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The 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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Original

The 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 / Afrin, Sadia; Giampieri, Francesca; Gasparrini, Massimiliano; Forbes Hernandez, Tamara; Cianciosi, Danila; Reboredo Rodríguez, Patricia; Amici, Adolfo; Quiles, José L.; Battino, Maurizio. - In: FOOD & FUNCTION. - ISSN 2042-6496. -ELETTRONICO. - 9:(2018), pp. 2145-2157. [10.1039/C8FO00164B]

Availability:

This version is available at: 11566/256175 since: 2022-05-25T12:19:33Z

Publisher:

Published DOI:10.1039/C8FO00164B

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1	Inhibitory effect of Manuka honey on human colon cancer HCT-116 and LoVo cells
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# 28 Abstract

29 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 30 luteolin, kaempferol, quercetin, gallic acid and syringic acid. The aim of this work was to 31 32 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 33 cells and 0-60 mg/mL for LoVo cells) for 48 h to measure apoptosis and cell cycle arrest as 34 well as apoptosis and cell cycle regulatory gene and protein expression. MH exhibited 35 profound inhibitory effects on cellular growth by reducing the proliferation ability, inducing 36 apoptosis and arresting cell cycle in a dose-dependent manner. Interestingly, MH treatment in 37 non-malignant cells did not exert any significant toxicity at similar concentration. The apoptosis 38 event was associated with increasing expression of p53, cleaved-PARP and caspase-3, and with 39 the activation of both intrinsic (caspase-9) and extrinsic (caspase-8) apoptotic pathways. MH 40 induced cell cycle arrest at S phase in HCT-116 cells, simultaneously, in LoVo cells, it arrested 41 at G2/M phase through the modulation of cell cycle regulator genes (cyclin D1, cyclin E, CDK2, 42 43 CDK4, p21, p27 and Rb). The expression of p-Akt was suppressed while the expression of pp38MAPK, p-Erk1/2 and endoplasmic stress markers (ATF6 and XBP1) was increased for 44 apoptosis induction. Overall, these findings indicate that MH could be a promising preventive or 45 curative food therapy for colon cancer. 46

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

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### 52 **1. Introduction**

Globally, colorectal cancer (CRC) is the third most widespread cancer in both men and women; 53 over 1 million new cases are diagnosed each year accounting for 9.7% of all cancers apart and 54 consequently more than 693,933 deaths per annum corresponding to 8.5% of the total number of 55 cancer deaths.<sup>1</sup> Notably, about 50% CRC patients develop the recurrent disease<sup>2</sup> indicating that 56 57 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, 58 the expenditure of CRC treatment is estimated to exceed \$17 billion in the healthcare system by 59  $2020.^{3}$ 60

Apoptosis process plays a critical role in cancer pathogenesis: indeed, a failure to undergo 61 apoptosis results in the development and progression of cancer. The intrinsic apoptotic pathway 62 involves distraction of the mitochondria membrane potential promoted by regulating apoptotic 63 protein and responses against several intracellular stresses, while extrinsic apoptotic pathway 64 activates by binding with cell surface receptor their specific ligands.<sup>4</sup> Several molecules and 65 processes modulate the apoptosis process, largely through caspase cascade activation, which 66 breakdown specific proteins in the cytoplasm and nucleus triggering cell death.<sup>4</sup> Apoptosis 67 effects may be induced in cancer cells by regulating of cell cycle progression.<sup>5</sup> Dysregulation of 68 cell cycle is one of the features of carcinogenesis which contributes to the abounded proliferation 69 in human cancer.<sup>6</sup> 70

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,<sup>7</sup> representing an attractive therapeutic target for anticancer

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therapy. The phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) signaling pathway can 75 be activated by several growth factors and by G-protein-coupled receptors upon stimulation with 76 phospholipids and chemokines that can activate the signals for cell survival and proliferation, 77 cell cycle and anti-apoptotic effects leading to CRC carcinogenesis.<sup>8</sup> The mitogen-activated 78 protein kinase (MAPK) can convert various extracellular signals into intracellular responses 79 80 through serial phosphorylation cascades. The major MAPK families containing extracellularsignal-regulated kinase 1/2 (Erk1/2), Jun N-terminal kinase (JNK), and p38 MAPK, are well-81 known to transmit, integrate and triggers signals from various stimuli for controlling 82 inflammation, cellular proliferation and differentiation as well as apoptosis in different cancers.<sup>9</sup> 83 The endoplasmic reticulum (ER) is the principle site for protein maturation, folding and 84 secretion, and maintenance of phospholipid biosynthesis and calcium homeostasis. ER stress 85 induced cancer cell death has raised growing attention because it seems to increase the efficacy 86 of chemotherapeutic mediators by eradicating the damaged cells via activation of both intrinsic 87 and extrinsic apoptosis pathways.<sup>10</sup> 88

