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Inhibitory effect of Manuka honey on human colon cancer HCT-116 and LoVo cells growth. Part 1: Suppression of proliferation, promotion of apoptosis and arrest of cell cycle

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Abstract

Numerous investigations have been made on plant phenolic compounds and cancer prevention in recent decades. Manuka honey (MH) represents a good source of phenolic compounds such as luteolin, kaempferol, quercetin, gallic acid and syringic acid. The aim of this work was to evaluate the chemopreventive effects of MH on human colon cancer HCT-116 and LoVo cells. Both cells were exposed to different concentration of MH (0-20 mg/mL for HCT-116 cells and 0-60 mg/mL for LoVo cells) for 48 h to measure apoptosis and cell cycle arrest as well as apoptosis and cell cycle regulatory gene and protein expression. MH exhibited profound inhibitory effects on cellular growth by reducing the proliferation ability, inducing apoptosis and arresting cell cycle in a dose-dependent manner. Interestingly, MH treatment in non-malignant cells did not exert any significant toxicity at similar concentration. The apoptosis event was associated with increasing expression of p53, cleaved-PARP and caspase-3, and with the activation of both intrinsic (caspase-9) and extrinsic (caspase-8) apoptotic pathways. MH induced cell cycle arrest at S phase in HCT-116 cells, simultaneously, in LoVo cells, it arrested at G2/M phase through the modulation of cell cycle regulator genes (cyclin D1, cyclin E, CDK2, CDK4, p21, p27 and Rb). The expression of p-Akt was suppressed while the expression of p-p38MAPK, p-Erk1/2 and endoplasmic stress markers (ATF6 and XBP1) was increased for apoptosis induction. Overall, these findings indicate that MH could be a promising preventive or curative food therapy for colon cancer.

Keywords: Manuka honey, phenolic compounds, apoptosis, cell cycle arrest, endoplasmic reticulum stress, chemoprevention.

1. Introduction

Globally, colorectal cancer (CRC) is the third most widespread cancer in both men and women; over 1 million new cases are diagnosed each year accounting for 9.7% of all cancers apart and consequently more than 693,933 deaths per annum corresponding to 8.5% of the total number of cancer deaths.[1] Notably, about 50% CRC patients develop the recurrent disease[2] indicating that presently existing treatments are not able to control this deadly disease. Furthermore, CRC has a significant impact on medical care as well as on the global economy. In fact in the USA alone, the expenditure of CRC treatment is estimated to exceed $17 billion in the healthcare system by 2020.[3]

Apoptosis process plays a critical role in cancer pathogenesis: indeed, a failure to undergo apoptosis results in the development and progression of cancer. The intrinsic apoptotic pathway involves distraction of the mitochondria membrane potential promoted by regulating apoptotic protein and responses against several intracellular stresses, while extrinsic apoptotic pathway activates by binding with cell surface receptor their specific ligands.[4] Several molecules and processes modulate the apoptosis process, largely through caspase cascade activation, which breakdown specific proteins in the cytoplasm and nucleus triggering cell death.[4] Apoptosis effects may be induced in cancer cells by regulating of cell cycle progression.[5] Dysregulation of cell cycle is one of the features of carcinogenesis which contributes to the abounded proliferation in human cancer.[6]

The epidermal growth factor receptor (EGFR) and in a smaller extent human epidermal growth factor receptor 2 (HER2) are key surface receptors which can activate or overexpress a number of oncogenic pathways which play an important function in colon cancer proliferation, survival, angiogenesis, invasion and metastasis,[7] representing an attractive therapeutic target for anticancer

therapy. The phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) signaling pathway can be activated by several growth factors and by G-protein-coupled receptors upon stimulation with phospholipids and chemokines that can activate the signals for cell survival and proliferation, cell cycle and anti-apoptotic effects leading to CRC carcinogenesis.[8] The mitogen-activated protein kinase (MAPK) can convert various extracellular signals into intracellular responses through serial phosphorylation cascades. The major MAPK families containing extracellular-signal-regulated kinase 1/2 (Erk1/2), Jun N-terminal kinase (JNK), and p38 MAPK, are well-known to transmit, integrate and triggers signals from various stimuli for controlling inflammation, cellular proliferation and differentiation as well as apoptosis in different cancers.[9]

The endoplasmic reticulum (ER) is the principle site for protein maturation, folding and secretion, and maintenance of phospholipid biosynthesis and calcium homeostasis. ER stress induced cancer cell death has raised growing attention because it seems to increase the efficacy of chemotherapeutic mediators by eradicating the damaged cells via activation of both intrinsic and extrinsic apoptosis pathways.[10]

The use of various natural and synthetic drugs for CRC prevention has attained remarkable attention in recent years; in this context natural food products may represent a valid alternative, because of their chemopreventive or chemotherapeutic properties.[11, 12] Honey is a good source of natural therapeutic molecules with antibacterial, wound healing, antioxidant, anti-inflammatory, and anticancer properties.[13-15] Manuka honey (MH) is a particular type of honey collected from the *Leptospermum scoparium* tree (Family: Myrtaceae), which has been widely investigated for its antioxidant, antibacterial and wound healing activities as well as for its physiochemical properties and attractive biological and pharmacological compounds.[13, 14] However, there has been a sporadic effort on the anticancer activity of whole honey on human colon cancer *in vitro*[15]

and only two investigations have reported the antiproliferative effect of MH on colon cancer cells.[16, 17]

The present study was designed to characterize the anti-proliferative and apoptotic activities of MH in human colon adenocarcinoma cells (HCT-116) and Dukes' type C, grade IV, colon metastasis cells (LoVo). Furthermore, the effect of MH on the cell cycle, MAPK signaling and ER stress highlighting the underlying molecular mechanism involved in these cell types were also investigated.

2. Materials and methods

2.1. Honey samples and reagents

MH samples originating from New Zealand were imported to Italy by EfitSrl and kept at 4°C until analysis. The samples were collected from mid spring to late summer in 2014. All reagents were purchased from Sigma-Aldrich (Milan, Italy), Extrasynthese (Genay, France) and SAFC (St. Louis, MO, USA). The primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), while goat anti-rabbit IgG peroxidase secondary antibody was purchased from Sigma-Aldrich (Milan, Italy).

2.2. Extraction, identification and quantification procedure for phenolic compounds of MH

2.2.1. Solid-phase extraction (SPE) and HPLC conditions

MH was pre-concentrated with Strata X-A cartridges 33u Polymeric Strong Anion sorbent (60 mg, 3 mL size) from Phenomenex (Torrance, CA, USA). The SPE method was carried out according to a slight modification as previously reported.[18, 19] MH (3 g) were mixed with ultrapure water (12 mL) and then the solution was adjusted to pH=2 with concentrated HCl. The

fluid samples were centrifuged at 8000 x g for 10 min to remove the solid particles. The supernatants were loaded onto the previously conditioned cartridges (under these conditions: methanol (3 mL), equilibrated as follows: acidified ultrapure water (pH = 2; 3 mL)). After loading, these cartridges were washed with acidified ultrapure water (pH = 2; 4 mL) to remove sugars and other polar compounds of honey that were not absorbed on the sorbents. Then, phenolic compounds retained on the cartridges were eluted with formic acid: methanol (1:9, v/v; 5 mL). The eluate was evaporated down (25 °C, 5 psi) and then reconstituted in methanol with 2% acetic acid (2 mL). All solutions were filtered through a 0.22-µm filter prior to HPLC injection. HPLC measurements were made by using a Thermo Scientific System equipped with a Spectra SYSTEM P 4000 pump, a Spectra SYSTEM AS 3000 auto sampler and a Finnigan Surveyor PDA Plus Detector. Chromatographic separations were performed with a Luna C18 analytical column (150 x 3 mm ID, 3-µm particle) with a guard column (4 x 3 mm ID) containing the same packing material, both from Phenomenex (Torrance, CA, USA).

2.2.2. Determination of flavonols and phenolic acids

An aliquot (20 µL) was injected into the column and eluted at 35°C with a constant flow rate of 0.4 mL/min. The mobile phase was composed of water/formic acid/acetonitrile (87:10:3), v/v/v; Component A) and water/formic acid/acetonitrile (40:10:50, v/v/v; Component B) for flavonols and for phenolic acids the mobile phase was composed of 2% (v/v) acetic acid in water (Component A) and of acetic acid in water and acetonitrile (1:49:50, v/v/v; Component B). The following gradient was used for flavonols: 90% A, changed to 75% A in 10 min, changed to 69% A in 5 min, changed to 60% A in 5 min, changed to 50% A in 10 min, changed to 0% in 10 min, held for 5 min and, finally changed to 90% A in 5 min giving an analysis time of 50 min.

Similarly, for phenolic acid the following gradient was used: 90% A for 10 min, changed to 45% A in 50 min, changed to 2% A in 10 min, changed to 90% A in 2 min and, finally held for 10 min giving an analysis time of 82 min. A photodiode-array detector was employed in full-scan mode (range between 200 and 600 nm) for the determination of phenolic acids and flavonols.

