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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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28 **Abstract**

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

47

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

50

51

52 **1. Introduction**

53 Globally, colorectal cancer (CRC) is the third most widespread cancer in both men and women;
54 over 1 million new cases are diagnosed each year accounting for 9.7% of all cancers apart and
55 consequently more than 693,933 deaths per annum corresponding to 8.5% of the total number of
56 cancer deaths.¹ Notably, about 50% CRC patients develop the recurrent disease² indicating that
57 presently existing treatments are not able to control this deadly disease. Furthermore, CRC has a
58 significant impact on medical care as well as on the global economy. In fact in the USA alone,
59 the expenditure of CRC treatment is estimated to exceed \$17 billion in the healthcare system by
60 2020.³

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

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

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

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

98 and only two investigations have reported the antiproliferative effect of MH on colon cancer
99 cells.^{16, 17}

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.

105

106 **2. Materials and methods**

107 2.1. Honey samples and reagents

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

114

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.^{18, 19} MH (3 g) were mixed with
120 ultrapure water (12 mL) and then the solution was adjusted to pH=2 with concentrated HCl. The

121 fluid samples were centrifuged at 8000 x g for 10 min to remove the solid particles. The
122 supernatants were loaded onto the previously conditioned cartridges (under these conditions:
123 methanol (3 mL), equilibrated as follows: acidified ultrapure water (pH = 2; 3 mL)). After
124 loading, these cartridges were washed with acidified ultrapure water (pH = 2; 4 mL) to remove
125 sugars and other polar compounds of honey that were not absorbed on the sorbents. Then,
126 phenolic compounds retained on the cartridges were eluted with formic acid: methanol (1:9, v/v;
127 5 mL). The eluate was evaporated down (25 °C, 5 psi) and then reconstituted in methanol with
128 2% acetic acid (2 mL). All solutions were filtered through a 0.22- μ m filter prior to HPLC
129 injection. HPLC measurements were made by using a Thermo Scientific System equipped with a
130 Spectra SYSTEM P 4000 pump, a Spectra SYSTEM AS 3000 auto sampler and a Finnigan
131 Surveyor PDA Plus Detector. Chromatographic separations were performed with a Luna C18
132 analytical column (150 x 3 mm ID, 3- μ m particle) with a guard column (4 x 3 mm ID)
133 containing the same packing material, both from Phenomenex (Torrance, CA, USA).

134

135 2.2.2. Determination of flavonols and phenolic acids

136 An aliquot (20 μ L) was injected into the column and eluted at 35°C with a constant flow rate of
137 0.4 mL/min. The mobile phase was composed of water/formic acid/acetonitrile (87:10:3), v/v/v;
138 Component A) and water/formic acid/acetonitrile (40:10:50, v/v/v; Component B) for flavonols
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
141 following gradient was used for flavonols: 90% A, changed to 75% A in 10 min, changed to 69%
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.

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

148

149 2.3. Cell culture

150 Human colon adenocarcinoma (HCT-116), Dukes' type C, grade IV, colon metastasis (LoVo)
151 and healthy human dermal fibroblast (HDF) cell lines were purchased from the American Type
152 Culture Collection (ATCC, Manassas, VA, USA). Media and reagents for cell culture were
153 obtained from Carlo Erba Reagents (Milan, Italy). McCoy's 5A media was used for HCT-116
154 cell culture, F-12K media were used for LoVo cells culture and DMEM media were used for
155 HDF cells culture. All the media was prepared with of 10% heat-inactivated fetal bovine serum,
156 100 IU/mL penicillin and 100 µg /mL streptomycin. All cell lines were maintained in an
157 incubator at 37°C in a humidified atmosphere (95% air, 5% CO₂). For the subsequent
158 experiment, cells were used between the 6th and 10th passages.