The use of various natural and synthetic drugs for CRC prevention has attained remarkable 89 attention in recent years; in this context natural food products may represent a valid alternative, 90 because of their chemopreventive or chemotherapeutic properties.<sup>11, 12</sup> Honey is a good source of 91 natural therapeutic molecules with antibacterial, wound healing, antioxidant, anti-inflammatory, 92 and anticancer properties.<sup>13-15</sup> Manuka honey (MH) is a particular type of honey collected from 93 94 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 95 properties and attractive biological and pharmacological compounds.<sup>13, 14</sup> However, there has 96 been a sporadic effort on the anticancer activity of whole honey on human colon cancer in vitro<sup>15</sup> 97

and only two investigations have reported the antiproliferative effect of MH on colon cancer
cells.<sup>16, 17</sup>

100 The present study was designed to characterize the anti-proliferative and apoptotic activities of 101 MH in human colon adenocarcinoma cells (HCT-116) and Dukes' type C, grade IV, colon 102 metastasis cells (LoVo). Furthermore, the effect of MH on the cell cycle, MAPK signaling and 103 ER stress highlighting the underlying molecular mechanism involved in these cell types were 104 also investigated.

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#### 106 **2. Materials and methods**

107 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).

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115 2.2. Extraction, identification and quantification procedure for phenolic compounds of MH

116 2.2.1. Solid-phase extraction (SPE) and HPLC conditions

117 MH was pre-concentrated with Strata X-A cartridges 33u Polymeric Strong Anion sorbent (60 118 mg, 3 mL size) from Phenomenex (Torrance, CA, USA). The SPE method was carried out 119 according to a slight modification as previously reported.<sup>18, 19</sup> MH (3 g) were mixed with 120 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 121 supernatants were loaded onto the previously conditioned cartridges (under these conditions: 122 123 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 124 sugars and other polar compounds of honey that were not absorbed on the sorbents. Then, 125 126 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 127 128 2% acetic acid (2 mL). All solutions were filtered through a 0.22-µm filter prior to HPLC 129 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 130 Surveyor PDA Plus Detector. Chromatographic separations were performed with a Luna C18 131 analytical column (150 x 3 mm ID,  $3-\mu$ m particle) with a guard column (4 x 3 mm ID) 132 containing the same packing material, both from Phenomenex (Torrance, CA, USA). 133

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135 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 136 137 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 138 139 and for phenolic acids the mobile phase was composed of 2% (v/v) acetic acid in water 140 (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% 141 142 A in 5 min, changed to 60% A in 5 min, changed to 50% A in 10 min, changed to 0% in 10 min, 143 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.

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149 2.3. Cell culture

Human colon adenocarcinoma (HCT-116), Dukes' type C, grade IV, colon metastasis (LoVo) 150 and healthy human dermal fibroblast (HDF) cell lines were purchased from the American Type 151 152 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-116 153 cell culture, F-12K media were used for LoVo cells culture and DMEM media were used for 154 HDF cells culture. All the media was prepared with of 10% heat-inactivated fetal bovine serum, 155 100 IU/mL penicillin and 100 µg /mL streptomycin. All cell lines were maintained in an 156 incubator at 37°C in a humidified atmosphere (95% air, 5% CO<sub>2</sub>). For the subsequent 157 experiment, cells were used between the 6<sup>th</sup> and10<sup>th</sup> passages. 158

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### 160 2.4. Cell viability assay

161 Cells were seeded at a density of  $5 \times 10^3$  cells/well into 96-well plates using the specific complete 162 growth medium. To allow cell attachment, they were incubated overnight. After overnight 163 incubation, the HCT-116 cells were treated with 0 to 20 mg/mL of MH for 24 to 72 h. Similarly, 164 LoVo and HDF cells were treated with 0 to 60 mg/mL of MH for 24 to 72 h. After the incubation 165 time, 30 µL of RPMI medium containing 2 mg/mL of the 3-(4,5-dimethylthiazol-2-yl)-2,5-166 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.