2.3. Cell culture

Human colon adenocarcinoma (HCT-116), Dukes' type C, grade IV, colon metastasis (LoVo) and healthy human dermal fibroblast (HDF) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Media and reagents for cell culture were obtained from Carlo Erba Reagents (Milan, Italy). McCoy's 5A media was used for HCT-116 cell culture, F-12K media were used for LoVo cells culture and DMEM media were used for HDF cells culture. All the media was prepared with of 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 µg /mL streptomycin. All cell lines were maintained in an incubator at 37°C in a humidified atmosphere (95% air, 5% CO_2). For the subsequent experiment, cells were used between the 6^{th} and10^{th} passages.

2.4. Cell viability assay

Cells were seeded at a density of 5×10^3 cells/well into 96-well plates using the specific complete growth medium. To allow cell attachment, they were incubated overnight. After overnight incubation, the HCT-116 cells were treated with 0 to 20 mg/mL of MH for 24 to 72 h. Similarly, LoVo and HDF cells were treated with 0 to 60 mg/mL of MH for 24 to 72 h. After the incubation time, 30 µL of RPMI medium containing 2 mg/mL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added and cells were incubated for other 2 to 4 h. The

generated formazan crystals were dissolved by adding 100 µL of dimethyl sulfoxide in each well and measured by a microplate reader (Thermo Scientific Multiskan EX, Monza, Italy) at 590 nm. The proportion of viable cells was computed as absorbance of treated cells/absorbance of untreated cells x100.

2.5. Determination of apoptotic cells by Tali® Image-Based Cytometer

Apoptotic cells were identified by the Tali™ Apoptosis Assay Kit–Annexin V Alexa Fluor® 488 (Invitrogen™, Life Techonoliges, Milan, Italy) as reported earlier.[14] Cells were seeded at a density of 1.5×10^5 cells/well into 6 well and treated for 48 h with the MH (0, 10, 15 and 20 mg/mL for HCT-116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells). Cells were harvested 48 h post-treatment and centrifuged for 15 min at 1500 rpm at 4°C. After removing the supernatant and re-suspending the cells with 100 µL of Annexin binding buffer (ABB), 5 µL of Annexin V Alexa Fluor® 488 was added to each 100 µL of re-suspended cells. The mixture of cell and Annexin V Alexa Fluor® 488 was incubated at room temperature into the dark for 20 min and then again centrifuged. After removing the excess mixture and re-suspending with 100 µL of ABB, samples were incubated at room temperature into the dark for 1 to 5 min after adding 1 µL of Tali™ propidium iodide (PI). For each sample, 25 µL of cell suspension was loaded into one Tali™ Cellular Analysis Slide's chamber and analyzed in the Tali™ Image-Based Cytometer (Invitrogen™, Life Techonoliges, Milan, Italy). The instrument works at different excitation/emission wavelengths: 530/580 nm and 458/495 nm for the PI and Annexin V, respectively. The Tali™ Image-Based Cytometer was evaluated the live, apoptotic and dead cells. The annexin V-negative/PI negative cells were identified as viable cells by the cytometer software whereas the annexin-V positive/ PI negative cells were recognized as apoptotic cells.

Similarly, the annexin V positive/ PI positive cells were identified as dead cells. All data were reported as a mean value of three independent analyses ± standard deviation (SD).

2.6. Cell cycle analysis by Tali® Image-Based Cytometer

Cell cycle analysis was determined by the Tali® Cell Cycle Kit (Invitrogen™, Life Techonoliges, Milan, Italy) as previously described.[20] Cells were cultured in 6-well plates, at a density of 4×10^5 cells/ well and incubated with various concentration of MH 0, 10, 15 and 20 mg/mL for HCT-116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells for 48 h. Then, cells were trypsinized and centrifuged for 5 min at 500 x *g* after removing the excess media, resuspending with PBS, centrifuging for 5 min at 500 x *g* and transferring the cells into ice. Briefly, the cells were harvested and fixed with 70% cold ethanol at -20°C overnight. The fixed cells were washed twice with PBS, re-suspended in 100 µL PBS-based PI (Invitrogen™, Life Techonoliges, Milan, Italy) solution containing 0.1% Triton® X-100, 0.2 mg/ml RNase A (Invitrogen), and 20 µg/ml PI, and incubated for 30 min at room temperature protected from the light. For each sample, 25 µL of cell suspension was loaded into one Tali™ Cellular Analysis Slide's chamber (Invitrogen™, Life Techonoliges, Milan, Italy) and analyzed in the Tali™ Image-Based Cytometer. The results were expressed as the percentage of cells in each phase and all data were reported as a mean value of three independent analyses ± SD.

2.7. RNA isolation and quantitative real-time PCR analysis

HCT-116 and LoVo cells were treated with different concentrations of MH (0, 10, 15, 20 mg/mL for HCT-116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells) for 48 h. Total RNA content of cells was isolated using a PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according

to the manufacturer's protocol. RNA concentrations and purity were checked by using the microplate spectrophotometer system (BioTek Synergy HT, Winooski, USA). The sample ratio between 1.8 to 2.1 was only selected for gene expression assay. cDNA was synthesis from 100 ng RNA following reverse transcription according to the manufacturer's protocol (5X All-In-One RT MasterMix kit, Applied Biological Materials Inc. Canada). Real-time PCR was performed using EvaGreen 2X qPCR MasterMix (EvaGreen 2X qPCR MasterMix kit, Applied Biological Materials Inc. Canada) of forward and reverse primers (Supplementary Table 1) of cyclin D1, cyclin E, cyclin dependent kinase (CDK)2, CDK4, p21, p27, phosphorylated (p)-retinoblastoma (Rb), B-cell lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), cytochrome c (Cyt c), fatty acid synthetase ligand (FasL), caspase-8 and caspase-9 with 50 ng cDNA in a final volume 20 µL on a real-time PCR system (Corbett Life Science, Rotor-Gene 3000, Mortlake, Australia). RT-PCR control GADPH was amplified under the same PCR conditions for normalizing quantitative data. The $2^{-\Delta\Delta ct}$ method was used for calculating the fold change values.

2.8. Protein extraction and western blotting

HCT-116 and LoVo cells were treated with different concentrations of MH (0, 10, 15, 20 mg/mL for HCT-116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells) for 48 h. Protein lysates were prepared from cell pellets by using lysis buffer (120 mmol/L NaCl, 40 mmol/L Tris [pH 8], 0.1% NP40) with protease inhibitor cocktails (Sigma) and centrifuged at 13000 x g for 15 min. Proteins from cell supernatants were alienated on 8 or 10% polyacrylamide gel and then transferred into a nitrocellulose membrane, using the trans-blot SD semidry electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% non-fat-milk

with Tris HCl buffered saline with Tween 20 (TBST) for 1 h at room temperature. The primary antibodies p53, caspase-3, cleaved-PARP (c-PARP), phosphorylated (p)- p38MAPK, p-Erk1/2, EGFR, HER2, p-Akt, activating transcription factor 6 (ATF6), X-box-binding protein 1 (XBP1) and glyceraldehyde-3-phosphate dehydrogenase (GADPH) (1:500 dilutions) were used after overnight incubation at 4°C. Membranes were washed 3 times with TBST and incubated with their specific alkaline phosphatase conjugated secondary antibodies (1:80,000) for another 1h. Immunolabeled proteins were identified by using a chemiluminescence method (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany) and bands were quantified by image studio digits software 3.1 (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany).

2.9. Statistical analysis

The results are expressed as the mean values with SD of three independent experiments and the statistical analysis was assessed by using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). The significant differences are symbolized by letters that were acquired using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test ($p < 0.05$).