159

160 2.4. Cell viability assay

161 Cells were seeded at a density of 5×10^3 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

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

171

172 2.5. Determination of apoptotic cells by Tali[®] Image-Based Cytometer

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

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

192

193 2.6. Cell cycle analysis by Tali[®] Image-Based Cytometer

194 Cell cycle analysis was determined by the Tali[®] Cell Cycle Kit (Invitrogen[™], Life Techonoliges,
195 Milan, Italy) as previously described.²⁰ Cells were cultured in 6-well plates, at a density of 4×10^5
196 cells/ well and incubated with various concentration of MH 0, 10, 15 and 20 mg/mL for HCT-
197 116 cells and 0, 30, 40 and 50 mg/mL for LoVo cells for 48 h. Then, cells were trypsinized and
198 centrifuged for 5 min at 500 x g after removing the excess media, resuspending with PBS,
199 centrifuging for 5 min at 500 x g and transferring the cells into ice. Briefly, the cells were
200 harvested and fixed with 70% cold ethanol at -20°C overnight. The fixed cells were washed
201 twice with PBS, re-suspended in 100 μ L PBS-based PI (Invitrogen[™], Life Techonoliges, Milan,
202 Italy) solution containing 0.1% Triton[®] X-100, 0.2 mg/ml RNase A (Invitrogen), and 20 μ g/ml
203 PI, and incubated for 30 min at room temperature protected from the light. For each sample, 25
204 μ L of cell suspension was loaded into one Tali[™] Cellular Analysis Slide's chamber
205 (Invitrogen[™], Life Techonoliges, Milan, Italy) and analyzed in the Tali[™] Image-Based
206 Cytometer. The results were expressed as the percentage of cells in each phase and all data were
207 reported as a mean value of three independent analyses \pm SD.

208

209 2.7. RNA isolation and quantitative real-time PCR analysis

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

213 to the manufacturer's protocol. RNA concentrations and purity were checked by using the
214 microplate spectrophotometer system (BioTek Synergy HT, Winooski, USA). The sample ratio
215 between 1.8 to 2.1 was only selected for gene expression assay. cDNA was synthesis from 100
216 ng RNA following reverse transcription according to the manufacturer's protocol (5X All-In-
217 One RT MasterMix kit, Applied Biological Materials Inc. Canada). Real-time PCR was
218 performed using EvaGreen 2X qPCR MasterMix (EvaGreen 2X qPCR MasterMix kit, Applied
219 Biological Materials Inc. Canada) of forward and reverse primers (Supplementary Table 1) of
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
222 c (Cyt c), fatty acid synthetase ligand (FasL), caspase-8 and caspase-9 with 50 ng cDNA in a
223 final volume 20 μ L on a real-time PCR system (Corbett Life Science, Rotor-Gene 3000,
224 Mortlake, Australia). RT-PCR control GADPH was amplified under the same PCR conditions
225 for normalizing quantitative data. The $2^{-\Delta\Delta Ct}$ method was used for calculating the fold change
226 values.

227

228 2.8. Protein extraction and western blotting

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

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

245

246 2.9. Statistical analysis

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

252

253 **3. Results and Discussion**

254 3.1. Identification and quantification of phenolic compounds

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

259 acid, ellagic acid and *trans*-cinnamic acid. Gallic acid (36.57%) and syringic acid (32.55%) were
260 the main components (11.55 and 10.28 mg/100 g honey, respectively), while the other phenolic
261 acids were presented in low proportions ranging from 1.84% to 1.68% for 4-hydroxybenzoic
262 acid and caffeic acid (0.58 and 0.53 mg/100 g honey), respectively. In a recent study, Ahmed *et*
263 *al.* analyzed the phenolic composition of 17 multifloral honey samples.²¹ 4-Hydroxybenzoic acid
264 was the main phenolic compound identified in all the studied honeys, followed by gallic acid
265 with similar concentrations to those found in the present study. As flavonols, the highest
266 concentrations of quercetin, luteolin and kaempferol were found in this family (3.73, 2.62 and
267 1.17 mg/ 100 g of honey). They represented 11.81%, 8.30% and 3.70% of the total phenolic
268 content, respectively (Table 1). All these three compounds have also been identified in MH by
269 Marshall *et al.*²² and by Alvarez-Suarez *et al.*¹⁴ Finally, it is remarkable that kaempferol,
270 quercetin and myricetin possess antimicrobial properties, a well-known characteristic of MH.²³
271 In our previous work, MH exhibited high antioxidant capacity and there was a significant
272 correlation between its polyphenol content and its antioxidant parameters.¹⁶