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172 2.5. Determination of apoptotic cells by Tali<sup>®</sup> Image-Based Cytometer

Apoptotic cells were identified by the Tali<sup>™</sup> Apoptosis Assay Kit–Annexin V Alexa Fluor<sup>®</sup> 488 173 (Invitrogen<sup>TM</sup>, Life Techonoliges, Milan, Italy) as reported earlier.<sup>14</sup> Cells were seeded at a 174 density of  $1.5 \times 10^5$  cells/well into 6 well and treated for 48 h with the MH (0, 10, 15 and 20) 175 mg/mL for HCT-116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells). Cells were harvested 48 176 h post-treatment and centrifuged for 15 min at 1500 rpm at 4°C. After removing the supernatant 177 and re-suspending the cells with 100 µL of Annexin binding buffer (ABB), 5 µL of Annexin V 178 Alexa Fluor<sup>®</sup> 488 was added to each 100 µL of re-suspended cells. The mixture of cell and 179 Annexin V Alexa Fluor<sup>®</sup> 488 was incubated at room temperature into the dark for 20 min and 180 then again centrifuged. After removing the excess mixture and re-suspending with 100 µL of 181 ABB, samples were incubated at room temperature into the dark for 1 to 5 min after adding 1  $\mu$ L 182 of Tali<sup>™</sup> propidium iodide (PI). For each sample, 25 µL of cell suspension was loaded into one 183 Tali<sup>TM</sup> Cellular Analysis Slide's chamber and analyzed in the Tali<sup>TM</sup> Image-Based Cytometer 184 (Invitrogen<sup>TM</sup>, Life Techonoliges, Milan, Italy). The instrument works at different 185 186 excitation/emission wavelengths: 530/580 nm and 458/495 nm for the PI and Annexin V, respectively. The Tali<sup>™</sup> Image-Based Cytometer was evaluated the live, apoptotic and dead 187 188 cells. The annexin V-negative/PI negative cells were identified as viable cells by the cytometer 189 software whereas the annexin-V positive/ PI negative cells were recognized as apoptotic cells.

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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  $\pm$  standard deviation (SD).

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193 2.6. Cell cycle analysis by Tali<sup>®</sup> Image-Based Cytometer

Cell cycle analysis was determined by the Tali<sup>®</sup> Cell Cycle Kit (Invitrogen<sup>™</sup>, Life Techonoliges, 194 Milan, Italy) as previously described.<sup>20</sup> Cells were cultured in 6-well plates, at a density of  $4 \times 10^5$ 195 cells/ well and incubated with various concentration of MH 0, 10, 15 and 20 mg/mL for HCT-196 116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells for 48 h. Then, cells were trypsinized and 197 198 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 199 harvested and fixed with 70% cold ethanol at -20°C overnight. The fixed cells were washed 200 twice with PBS, re-suspended in 100 µL PBS-based PI (Invitrogen<sup>™</sup>, Life Techonoliges, Milan, 201 Italy) solution containing 0.1% Triton<sup>®</sup> X-100, 0.2 mg/ml RNase A (Invitrogen), and 20 µg/ml 202 PI, and incubated for 30 min at room temperature protected from the light. For each sample, 25 203 µL of cell suspension was loaded into one Tali<sup>TM</sup> Cellular Analysis Slide's chamber 204 (Invitrogen<sup>TM</sup>, Life Techonoliges, Milan, Italy) and analyzed in the Tali<sup>TM</sup> Image-Based 205 Cytometer. The results were expressed as the percentage of cells in each phase and all data were 206 reported as a mean value of three independent analyses  $\pm$  SD. 207

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209 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<sup>®</sup> RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according

to the manufacturer's protocol. RNA concentrations and purity were checked by using the 213 microplate spectrophotometer system (BioTek Synergy HT, Winooski, USA). The sample ratio 214 between 1.8 to 2.1 was only selected for gene expression assay. cDNA was synthesis from 100 215 ng RNA following reverse transcription according to the manufacturer's protocol (5X All-In-216 One RT MasterMix kit, Applied Biological Materials Inc. Canada). Real-time PCR was 217 218 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 219 220 cyclin D1, cyclin E, cyclin dependent kinase (CDK)2, CDK4, p21, p27, phosphorylated (p)-221 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 222 final volume 20 µL on a real-time PCR system (Corbett Life Science, Rotor-Gene 3000, 223 Mortlake, Australia ). RT-PCR control GADPH was amplified under the same PCR conditions 224 for normalizing quantitative data. The  $2^{-\Delta\Delta ct}$  method was used for calculating the fold change 225 226 values.

