, Mansor M, et al (2010). The inhibitory effects of Gelam honey and its extracts on nitric oxide and prostaglandin E2 in inflammatory tissues. *Fitoterapia*, **81**, 1196-201.

Khafif A, Schantz SP, Chou TC, et al (1998). Quantitation of chemopreventive synergism between (-)-epigallocatechin-3-gallate and curcumin in normal, premalignant and malignant human oral epithelial cells. *Carcinogenesis*, **19**, 419-24.

Khan KH, Yap TA, Cunningham D (2013). Targeting the PI3K-AKT-mTOR signaling network in cancer. *Chin J Cancer*, **32**, 253-65.

Klein EA, Assoian RK (2008). Transcriptional regulation of the cyclin D1 gene at a glance. *J Cell Sci*, **121**, 3853-7.

Kumar A, Pandurangan AK, Lu F, et al (2012). Chemopreventive sphingadienes downregulate Wnt signaling via PP2A/Akt/GSK3β pathway in colon cancer. *Carcinogenesis*, 1-38.

Lee HS, Cho HJ, Yu R, et al (2014). Mechanisms underlying apoptosis-inducing effects of kaempferol in HT-29 human colon cancer cells. *Int J Mol Sci*, **15**, 2722-37.

Majumdar APN, Nautiyal J, Banerjee S, et al (2009). Curcumin synergizes with resveratrol to inhibit colon cancer. *Nutr Cancer*, **61**, 544-53.

Mandal P, Misra TK, Ghosal M (2009). Free-readical scavenging activity and phytochemical analysis in the leaf and stem of Drymaria diandra Blume. *Int J Integ Biol*, **7**, 80-4.

Mologni L, Brussolo S, Ceccon M, et al (2012). Synergistic effects of combined Wnt/KRAS inhibition in colorectal cancer cells. *PLoS ONE*, **7**, 51449.

Moran A, Ortega P, Juan C, et al (2010). Differential colorectal carcinogenesis: Molecular Basis and clinical relevance. *World J Gastrointest Oncol*, **2**, 151-8.

Mouria M, Gukovskaya AS, Jung Y, et al (2002). Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome c release and apoptosis. *Int J Cancer*, **98**, 761-9.

Nakamura Y, Yogosawa S, Izutani Y, et al (2009). A combination of indol-3-carbinol and genistein synergistically induces apoptosis in human colon cancer HT-29 cells by inhibiting Akt phosphorylation and progression of autophagy. *Molec Cancer*, **8**, 100.

Palmer HJ, Paulson KE (1997). Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr Rev*, **55**, 353-61.

Park CH, Chang JY, Hahm ER, et al (2005). Quercetin, a potent inhibitor against β-catenin/Tcf signaling in SW480 colon cancer cells. *Biocheml Biophys Res Comm*, **328**, 227-34.

Reyes-Zurita FJ, Pachon-Pena G, Lizarraga D, et al (2011). The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer*, **11**, 154.

Shimuzu M, Deguchi A, Lim TE (2005). (-)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin Cancer Res*, **11**, 2735-46.

Shimuzu M, Deguchi A, Lim TE (2005). (-)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. *Clin Cancer Res*, **11**, 2735-46.

Shukla Y, Singh M (2007). Cancer preventive properties of ginger: A brief review. *Food Chem Toxicol*, **45**, 683-90.

Soerjomataram I, Oomen D, Lemmens V, et al (2010). Increased consumption of fruit and vegetables and future cancer incidence in selected European countries. *Eur J Cancer*, **46**, 2563-80.

Surh YJ (2002). Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: a short review. *Food Chem Toxicol*, **40**, 1091-7.

Tahir AA, Abdul Sani NF, Morad NA, et al. (2015). Combined ginger extract & Gelam honey modulate Ras/ERK and PI3K/AKT pathway genes in colon cancer HT29 cells. *Nutr J*, **14**, 31.

Teh PW, Hussein SZ, Abdullah S, et al (2012). Gelam and nenas honeys inhibit proliferation of HT29 colon cancer cells by inducing DNA damage and apoptosis while suppressing inflammation. *Asia Pac J Cancer Prev*, **13**, 1605-10.

The Cancer Genome Atlas Network (2012). Comprehensive molecular characterization of human colon and rectal cancer. *Nature*, **487**, 330-7.

Vara JAF, Casado E, Castro J, et al (2004). PI3K/Akt signalling pathway and cancer. *Cancer Treatment Rev*, **30**, 193-204.

Wong SY (2011). Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res*, **30**, 87.

Wu KK, Wang XJ, Cheng SL, et al (2013). Dysregulation and crosstalk of cellular signaling pathways in colon carcinogenesis. *Crit Rev Oncol/Hematol*, **86**, 251-77.

Zainal AO, Nor Saleha IT (2011). National cancer registry report Malaysia Cancer statistics 2007. ministry of health Malaysia.

Zeng J, Liu D, Qiu Z, et al (2014). GSK3β overexpression indicates poor prognosis and its inhibition reduces cell proliferation and survival of non-small cell lung cancer cells. *PLoS ONE*, **9**, 91231.

Zhang J, Liu Z, Huang Y, et al (2013). GSK3β overexpression indicated poor prognosis and its inhibition reduces cell proliferation and survival of non-small cell lung cancer cells. *PLoS ONE*, **9**, 91231.