3. Results and Discussion

3.1. Identification and quantification of phenolic compounds

The phenolic compounds identified in MH are reported in Table 1. Two different families were determined: flavonols including rutin, myricetin, fisetin, quercetin, luteolin, apigenin, kaempferol and isorhamnetin, and phenolic acids including gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, *trans*-ferulic

acid, ellagic acid and *trans*-cinnamic acid. Gallic acid (36.57%) and syringic acid (32.55%) were the main components (11.55 and 10.28 mg/100 g honey, respectively), while the other phenolic acids were presented in low proportions ranging from 1.84% to 1.68% for 4-hydroxybenzoic acid and caffeic acid (0.58 and 0.53 mg/100 g honey), respectively. In a recent study, Ahmed *et al.* analyzed the phenolic composition of 17 multifloral honey samples.[21] 4-Hydroxybenzoic acid was the main phenolic compound identified in all the studied honeys, followed by gallic acid with similar concentrations to those found in the present study. As flavonols, the highest concentrations of quercetin, luteolin and kaempferol were found in this family (3.73, 2.62 and 1.17 mg/ 100 g of honey). They represented 11.81%, 8.30% and 3.70% of the total phenolic content, respectively (Table 1). All these three compounds have also been identified in MH by Marshall *et al.*[22] and by Alvarez-Suarez *et al.*[14] Finally, it is remarkable that kaempferol, quercetin and myricetin possess antimicrobial properties, a well-known characteristic of MH.[23] In our previous work, MH exhibited high antioxidant capacity and there was a significant correlation between its polyphenol content and its antioxidant parameters.[16]

3.2. Anti-proliferative effects of MH on HCT-116 and LoVo cells

Our result documented the capability of MH in the suppression of the colon cancer cell proliferation at different concentrations and times. As shown in Fig. 1A and Fig. 1B, the percentage of viable cells was reduced in the treated HCT-116 and LoVo cells compared to untreated cells in a dose- and time-dependent manner. In HCT-116 and LoVo cells, the IC_{50} (concentrations necessary for 50% inhibition of cell growth) of MH were 21.98 mg/mL and 62.85 mg/mL at 24 h, 15.10 mg/mL and 40.97 mg/mL at 48 h, and 13.35 mg/mL and 22.73 mg/mL at 72 h, respectively (Fig. 1A, 1B). The range of concentrations used to treat the LoVo

cells was higher than the range used for HCT-116 because of its metastatic nature. In LoVo cells, at lower concentration, there was no significant cytotoxic effect. Similar concentrations of MH treatment from 24 to 72 h were not induced any significant toxicity in non-cancer cells (Fig. 1C). Honey represents a good source of sugar. To evaluate if the cytotoxic effect of MH is associated with its sugar content, we performed the cytotoxic effect of artificial honey (1.5 g of sucrose, 7.5 g of maltose, 40.5 g of fructose and 33.5 g glucose in 17 mL of purified water) on both cell lines at similar concentrations and time duration. We observed that artificial honey didn't induce any toxic effects on HCT-116 and LoVo cells until 48 h. At 72 h, it induced less toxic effect at higher concentrations (Supplemental Fig. 1).

By using MH, Fernandez-Cabezudo *et al.*[17] reported the IC_{50} value of 20 and 10 mg/mL in the colon cancer CT-29 cells at 24 and 72 h. These results are quite similar to our results in HCT-116 cells. Based on the viability data on other types of honeys, Gelam honey induced IC_{50} values were 39 mg/mL and 80 mg/mL, Nenas honey induced IC_{50} values were 85.5 mg/mL and Indian commercial honey induced IC_{50} were 35 to 40 mg/mL on human colon cancer HT-29, HCT-15 and HCT-116 cells at 24 h;[24-26] these concentrations were more compared to MH in the present study. These variations are mainly due to honey composition, specifically on different types of flavonoids and phenolic acids, which are known as chemopreventive agents.[15] According to different investigations, phenolic compounds such as quercetin, luteolin, kaempferol, gallic acid and caffeic acid that are also present in the MH (Table 1), play an essential function in the suppression of cancer cell proliferation.[11, 15, 17]

Overall these results confirmed that MH induced no cytotoxic effects in non-cancer cells at the concentration that had high cytotoxic effects of both colon cancer cells, while the toxicity of MH is not associated with its sugar content. Furthermore, we tried to find out whether the anti-

proliferative effects were associated with promoting apoptosis and arresting cell cycle. According to the above observation, the MH concentrations were 10, 15 and 20 mg/mL for HCT-116 and, 30, 40 and 50 mg/mL of Lovo cells were selected for further experiments. In all cases, 48 h were used for treatment duration. The selected concentrations correspond to those concentrations at which approximately 70% to 40% cells were viable.

3.3. MH induces apoptosis on HCT 116 and LoVo cells

Apoptosis induction is a broadly accepted method for controlling the growth and development of cancer cells. Flow cytometry analysis confirmed the apoptotic cell death induced by MH (Fig. 2). The number of apoptotic cells was significantly ($p < 0.05$) increased from up to 3.38 fold in HCT-116 cells (Fig. 2A) and up to 4.95 fold in LoVo cells (Fig. 2B), respectively compared to untreated cells. The highest number of apoptotic cells was observed at 15 mg/mL dose for HCT-116 cells and 40 to 50 mg/mL dose for LoVo cells (Fig. 2); however the induction of apoptosis was more robust in LoVo cells than in HCT-116 cells treated with MH. By using the same technique, by-products from beeswax induced apoptotic cell death in liver cancer cells.[28] Furthermore, phenolic rich plant extract increased apoptotic cells percentage in DLD-1, HCT-116 and HT-29 colon cancer cells compared to untreated cells.[29-31]

The molecular mechanism by which MH induced apoptosis was investigated by western blot analysis. Exposure of HCT-116 and LoVo cells to MH caused significant ($p < 0.05$) increase in protein expression of p53, caspase-3 and c-PARP in a dose-dependent way (Fig. 2C, 2D). It is well known that p53 acts as a tumor suppressor gene and plays a vital role for suppressing tumor growth by inducing apoptosis and arresting cell cycle, while activation of caspase-3 and its downstream target PARP mediated apoptosis by chromatin condensation and DNA

fragmentation.[4] In HCT-116 cells, the expression of p53 was increased from 1.11 to 1.58 fold, caspase-3 was increased from 1.22 to 1.75 fold and c-PARP was increased from 1.21 to 1.86 fold after MH treatment (Fig. 2C). Similarly, in LoVo cells, the protein expression of p53 was increased from 1.63 to 2.21 fold, caspase-3 was increased from 1.25 to 2.06 fold and c-PARP was increased from 1.18 to 1.80 fold after MH treatment (Fig. 2D). Previously, in colon cancer HCT-15 and HT-29 cells, treated with Indian commercial honey, apoptosis was activated through increasing p53, c-PARP, Bax, caspase-3 and decreasing Bcl-2 expression.[26] In addition, MH is able to induce apoptotic cell death in colon cancer (CT29), breast cancer (MCF-7) and melanoma (B16.F1) cells by activating PARP, caspase-3 and decreasing Bcl-2 expression.[17]

3.4. MH induces intrinsic and extrinsic apoptosis on HCT 116 and LoVo cells

To determine whether the perceptible induction of apoptosis was associated with the activation of intrinsic and extrinsic pathways, we further evaluated the mRNA expression of apoptotic marker Bcl-2, Bax, Cyto c, FasL, caspase-8 and caspase-9 by real time PCR. It was observed that MH treatment significantly decreased the expression of Bcl-2 (up to 0.76 and 0.60 fold), while the expression of Bax (up to 1.84 and 2.88 fold), Cyto c (up to 1.48 and 2.70 fold), FasL (up to 1.63 and 2.15 fold), caspase-8 (up to 1.51 and 1.96 fold) and caspase-9 (up to 1.30 and 1.90 fold) were increased in both HCT-116 and Lovo cells compared to untreated cells, and the effects were dose-dependently (Fig. 3).

Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) proteins play a vital role for controlling the outer mitochondrial membrane permeabilization and leakage of apoptogenic protein (Cyto C) and other apoptotic factors, which in turn activate caspase-9 in the intrinsic apoptotic pathway.[4] In the extrinsic apoptotic pathway, caspase-8 is activated through binding death receptor (Fas or

tumor necrosis factor) associated specific ligands.[4] Based on these observation, our results indicated that MH induced intrinsic apoptosis by decreasing Bcl-2, at the same time increasing Bax, Cyto c and caspase-9 mRNA expressions (Fig. 3). The extrinsic apoptosis was evaluated by increasing FasL and caspase-8 mRNA expressions in both colon cancer cell lines (Fig. 3). These results were consistent with the previous report by other natural bioactive compounds such as chrysin, quercetin, flavokawain C, ellagic acid, agrimonolide etc. that induced intrinsic and extrinsic apoptotic cell death in several cancer cells by activating p53, caspase-3, caspase-8, caspase-9, c-PRAP, releasing Cyto C and increasing proapoptotic protein expression (Bax, Bad, Bak and DR5) as well as decreasing antiapoptotic proteins (Bcl-2 and Bcl-xL). [5, 27, 29-34]

3.5. MH induces cell cycle arrest of HCT 116 and LoVo cells

Cell proliferation is correlated with the regulation of cell cycle progression. Therefore, we determined the effects of MH on cell cycle arrest on HCT-116 and LoVo cells by Tali™ Image-based Cytometer. MH treatment increased the accumulation of cells at Sub-G1 phase of about 6% at high concentration (20 mg/mL) in HCT-116 cells, at the same time, in LoVo cells, it was 20% at high concentration (50 mg/mL) (Fig. 4). In HCT-116 cells, the percentage of cells in the S phase was significantly ($p < 0.05$) increased from up to 43% after MH treatment compared to untreated cells (23 to 26%), while the percentage of cells was significantly ($p < 0.05$) decreased in the G0/G1 and G2/M phase (Fig. 4A). At the same time, in LoVo cells, the percentage of cells in the G2/M phase was significantly ($p < 0.05$) increased up to 40% after MH treatment compared to untreated cells (20 to 24%), while the percentage of cells in the G0/G1 and S phase was significantly ($p < 0.05$) decreased (Fig. 4B).