273

274 3.2. Anti-proliferative effects of MH on HCT-116 and LoVo cells

275 Our result documented the capability of MH in the suppression of the colon cancer cell
276 proliferation at different concentrations and times. As shown in Fig. 1A and Fig. 1B, the
277 percentage of viable cells was reduced in the treated HCT-116 and LoVo cells compared to
278 untreated cells in a dose- and time-dependent manner. In HCT-116 and LoVo cells, the IC₅₀
279 (concentrations necessary for 50% inhibition of cell growth) of MH were 21.98 mg/mL and
280 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
281 mg/mL at 72 h, respectively (Fig. 1A, 1B). The range of concentrations used to treat the LoVo

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

291 By using MH, Fernandez-Cabezudo *et al.*¹⁷ reported the IC₅₀ value of 20 and 10 mg/mL in the
292 colon cancer CT-29 cells at 24 and 72 h. These results are quite similar to our results in HCT-
293 116 cells. Based on the viability data on other types of honeys, Gelam honey induced IC₅₀ values
294 were 39 mg/mL and 80 mg/mL, Nenas honey induced IC₅₀ values were 85.5 mg/mL and Indian
295 commercial honey induced IC₅₀ were 35 to 40 mg/mL on human colon cancer HT-29, HCT-15
296 and HCT-116 cells at 24 h;²⁴⁻²⁶ these concentrations were more compared to MH in the present
297 study. These variations are mainly due to honey composition, specifically on different types of
298 flavonoids and phenolic acids, which are known as chemopreventive agents.¹⁵ According to
299 different investigations, phenolic compounds such as quercetin, luteolin, kaempferol, gallic acid
300 and caffeic acid that are also present in the MH (Table 1), play an essential function in the
301 suppression of cancer cell proliferation.^{11, 15, 17}

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

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

310

311 3.3. MH induces apoptosis on HCT 116 and LoVo cells

312 Apoptosis induction is a broadly accepted method for controlling the growth and development of
313 cancer cells. Flow cytometry analysis confirmed the apoptotic cell death induced by MH (Fig. 2).
314 The number of apoptotic cells was significantly ($p < 0.05$) increased from up to 3.38 fold in
315 HCT-116 cells (Fig. 2A) and up to 4.95 fold in LoVo cells (Fig. 2B), respectively compared to
316 untreated cells. The highest number of apoptotic cells was observed at 15 mg/mL dose for HCT-
317 116 cells and 40 to 50 mg/mL dose for LoVo cells (Fig. 2); however the induction of apoptosis
318 was more robust in LoVo cells than in HCT-116 cells treated with MH. By using the same
319 technique, by-products from beeswax induced apoptotic cell death in liver cancer cells.²⁸
320 Furthermore, phenolic rich plant extract increased apoptotic cells percentage in DLD-1, HCT-
321 116 and HT-29 colon cancer cells compared to untreated cells.²⁹⁻³¹

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

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

337

338 3.4. MH induces intrinsic and extrinsic apoptosis on HCT 116 and LoVo cells

339 To determine whether the perceptible induction of apoptosis was associated with the activation
340 of intrinsic and extrinsic pathways, we further evaluated the mRNA expression of apoptotic
341 marker Bcl-2, Bax, Cyto c, FasL, caspase-8 and caspase-9 by real time PCR. It was observed that
342 MH treatment significantly decreased the expression of Bcl-2 (up to 0.76 and 0.60 fold), while
343 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
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)
345 were increased in both HCT-116 and Lovo cells compared to untreated cells, and the effects
346 were dose-dependently (Fig. 3).