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228 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 236 antibodies p53, caspase-3, cleaved-PARP (c-PARP), phosphorylated (p)- p38MAPK, p-Erk1/2, 237 EGFR, HER2, p-Akt, activating transcription factor 6 (ATF6), X-box-binding protein 1 (XBP1) 238 and glyceraldehyde-3-phosphate dehydrogenase (GADPH) (1:500 dilutions) were used after 239 overnight incubation at 4°C. Membranes were washed 3 times with TBST and incubated with 240 241 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 242 Scanner, LICOR, Bad Homburg, Germany) and bands were quantified by image studio digits 243 244 software 3.1 (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany).

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246 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).

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### 253 **3. Results and Discussion**

254 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, 4hydroxybenzoic 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 259 the main components (11.55 and 10.28 mg/100 g honey, respectively), while the other phenolic 260 acids were presented in low proportions ranging from 1.84% to 1.68% for 4-hydroxybenzoic 261 acid and caffeic acid (0.58 and 0.53 mg/100 g honey), respectively. In a recent study, Ahmed et 262 *al.* analyzed the phenolic composition of 17 multifloral honey samples.<sup>21</sup> 4-Hydroxybenzoic acid 263 264 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 265 concentrations of quercetin, luteolin and kaempferol were found in this family (3.73, 2.62 and 266 1.17 mg/ 100 g of honey). They represented 11.81%, 8.30% and 3.70% of the total phenolic 267 content, respectively (Table 1). All these three compounds have also been identified in MH by 268 Marshall et al.<sup>22</sup> and by Alvarez-Suarez et al.<sup>14</sup> Finally, it is remarkable that kaempferol, 269 quercetin and myricetin possess antimicrobial properties, a well-known characteristic of MH.<sup>23</sup> 270 In our previous work, MH exhibited high antioxidant capacity and there was a significant 271 correlation between its polyphenol content and its antioxidant parameters.<sup>16</sup> 272

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274 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<sub>50</sub> (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, 282 at lower concentration, there was no significant cytotoxic effect. Similar concentrations of MH 283 treatment from 24 to 72 h were not induced any significant toxicity in non-cancer cells (Fig. 1C). 284 Honey represents a good source of sugar. To evaluate if the cytotoxic effect of MH is associated 285 with its sugar content, we performed the cytotoxic effect of artificial honey (1.5 g of sucrose, 7.5 286 287 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 288 toxic effects on HCT-116 and LoVo cells until 48 h. At 72 h, it induced less toxic effect at higher 289 290 concentrations (Supplemental Fig. 1).

By using MH, Fernandez-Cabezudo et al.<sup>17</sup> reported the IC<sub>50</sub> value of 20 and 10 mg/mL in the 291 colon cancer CT-29 cells at 24 and 72 h. These results are quite similar to our results in HCT-292 116 cells. Based on the viability data on other types of honeys, Gelam honey induced IC<sub>50</sub> values 293 were 39 mg/mL and 80 mg/mL, Nenas honey induced IC<sub>50</sub> values were 85.5 mg/mL and Indian 294 commercial honey induced IC<sub>50</sub> were 35 to 40 mg/mL on human colon cancer HT-29, HCT-15 295 and HCT-116 cells at 24 h;<sup>24-26</sup> these concentrations were more compared to MH in the present 296 297 study. These variations are mainly due to honey composition, specifically on different types of flavonoids and phenolic acids, which are known as chemopreventive agents.<sup>15</sup> According to 298 different investigations, phenolic compounds such as quercetin, luteolin, kaempferol, gallic acid 299 300 and caffeic acid that are also present in the MH (Table 1), play an essential function in the suppression of cancer cell proliferation.<sup>11, 15, 17</sup> 301

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 antiproliferative 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.