Several studies reported that honey or its various phenolic and flavonoids compounds have the ability to arrest the cell cycle at different phases. The different Indian commercial honey increased the accumulation of cells at Sub-G1 phase and arrested the cell cycle at G0/G1 phase in colon cancer HCT-15 and HT-29 cells.[26] Similarly, other bioactive compounds from the plants have the ability to arrest the cell cycle at S and G2/M phase in different cancer cell lines.[5, 31, 35, 36]

3.6. Effect of MH on cyclin, CDK, $p21^{waf1/Cip1}$and $p27^{kip1}$ mRNA expression in HCT-116 and LoVo cells

After demonstrating that MH is able to arrest the cell cycle, we examined the effect of MH on cell cycle regulatory proteins (cyclin D1 and cyclin E), cyclin dependent kinases (CDK2 and CDK4), cyclin dependent kinase inhibitor ($p21^{waf1/Cip1}$ and $p27^{kip1}$) and p-Rb expression by real time PCR (Fig. 5). In the cell cycle, the transformation from one phase to another phase is controlled by several checkpoint genes such as CDKs via uniting with their relevant administrative subunits cyclins that can activate different downstream targets.[37] Furthermore, CDK-cyclin complexes are inversely regulated by CDK inhibitors (p21Cip and p27Kip), which in turn dephosphorylate Rb proteins for the transcriptional activation of cell cycle regulated genes.[37] We found that MH treatment significantly ($p < 0.05$) reduced cyclin D1 (0.76 to 0.65 fold), cyclin E (0.81 to 0.62 fold), CDK2 (0.81 to 0.55 fold), CDK4 (0.70 to 0.49 fold) and p-Rb (0.76 to 0.61 fold) mRNA expression dose dependently in HCT-116 cells compared to untreated cells (Fig. 5A). Consistent with CDKs reduction, the mRNA levels of $p21^{waf1/Cip1}$ (1.18 to 1.47 fold) and $p27^{kip1}$ (1.20 to 1.46 fold) dramatically elevated following the MH treatment in HCT-116 cells (Fig. 5A). The treatment of HCT-116 and HT-29 cells with flavokawain C and bay leaf extract arrested the cell cycle at S phase through downregulating the expression of CDK2 and CDK4 while upregulating $p21^{waf1/Cip1}$ and $p27^{kip1}$ and hypophosphorylation of Rb.[5, 36] In another

study pinostilbene was shown to inhibit the expression of cyclin E, p-Rb and increase the expression of $p21^{waf1/Cip1}$ in HCT-116 cells for inducing the arrest of cell cycle at S phase.[38] Furthermore, in LoVo cells we found that MH treatment markedly increased the $p21^{waf1/Cip1}$ (1.47 to 1.75 fold) and $p27^{kip1}$ (1.33 to 2.37 fold) mRNA expression, while cyclin D1 (0.77 to 0.61 fold) expression was significantly suppressed compared to untreated cells (Fig. 5B). However, no changes of other mRNA expression were observed (data not shown). These results were consistent with previous report showing that natural compounds treatment suppressed the colon cancer cells progression through arresting the cell cycle at G2/M phase via inhibiting cyclin D1 expression[39] as well as increasing p21 expression.[35]

Based on these results, it can be summarized that MH suppressed HCT-116 cells growth through arresting the cell cycle at S phase, upregulating p21 and p27, and downregulating CDK2, CDK4, cyclin D1, cyclin E and p-RB expression. In Lovo cells, MH arrested the cell cycle at G2/M phase and the mechanism was associated with increasing expression of p21 and p27 and decreasing cyclin D1 expression, respectively.

3.7. Effect of MH on EGFR and MAPK signaling pathways on HCT-116 and LoVo cells

We examined the effects of MH on membrane protein EGFR and HER2 and their downstream signaling protein p-Akt on HCT-116 and LoVo cells. As shown in Fig. 6, treatment with MH dose-dependently suppressed the expression levels of EGFR up to 0.52 fold for HCT-116 cells and up to 0.40 fold for LoVo cells. Similarly, the expression of HER2 was also suppressed up to 0.51 fold in HCT-116 cells and up to 0.38 fold for LoVo cells, respectively (Fig. 6A, 6B). Additionally, MH treatment at high concentration significantly suppressed the expression of p-Akt up to 0.22 fold for HCT-116 and up to 0.37 folds LoVo cells (Fig. 6A, 6B). Tahir *et al.*

reported that the Gelam honey alone or combined with ginger suppresses the expression of PI3K/Akt pathways in colon cancer cells HT-29.[25] Furthermore, caffeic acid phenethyl ester, a compound derived from honey bee propolis, suppresses total and phosphorylated EGFR in breast cancer cells.[40] Similarly, flavokawain C and protein extract from plant sources induce apoptosis in HCT-116 and DLD-1 colon cancer cells by suppressing Akt expression.[5, 30] After polyphenol treatment, a reduction has been observed in EGFR expression by decreasing the transcription factor Egr-1 in colon cancer Caco-2 and HT-29 cells.[41] In this work, we found that MH suppressed the expression of EGFR, HER2 and p-Akt (Fig. 6A, 6B), while EGFR was suppressed in a more remarkable manner in LoVo cells while p-Akt was highly suppressed in HCT-116 cells after MH.

We further investigated the involvement of p-p38MAPK and p-Erk1/2 pathways in the apoptotic properties of MH. MH increased the phosphorylation of p38MAPK and Erk1/2 protein from 1.82 to 2.63 fold and 1.49 to 2.60 fold for HCT-116 cells at dose 10 to 20 mg/mL and 1.50 to 2.48 fold and 2.11 to 3.29 fold for LoVo cells at dose 30 to 40 mg/mL, respectively (Fig. 6A, 6B). Similarly, quercetin (common flavonoids of MH) increases MAPK activation through the activation of p-Erk, p-JNK and p-38MAPK in colon cancer CT26 cells, leading to apoptosis.[27] Furthermore, natural compounds from several plant sources increase the expression of p-Erk1/2 and p-p38MAPK in colon cancer HCT-116, DLD-1 and HT-29 cells for inducing cell death by apoptosis.[5, 30, 33]

These findings highlight that MH induces apoptosis through the alteration of intrinsic and extrinsic apoptotic markers by activation of p-p38MAPK and p-Erk1/2 pathway in HCT-116 and LoVo cells.

3.8. MH induces endoplasmic reticulum stress in HCT-116 and LoVo cells

To further find out the probable apoptosis effects activated by MH, we investigated the protein level of ER stress-associated molecules, ATF6 and XBP1 by western blotting. As shown in Fig. 6C, 6D, the lower concentration of MH did not cause an upregulation of ATF6 and XBP1 expressions in both cell lines, but after treatment with higher concentrations the expression of these two proteins was unregulated 1.77 to 2.15 fold and 1.16 to 1.75 fold in HCT-116 cells and 1.42 to 2.56 fold and 1.91 to 2.62 fold in Lovo cells compared to untreated cells, respectively. This finding indicated that MH leads to ER stress inducing HCT-116 cell death by increasing the ATF6 expression which further activates the XBP1 expression. Earlier studies indicated that the ER stress-induced cancer cell death, by activating ATF6 and XBP1 expression in colon cancer HCT-116 and HT-29 cells, has been assessed by other natural compounds.[42, 43]

4. Conclusion

This study explored the anti-proliferative and apoptotic effects of MH in two genetically well recognized human colon adenocarcinoma cell lines HCT-116 and LoVo. Despite its high phenolic constitutes, only a few studies addressed the chemopreventive effects of MH. Exposure to MH inhibited the cells proliferation ability, induced apoptosis and blocked cell cycle progression in both colon cancer cell types. This event was accompanied by increasing the mRNA expression of both intrinsic and extrinsic apoptotic markers such as caspase-8, -9, Bax, Cyto C and FasL while the expression of Bcl-2 was decreased. Consequently, protein expression of p53, caspase-3 and c-PARP also confirmed the apoptosis induction after MH treatment. In addition, MH induced cell cycle arrest at the S phase in HCT-116 cells and molecular mechanism behind of this was the increased expression of p21 and p27, while cyclin D1, cyclin

E, CDK2, CDK4 and p-Rb expression was decreased. Furthermore, in LoVo cells, MH induced G2/M phase arrest through increasing p21 and p27 expression and only suppressed cyclin D1 expression was observed which activate apoptosis through leading to growth reduction. In addition, we also demonstrated that MH induced apoptotic effect was related with increased ER stress associated cells death by elevating ATF6 and XBP1 expression, suppressed of EGFR, HER2 and p-Akt pathway and the expression of p-p38MAPK and the p-Erk1/2 pathway was increased. Interestingly, MH treatment in non-malignant cells does not exert any significant toxicity. These interesting and promising results encourage our knowledge about chemopreventive effects of honey and could be useful for further studies to highlight the phenolic compounds of MH and the possible molecular mechanisms as well as for *in vivo* studies against colon cancer.