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

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

360

361 3.5. MH induces cell cycle arrest of HCT 116 and LoVo cells

362 Cell proliferation is correlated with the regulation of cell cycle progression. Therefore, we
363 determined the effects of MH on cell cycle arrest on HCT-116 and LoVo cells by Tali™ Image-
364 based Cytometer. MH treatment increased the accumulation of cells at Sub-G1 phase of about
365 6% at high concentration (20 mg/mL) in HCT-116 cells, at the same time, in LoVo cells, it was
366 20% at high concentration (50 mg/mL) (Fig. 4). In HCT-116 cells, the percentage of cells in the
367 S phase was significantly ($p < 0.05$) increased from up to 43% after MH treatment compared to
368 untreated cells (23 to 26%), while the percentage of cells was significantly ($p < 0.05$) decreased
369 in the G0/G1 and G2/M phase (Fig. 4A). At the same time, in LoVo cells, the percentage of cells
370 in the G2/M phase was significantly ($p < 0.05$) increased up to 40% after MH treatment
371 compared to untreated cells (20 to 24%), while the percentage of cells in the G0/G1 and S phase
372 was significantly ($p < 0.05$) decreased (Fig. 4B).

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

378

379 3.6. Effect of MH on cyclin, CDK, p21^{waf1/Cip1} and p27^{kip1} 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
383 CDK4), cyclin dependent kinase inhibitor (p21^{waf1/Cip1} and p27^{kip1}) and p-Rb expression by real
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
386 administrative subunits cyclins that can activate different downstream targets.³⁷ Furthermore,
387 CDK-cyclin complexes are inversely regulated by CDK inhibitors (p21Cip and p27Kip), which
388 in turn dephosphorylate Rb proteins for the transcriptional activation of cell cycle regulated
389 genes.³⁷ We found that MH treatment significantly ($p < 0.05$) reduced cyclin D1 (0.76 to 0.65
390 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
391 (0.76 to 0.61 fold) mRNA expression dose dependently in HCT-116 cells compared to untreated
392 cells (Fig. 5A). Consistent with CDKs reduction, the mRNA levels of p21^{waf1/Cip1} (1.18 to 1.47
393 fold) and p27^{kip1} (1.20 to 1.46 fold) dramatically elevated following the MH treatment in HCT-
394 116 cells (Fig. 5A). The treatment of HCT-116 and HT-29 cells with flavokawain C and bay leaf
395 extract arrested the cell cycle at S phase through downregulating the expression of CDK2 and
396 CDK4 while upregulating p21^{waf1/Cip1} and p27^{kip1} and hypophosphorylation of Rb.^{5, 36} In another

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.³⁸
399 Furthermore, in LoVo cells we found that MH treatment markedly increased the p21^{waf1/Cip1} (1.47
400 to 1.75 fold) and p27^{kip1} (1.33 to 2.37 fold) mRNA expression, while cyclin D1 (0.77 to 0.61
401 fold) expression was significantly suppressed compared to untreated cells (Fig. 5B). However,
402 no changes of other mRNA expression were observed (data not shown). These results were
403 consistent with previous report showing that natural compounds treatment suppressed the colon
404 cancer cells progression through arresting the cell cycle at G2/M phase via inhibiting cyclin D1
405 expression³⁹ as well as increasing p21 expression.³⁵
406 Based on these results, it can be summarized that MH suppressed HCT-116 cells growth through
407 arresting the cell cycle at S phase, upregulating p21 and p27, and downregulating CDK2, CDK4,
408 cyclin D1, cyclin E and p-RB expression. In Lovo cells, MH arrested the cell cycle at G2/M
409 phase and the mechanism was associated with increasing expression of p21 and p27 and
410 decreasing cyclin D1 expression, respectively.