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311 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 312 313 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 314 HCT-116 cells (Fig. 2A) and up to 4.95 fold in LoVo cells (Fig. 2B), respectively compared to 315 untreated cells. The highest number of apoptotic cells was observed at 15 mg/mL dose for HCT-316 116 cells and 40 to 50 mg/mL dose for LoVo cells (Fig. 2); however the induction of apoptosis 317 318 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.<sup>28</sup> 319 Furthermore, phenolic rich plant extract increased apoptotic cells percentage in DLD-1, HCT-320 116 and HT-29 colon cancer cells compared to untreated cells.<sup>29-31</sup> 321

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

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fragmentation.<sup>4</sup> In HCT-116 cells, the expression of p53 was increased from 1.11 to 1.58 fold, 328 caspase-3 was increased from 1.22 to 1.75 fold and c-PARP was increased from 1.21 to 1.86 fold 329 after MH treatment (Fig. 2C). Similarly, in LoVo cells, the protein expression of p53 was 330 increased from 1.63 to 2.21 fold, caspase-3 was increased from 1.25 to 2.06 fold and c-PARP 331 was increased from 1.18 to 1.80 fold after MH treatment (Fig. 2D). Previously, in colon cancer 332 333 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.<sup>26</sup> In addition, 334 MH is able to induce apoptotic cell death in colon cancer (CT29), breast cancer (MCF-7) and 335 melanoma (B16.F1) cells by activating PARP, caspase-3 and decreasing Bcl-2 expression.<sup>17</sup> 336

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338 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 339 of intrinsic and extrinsic pathways, we further evaluated the mRNA expression of apoptotic 340 marker Bcl-2, Bax, Cyto c, FasL, caspase-8 and caspase-9 by real time PCR. It was observed that 341 MH treatment significantly decreased the expression of Bcl-2 (up to 0.76 and 0.60 fold), while 342 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 343 344 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 345 346 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.<sup>4</sup> In the extrinsic apoptotic pathway, caspase-8 is activated through binding death receptor (Fas or

tumor necrosis factor) associated specific ligands.<sup>4</sup> Based on these observation, our results 351 indicated that MH induced intrinsic apoptosis by decreasing Bcl-2, at the same time increasing 352 Bax, Cyto c and caspase-9 mRNA expressions (Fig. 3). The extrinsic apoptosis was evaluated by 353 increasing FasL and caspase-8 mRNA expressions in both colon cancer cell lines (Fig. 3). These 354 results were consistent with the previous report by other natural bioactive compounds such as 355 356 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, 357 caspase-9, c-PRAP, releasing Cyto C and increasing proapoptotic protein expression (Bax, Bad, 358 Bak and DR5) as well as decreasing antiapoptotic proteins (Bcl-2 and Bcl-xL). <sup>5, 27, 29-34</sup> 359

360

361 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 362 determined the effects of MH on cell cycle arrest on HCT-116 and LoVo cells by Tali™ Image-363 based Cytometer. MH treatment increased the accumulation of cells at Sub-G1 phase of about 364 6% at high concentration (20 mg/mL) in HCT-116 cells, at the same time, in LoVo cells, it was 365 20% at high concentration (50 mg/mL) (Fig. 4). In HCT-116 cells, the percentage of cells in the 366 367 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 368 in the G0/G1 and G2/M phase (Fig. 4A). At the same time, in LoVo cells, the percentage of cells 369 370 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 371 372 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.<sup>26</sup> 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.<sup>5, 31, 35, 36</sup>

379 3.6. Effect of MH on cyclin, CDK, p21<sup>waf1/Cip1</sup> and p27<sup>kip1</sup> mRNA expression in HCT-116 and
380 LoVo cells

381 After demonstrating that MH is able to arrest the cell cycle, we examined the effect of MH on 382 cell cycle regulatory proteins (cyclin D1 and cyclin E), cyclin dependent kinases (CDK2 and CDK4), cyclin dependent kinase inhibitor (p21<sup>waf1/Cip1</sup> and p27<sup>kip1</sup>) and p-Rb expression by real 383 384 time PCR (Fig. 5). In the cell cycle, the transformation from one phase to another phase is 385 controlled by several checkpoint genes such as CDKs via uniting with their relevant administrative subunits cyclins that can activate different downstream targets.<sup>37</sup> Furthermore, 386 387 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 388 genes.<sup>37</sup> We found that MH treatment significantly (p < 0.05) reduced cyclin D1 (0.76 to 0.65) 389 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 390 (0.76 to 0.61 fold) mRNA expression dose dependently in HCT-116 cells compared to untreated 391 cells (Fig. 5A). Consistent with CDKs reduction, the mRNA levels of p21<sup>waf1/Cip1</sup> (1.18 to 1.47 392 fold) and p27kip1 (1.20 to 1.46 fold) dramatically elevated following the MH treatment in HCT-393 116 cells (Fig. 5A). The treatment of HCT-116 and HT-29 cells with flavokawain C and bay leaf 394 395 extract arrested the cell cycle at S phase through downregulating the expression of CDK2 and CDK4 while upregulating p21<sup>waf1/Cip1</sup> and p27<sup>kip1</sup> and hypophosphorylation of Rb.<sup>5, 36</sup> In another 396