Conflict of interest

The authors declare no conflicts of interest.

Abbreviation

ABB, annexin binding buffer; Akt, protein kinase B; ATF6, activating transcription factor 6; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; CRC, colorectal cancer; CDK, cyclin dependent kinase; Cyto c, cytochrome c; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; Erk1/2, extracellular-signal-regulated kinase 1/2; FasL, fatty acid synthetase ligand; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HDF, human dermal fibroblast; HER2, human epidermal growth factor receptor 2; JNK, jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MH, manuka honey; MTT, 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PE, plating efficiency; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; Rb, retinoblastoma; TBST, Tris HCl buffered saline with Tween 20; XBP1, X-box binding protein 1.

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Table 1

CAS number, retention time (RT, min), maximum absorption wavelengths (λ, nm) and concentration (mg/100g of honey) for the considered phenolic compounds determined in Manuka honey.

Phenolic compounds	**CAS number**	**RT (min)**	**λ (nm)**	**Concentration (mg/100g of honey)**
Flavonols				
Rutin	153-18-4	13.34	352	nd
Myricetin	529-44-2	17.07	368	nd
Fisetin	528-48-3	19.73	361	nd
Quercetin	117-39-5	24.65	371	3.73±1.83
Luteolin	491-70-3	25.69	347	2.62±0.60
Apigenin	520-36-5	31.91	337	0.06±0.01
Kaempferol	520-18-3	31.93	365	1.17±0.50
Isorhammetin	480-19-3	34.83	370	0.42±0.20
Total flavonol content				8.00
Phenolic acids				
Gallic acid	149-91-7	3.11	270	11.55±1.80
Protocatechuic acid	99-50-3	5.65	259	nd
4-Hydroxybenzoic acid	99-96-7	10.52	254	0.58±0.01
Vanillic acid	121-34-6	15.65	260	nd
Caffeic acid	331-39-5	16.54	322	0.53±0.02
Syringic acid	530-57-4	19.87	274	10.28±1.61
p-coumaric acid	501-98-4	27.46	308	0.12±0.03
trans-ferulic acid	537-98-4	33.19	322	0.19±0.01
Ellagic acid	476-66-4	38.08	251	nd
trans-cinnamic acid	140-10-3	55.88	273	0.33±0.07
Total phenolic acid content				23.58

nd: not detected. Data are means (n=3).

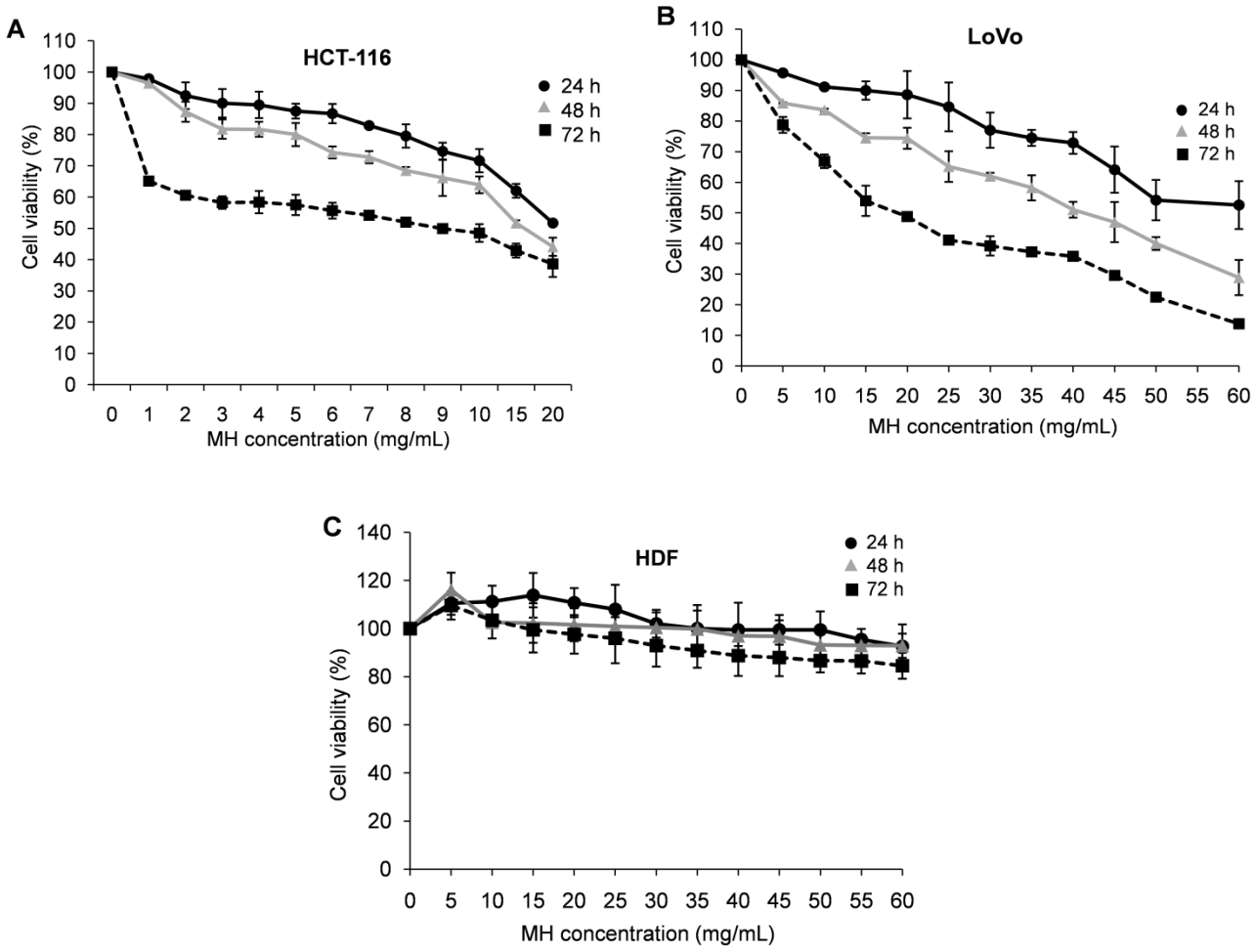


Fig. 1 Inhibition of cell proliferation by MH in HCT-116, LoVo and HDF cell lines. All cells were treated with different concentrations of MH for 24, 48 and 72 h. Cell viability was measured by using MTT assay and results were expressed as a % of viable (A) HCT-116, (B) LoVo and (C) HDF cells compared to untreated cells. All data are expressed as the mean ± standard deviation (SD) (n=3).