411

412 3.7. Effect of MH on EGFR and MAPK signaling pathways on HCT-116 and LoVo cells

413 We examined the effects of MH on membrane protein EGFR and HER2 and their downstream
414 signaling protein p-Akt on HCT-116 and LoVo cells. As shown in Fig. 6, treatment with MH
415 dose-dependently suppressed the expression levels of EGFR up to 0.52 fold for HCT-116 cells
416 and up to 0.40 fold for LoVo cells. Similarly, the expression of HER2 was also suppressed up to
417 0.51 fold in HCT-116 cells and up to 0.38 fold for LoVo cells, respectively (Fig. 6A, 6B).
418 Additionally, MH treatment at high concentration significantly suppressed the expression of p-
419 Akt up to 0.22 fold for HCT-116 and up to 0.37 folds LoVo cells (Fig. 6A, 6B). Tahir *et al.*

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

430 We further investigated the involvement of p-p38MAPK and p-Erk1/2 pathways in the apoptotic
431 properties of MH. MH increased the phosphorylation of p38MAPK and Erk1/2 protein from 1.82
432 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
433 fold and 2.11 to 3.29 fold for LoVo cells at dose 30 to 40 mg/mL, respectively (Fig. 6A, 6B).
434 Similarly, quercetin (common flavonoids of MH) increases MAPK activation through the
435 activation of p-Erk, p-JNK and p-38MAPK in colon cancer CT26 cells, leading to apoptosis.²⁷
436 Furthermore, natural compounds from several plant sources increase the expression of p-Erk1/2
437 and p-p38MAPK in colon cancer HCT-116, DLD-1 and HT-29 cells for inducing cell death by
438 apoptosis.^{5, 30, 33}

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

442

443 3.8. MH induces endoplasmic reticulum stress in HCT-116 and LoVo cells

444 To further find out the probable apoptosis effects activated by MH, we investigated the protein
445 level of ER stress-associated molecules, ATF6 and XBP1 by western blotting. As shown in Fig.
446 6C, 6D, the lower concentration of MH did not cause an upregulation of ATF6 and XBP1
447 expressions in both cell lines, but after treatment with higher concentrations the expression of
448 these two proteins was unregulated 1.77 to 2.15 fold and 1.16 to 1.75 fold in HCT-116 cells and
449 1.42 to 2.56 fold and 1.91 to 2.62 fold in Lovo cells compared to untreated cells, respectively.
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
452 ER stress-induced cancer cell death, by activating ATF6 and XBP1 expression in colon cancer
453 HCT-116 and HT-29 cells, has been assessed by other natural compounds.^{42, 43}

454

455 **4. Conclusion**

456 This study explored the anti-proliferative and apoptotic effects of MH in two genetically well
457 recognized human colon adenocarcinoma cell lines HCT-116 and LoVo. Despite its high
458 phenolic constitutes, only a few studies addressed the chemopreventive effects of MH.
459 Exposure to MH inhibited the cells proliferation ability, induced apoptosis and blocked cell cycle
460 progression in both colon cancer cell types. This event was accompanied by increasing the
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
463 of p53, caspase-3 and c-PARP also confirmed the apoptosis induction after MH treatment. In
464 addition, MH induced cell cycle arrest at the S phase in HCT-116 cells and molecular
465 mechanism behind of this was the increased expression of p21 and p27, while cyclin D1, cyclin

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

477

478 **Conflict of interest**

479 The authors declare no conflicts of interest.

480

481 **Abbreviation**

482 ABB, annexin binding buffer; Akt, protein kinase B; ATF6, activating transcription factor 6;
483 Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; CRC, colorectal cancer; CDK,
484 cyclin dependent kinase; Cyto c, cytochrome c; EGFR, epidermal growth factor receptor; ER,
485 endoplasmic reticulum; Erk1/2, extracellular-signal-regulated kinase 1/2; FasL, fatty acid
486 synthetase ligand; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HDF, human dermal
487 fibroblast; HER2, human epidermal growth factor receptor 2; JNK, jun N-terminal kinase;
488 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 (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
<i>p</i> -coumaric acid	501-98-4	27.46	308	0.12±0.03
<i>trans</i> -ferulic acid	537-98-4	33.19	322	0.19±0.01
Ellagic acid	476-66-4	38.08	251	nd
<i>trans</i> -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).