397 study pinostilbene was shown to inhibit the expression of cyclin E, p-Rb and increase the 398 expression of  $p21^{waf1/Cip1}$  in HCT-116 cells for inducing the arrest of cell cycle at S phase.<sup>38</sup>

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<sup>39</sup> as well as increasing p21 expression.<sup>35</sup>

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.

411

412 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 420 PI3K/Akt pathways in colon cancer cells HT-29.25 Furthermore, caffeic acid phenethyl ester, a 421 compound derived from honey bee propolis, suppresses total and phosphorylated EGFR in breast 422 cancer cells.<sup>40</sup> Similarly, flavokawain C and protein extract from plant sources induce apoptosis 423 in HCT-116 and DLD-1 colon cancer cells by suppressing Akt expression.<sup>5, 30</sup> After polyphenol 424 treatment, a reduction has been observed in EGFR expression by decreasing the transcription 425 factor Egr-1 in colon cancer Caco-2 and HT-29 cells.<sup>41</sup> In this work, we found that MH 426 suppressed the expression of EGFR, HER2 and p-Akt (Fig. 6A, 6B), while EGFR was 427 428 suppressed in a more remarkable manner in LoVo cells while p-Akt was highly suppressed in HCT-116 cells after MH. 429

We further investigated the involvement of p-p38MAPK and p-Erk1/2 pathways in the apoptotic 430 properties of MH. MH increased the phosphorylation of p38MAPK and Erk1/2 protein from 1.82 431 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 2.48 432 fold and 2.11 to 3.29 fold for LoVo cells at dose 30 to 40 mg/mL, respectively (Fig. 6A, 6B). 433 Similarly, quercetin (common flavonoids of MH) increases MAPK activation through the 434 activation of p-Erk, p-JNK and p-38MAPK in colon cancer CT26 cells, leading to apoptosis.<sup>27</sup> 435 436 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 437 apoptosis.<sup>5, 30, 33</sup> 438

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.

442

443 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 444 445 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 446 expressions in both cell lines, but after treatment with higher concentrations the expression of 447 448 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. 449 450 This finding indicated that MH leads to ER stress inducing HCT-116 cell death by increasing the 451 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 452 HCT-116 and HT-29 cells, has been assessed by other natural compounds.<sup>42,43</sup> 453

454

# 455 **4. Conclusion**

456 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 457 phenolic constitutes, only a few studies addressed the chemopreventive effects of MH. 458 459 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 460 461 mRNA expression of both intrinsic and extrinsic apoptotic markers such as caspase-8, -9, Bax, 462 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 463 addition, MH induced cell cycle arrest at the S phase in HCT-116 cells and molecular 464 465 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 466 G2/M phase arrest through increasing p21 and p27 expression and only suppressed cyclin D1 467 expression was observed which activate apoptosis through leading to growth reduction. In 468 addition, we also demonstrated that MH induced apoptotic effect was related with increased ER 469 stress associated cells death by elevating ATF6 and XBP1 expression, suppressed of EGFR, 470 471 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 472 toxicity. These interesting and promising results encourage our knowledge about 473 474 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 475 against colon cancer. 476

477

### 478 **Conflict of interest**

# 479 The authors declare no conflicts of interest.

480

# 481 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-

- 489 yl)-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PE, plating
- 490 efficiency; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; Rb, retinoblastoma;
- 491 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 ( $\lambda$ , 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
<i>p</i> -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  $\pm$  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<sup>®</sup> 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<sup>™</sup> apoptosis kit and the Tali<sup>™</sup> 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  $\mu$ m. 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  $\pm$  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  $\pm$  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<sup>®</sup> Cell Cycle Assay kit and Tali<sup>TM</sup> 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  $\pm$  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  $\pm$  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  $\pm$  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 <sup>kip1</sup>	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  $\pm$  standard deviation (SD) (n=3).