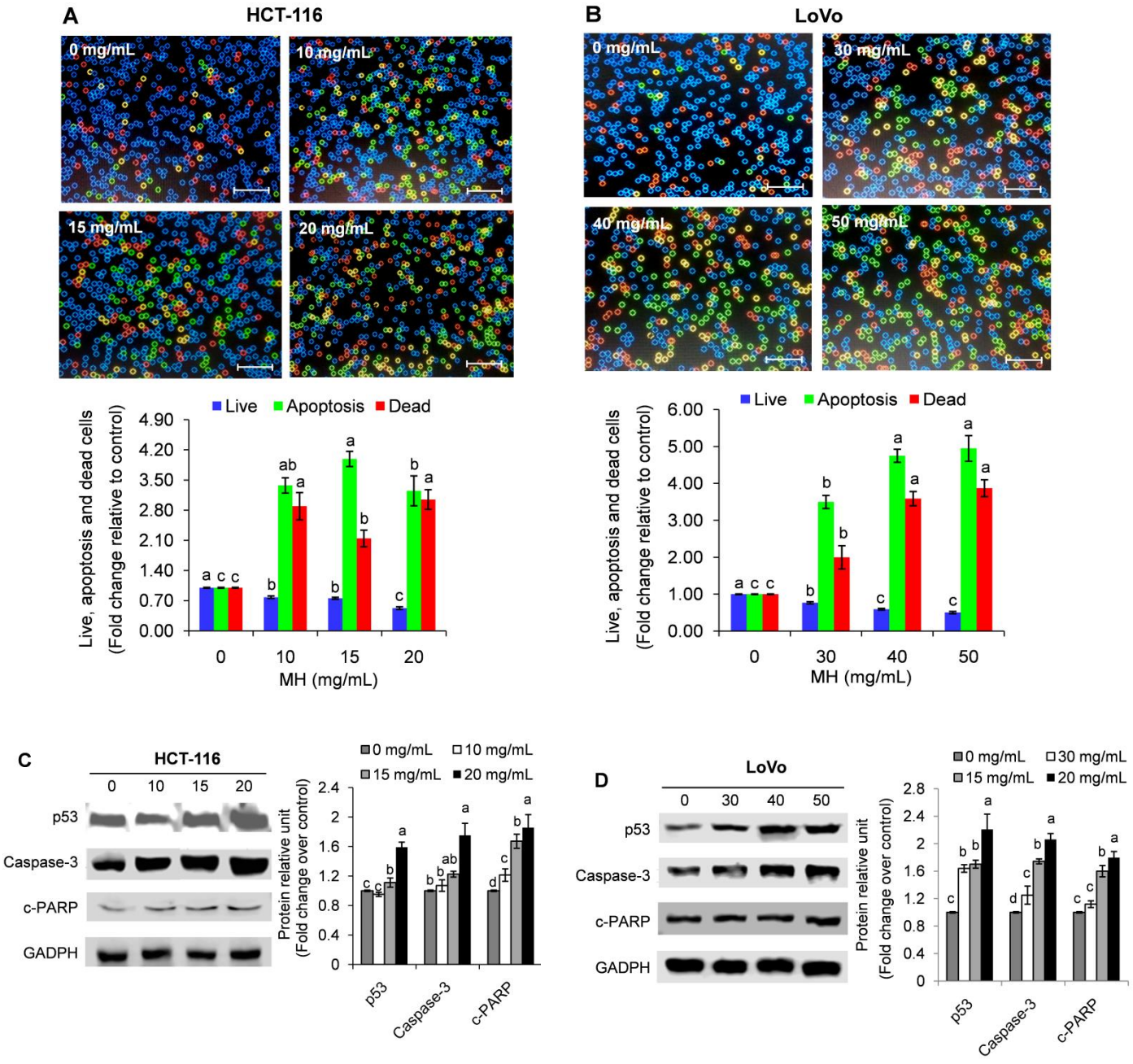


Fig. 2 Apoptosis induction by MH in HCT-116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. Annexin V Alexa Fluor® 488 and PI staining was used for determination of apoptotic effect of MH on (A) HCT-116 and (B) LoVo cells. Viable, death and apoptotic cells were calculated by using the Tali™ apoptosis kit and the Tali™ Image-based Cytometer. Representative

fluorescence image shows the effect of MH with or without treatment: blue colour corresponds to live cells, green colour corresponds to apoptotic cells and red and yellow colour corresponds to dead cells. Scale bar = 50 μm. Protein expression of apoptotic markers p53, caspase-3 and cleaved PARP (c-PARP) were determined by western blotting in (C) HCT-116 and (D) LoVo cells after MH treatment. GADPH was used as a loading control. All data shown were the mean ± SD (n=3). Different superscripts letter for each column indicated significant differences ($p < 0.05$).

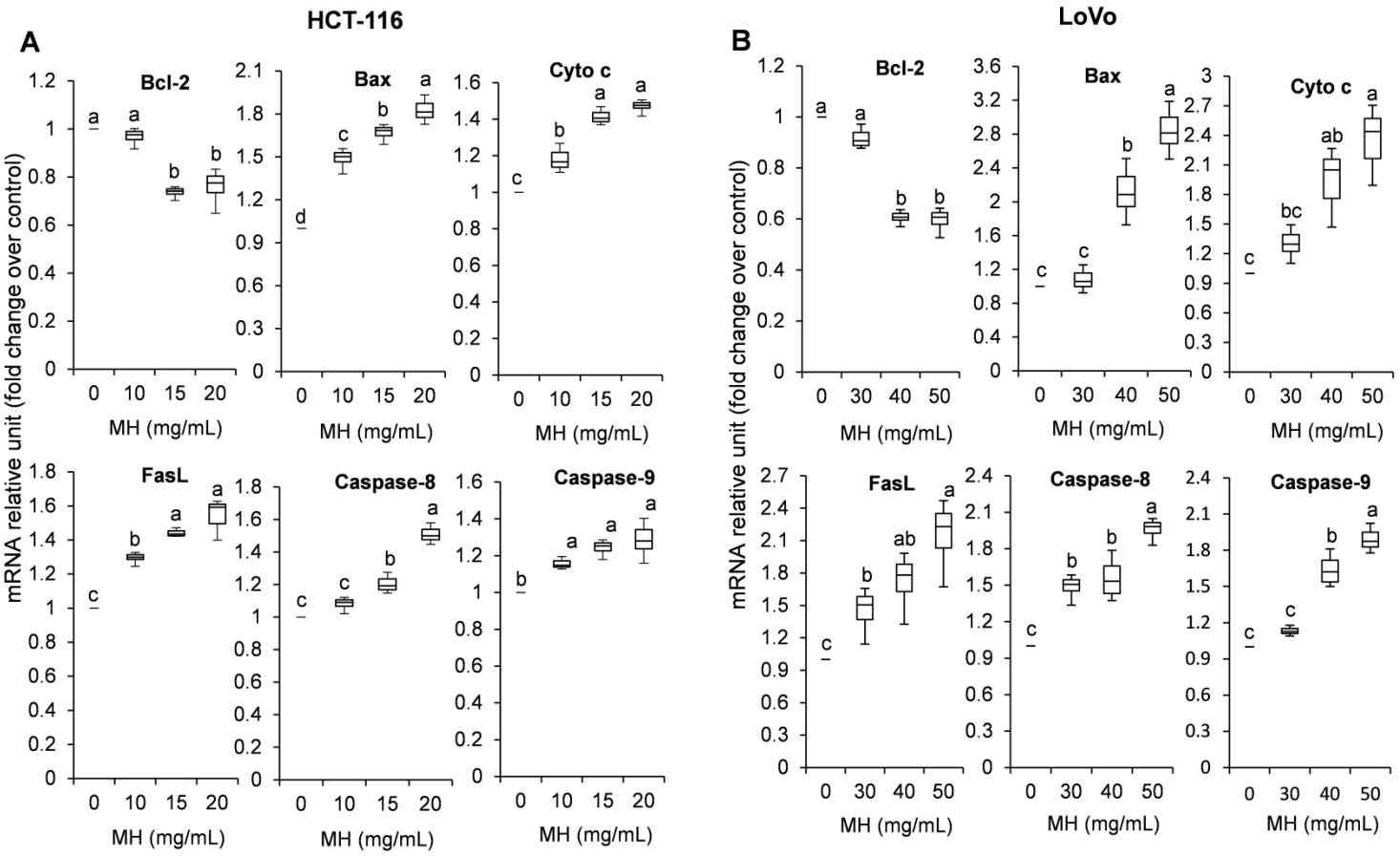


Fig. 3 MH induces intrinsic and extrinsic apoptosis on HCT 116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. mRNA expression of intrinsic and extrinsic apoptotic markers Bcl-2, Bax, Cyto c, FasL, caspase-8 and caspase-9 were analyzed by real-time PCR in (A) HCT-116 and (B) LoVo cells. GADPH was amplified under the same PCR conditions for normalize quantitative data. Results are expressed as median, first quartiles, third quartiles, minimum and maximum values. All data shown were the mean ± SD (n=6). Different superscripts letter for each column indicated significant differences ($p < 0.05$).