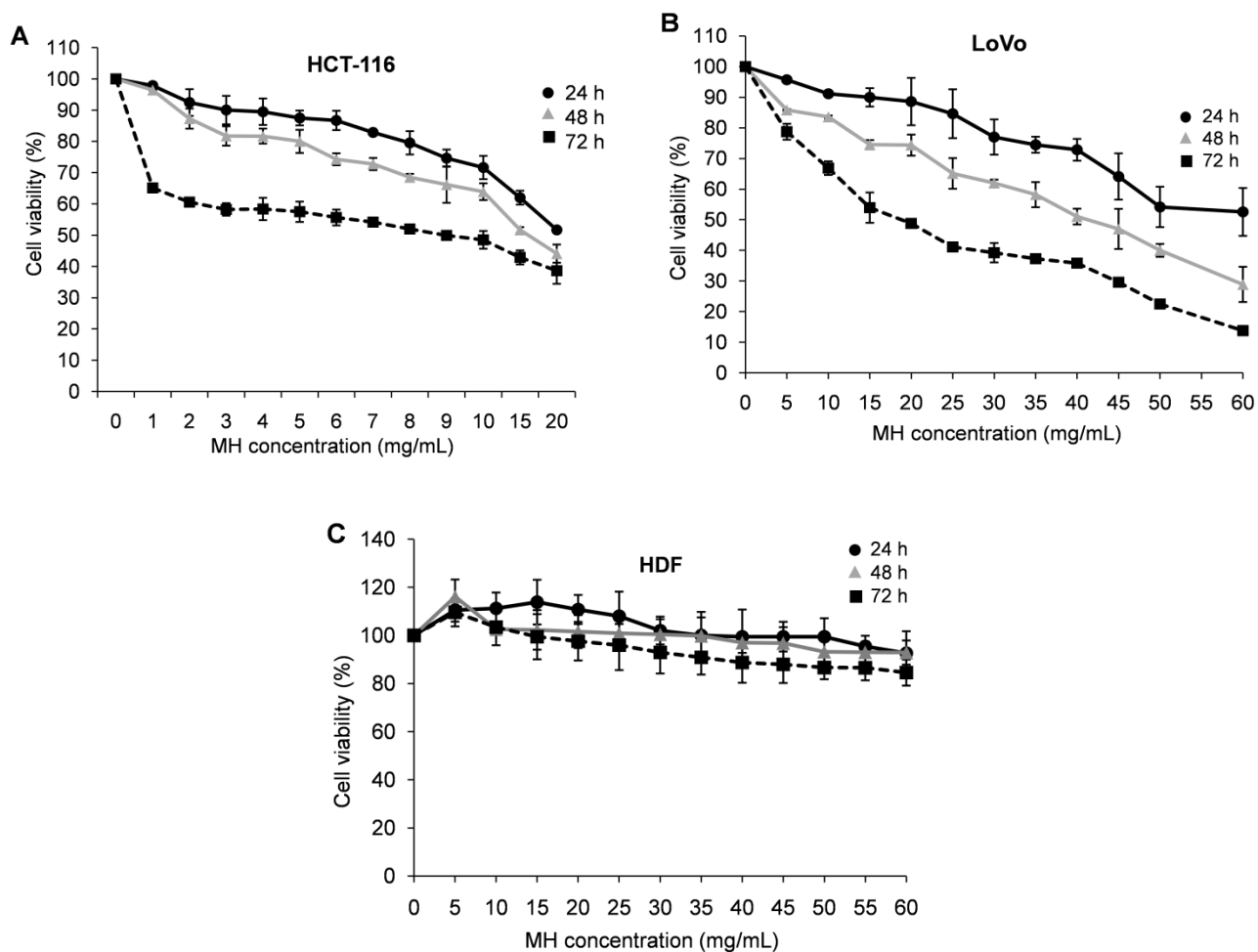


Fig. 1 Inhibition of cell proliferation by MH in HCT-116, LoVo and HDF cell lines. All cells were treated with different concentrations of MH for 24, 48 and 72 h. Cell viability was measured by using MTT assay and results were expressed as a % of viable (A) HCT-116, (B) LoVo and (C) HDF cells compared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