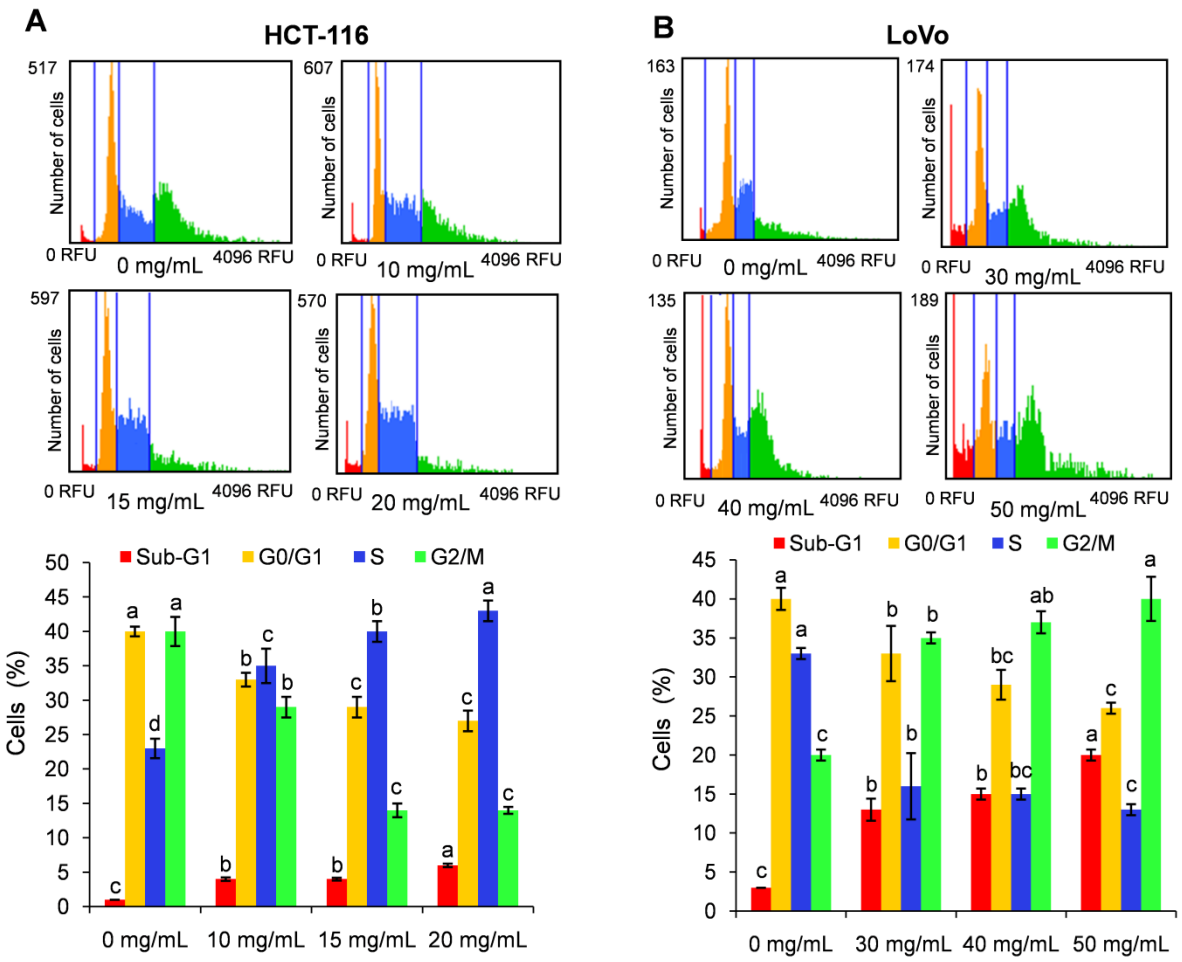


Fig. 4 Cell cycle alteration induces by MH in HCT-116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. The percentages of cells in each phase Sub-G1 (apoptotic cells), G0/G1, S and G2/M were calculate by the Tali® Cell Cycle Assay kit and Tali™ Image-based Cytometer. Representative fluorescence image of (A) HCT-116 and (B) LoVo cells cycle shows the effect of MH with or without treatment: red colour corresponds to Sub-G1 phase, yellow colour corresponds to G0/G1 phase, blue colour corresponds to S phase and green yellow colour corresponds to G2/M phase. All data shown were the mean ± SD (n=3). Different superscripts letter for each column indicated significant differences ($p < 0.05$).

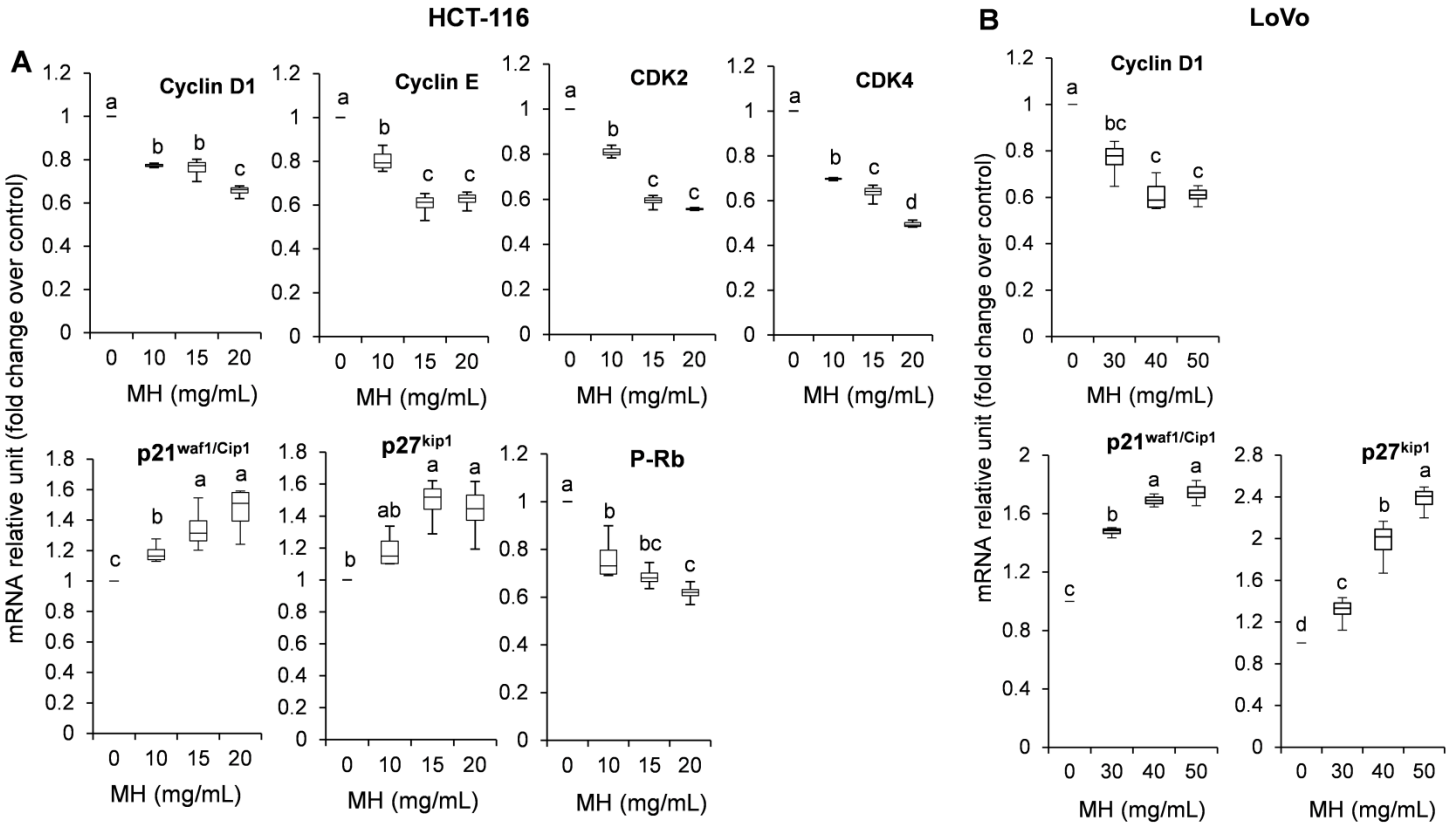


Fig. 5 MH induces alteration of cell cycle regulatory mRNA expression in HCT-116 and Lovo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. Cell cycle regulatory proteins (cyclin D1 and cyclin E), cyclin dependent kinases (CDK2 and CDK4), cyclin dependent kinase inhibitor ($p21^{waf1/Cip1}$ and $p27^{kip1}$) and p-Rb were analyzed by real-time PCR in (A) HCT-116 and (B) LoVo cells. GADPH was amplified under the same PCR conditions for normalize quantitative data. Results are expressed as median, first quartiles, third quartiles, minimum and maximum values. All data shown were the mean ± SD (n=6). Different superscripts letter for each column indicated significant differences ($p < 0.05$).

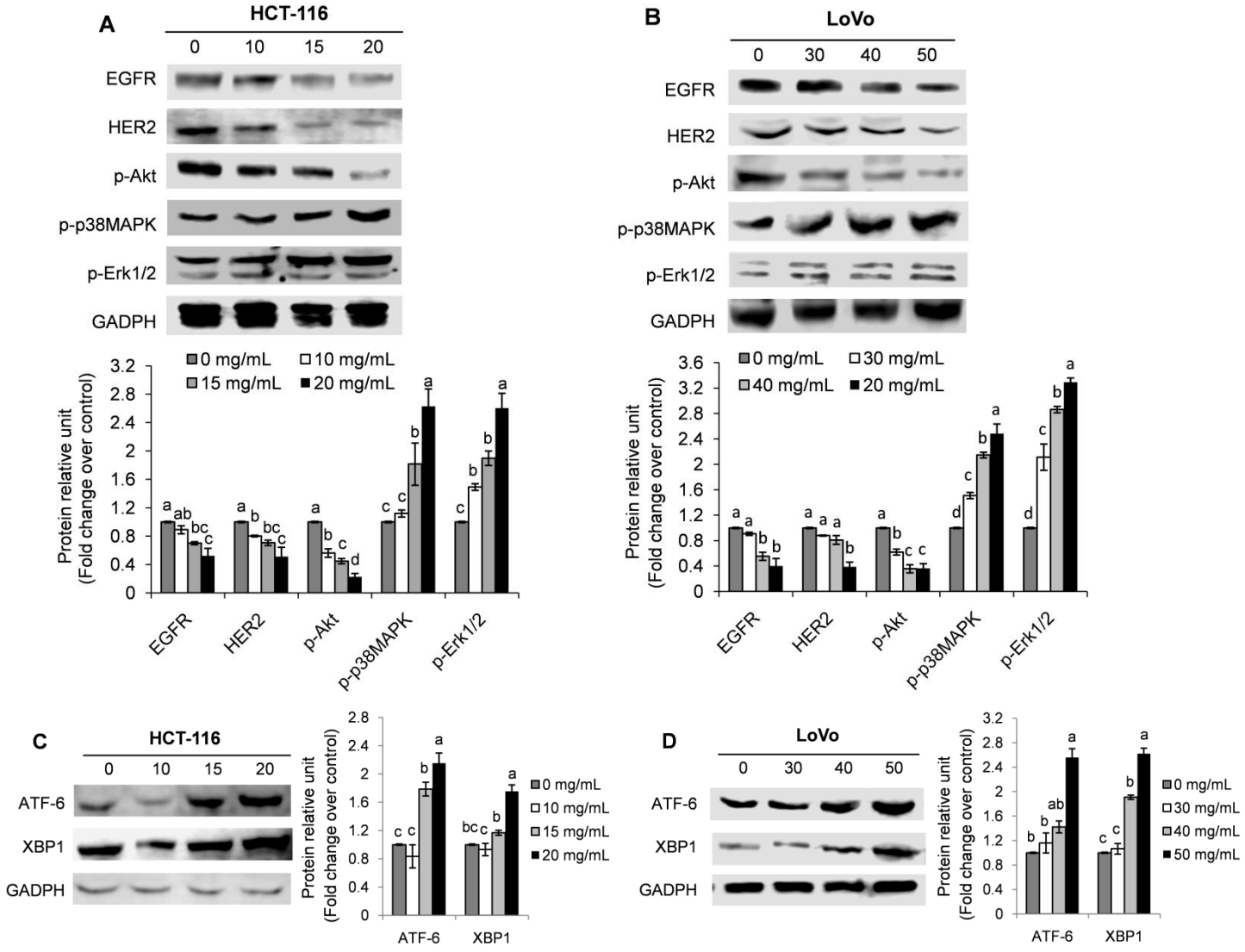
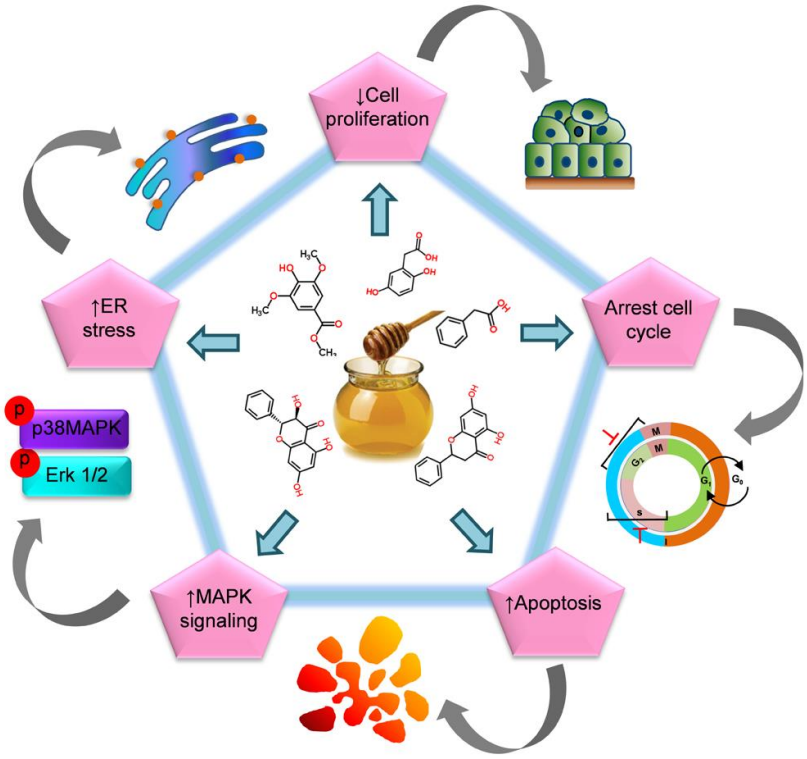


Fig. 6 Effect of MH on EGFR, MAPK and endoplasmic reticulum stress signaling in HCT-116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. (A) and (B), the protein involved on EGFR (EGFR, HER2 and p-Akt) and MAPK signaling (p-p38MAPK and p-Ekr1/2), and (C) and (D) the protein involved on endoplasmic reticulum stress (ATF6 and XBP1); that were analyzed by western blotting in HCT-116 and LoVo cells. GADPH was utilized as a loading control. All data are indicated as the mean ± SD (n=3). Different superscripts letter for each column indicated significant differences ($p < 0.05$).

Graphical Abstract

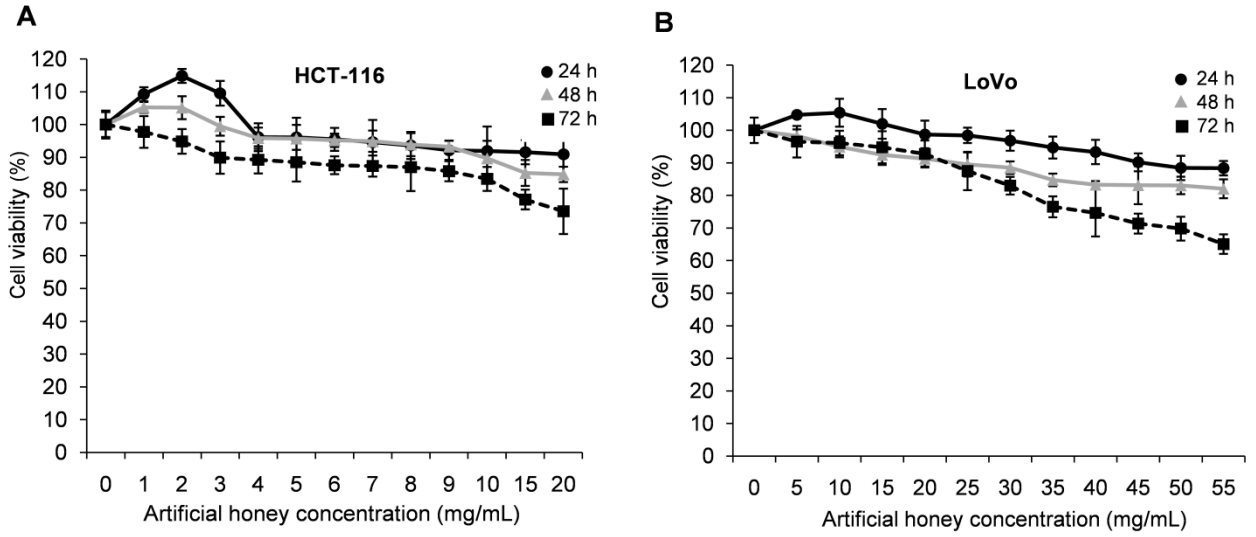
Manuka honey suppresses human colon cancer cells proliferation through promotion of apoptosis, arrest of cell cycle, activates MAPK signaling and induces endoplasmic reticulum stress.



Supplementary Table 1. Sequences of real-time PCR oligonucleotide primers.

Gene	Direction	Sequence (5′-3′)
Cyclin D1	Forward	GAACAAACAGATCATCCGCAA
	Reverse	TGCTCCTGGCAGGCACGGA
Cyclin E	Forward	GAGCCAGCCTTGGGACAATAA
	Reverse	GCACGTTGAGTTTGGGTAAACC
CDK2	Forward	TTTGCTGAGTGGTGACTCGCCG
	Reverse	CCGGCCCACTTGGGGAAAC
CDK4	Forward	CTTCCCGTCAGCACAGTTC
	Reverse	GGTCAGCATTTCCAGTAGC
$p21^{waf1/cip1}$	Forward	GCGATGGAACTTCGACTTTGT
	Reverse	GGGCTTCCTCTTGGAGAAGAT
$p27^{kip1}$	Forward	ATGTCAAACGTGCGAGTGTC
	Reverse	TCTCTGCAGTGCTTCTCCA
p-Rb	Forward	ATCCGAGGCAACTACAGCCTA
	Reverse	CCTTTCCAACCGTGGGAATAAT
Bcl-2	Forward	CCTGTGGATGACTGAGTACC
	Reverse	GAGACAGCCAGGAGAAATCA
Bax	Forward	GTTTCATCCAGGATCGAGCAG
	Reverse	CATCTTCTTCCAGATGGTGA
Cyt c	Forward	TTTGGATCCAATGGGTGATGTTGAG
	Reverse	CCATCCCTACGCATCCTTTAC

Fas L	Forward	GGATTGGGCCTGGGGATGTTTCA
	Reverse	TTGTGGCTCAGGGGCAGGTTGTTG
Caspase-8	Forward	AGAGTCTGTGCCCAAATCAAC
	Reverse	GCTGCTTCTCTCTTTGCTGAA
Caspase-9	Forward	TGTCCTACTCTACTTTCCCAGTTTT
	Reverse	GTGAGCCCACTGCTCAAAGAT
GADPH	Forward	GACCCCTTCATTGACCTCAACTACATG
	Reverse	GTGCACCACCCTGTTGCTGTAGCC



Supplementary Fig. 1 Inhibition of cell proliferation by artificial honey in (A) HCT-116 and (B) LoVo cell lines. Cells were treated with different concentrations of honey for 24, 48 and 72 h. Cell viability was measured by using MTT assay and results were expressed as a % of viable cells compared to untreated cells. All data are expressed as the mean ± standard deviation (SD) (n=3).