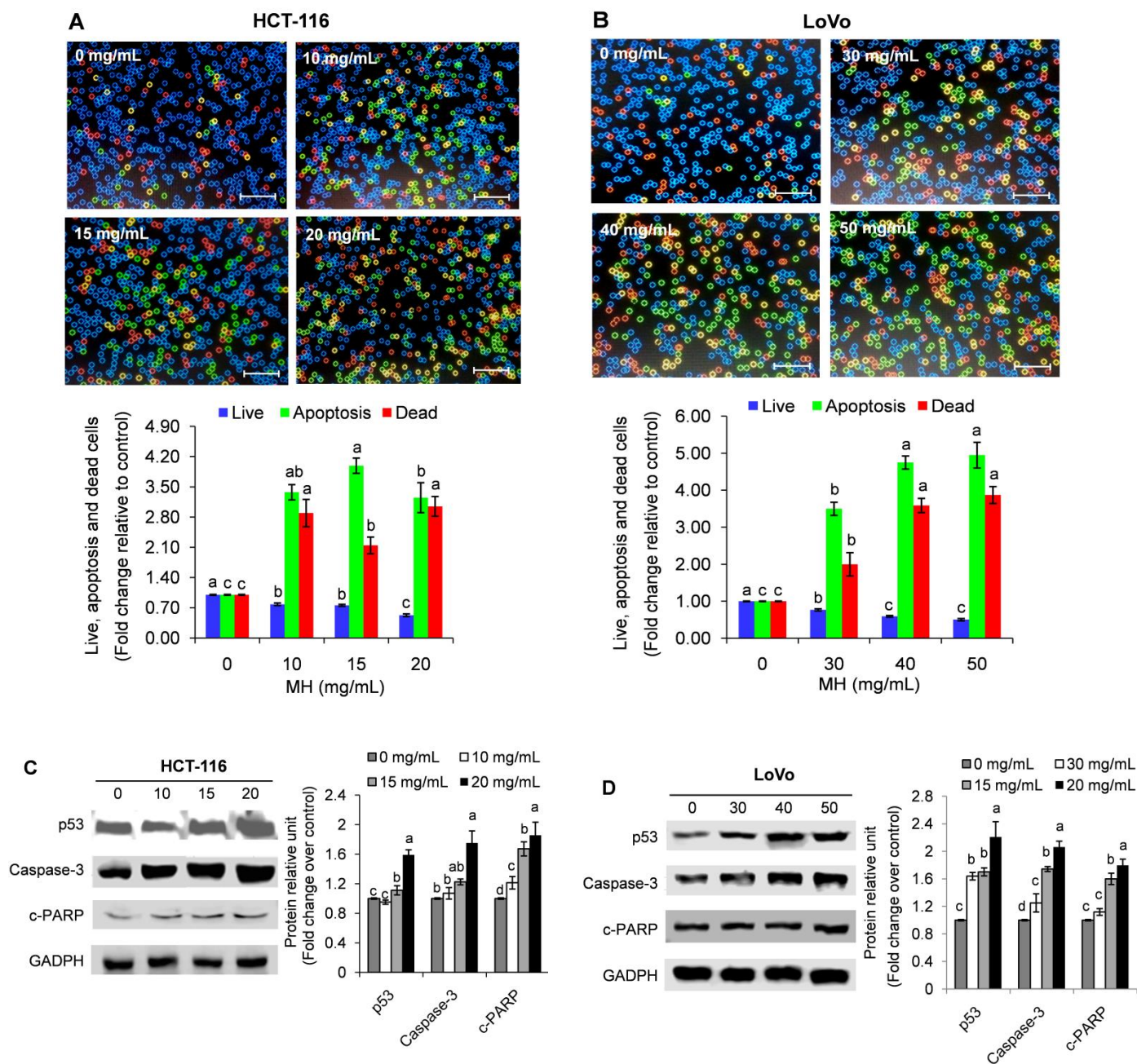


Fig. 2 Apoptosis induction by MH in HCT-116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. Annexin V Alexa Fluor® 488 and PI staining was used for determination of apoptotic effect of MH on (A) HCT-116 and (B) 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 μ 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. GAPDH 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 ($p < 0.05$).

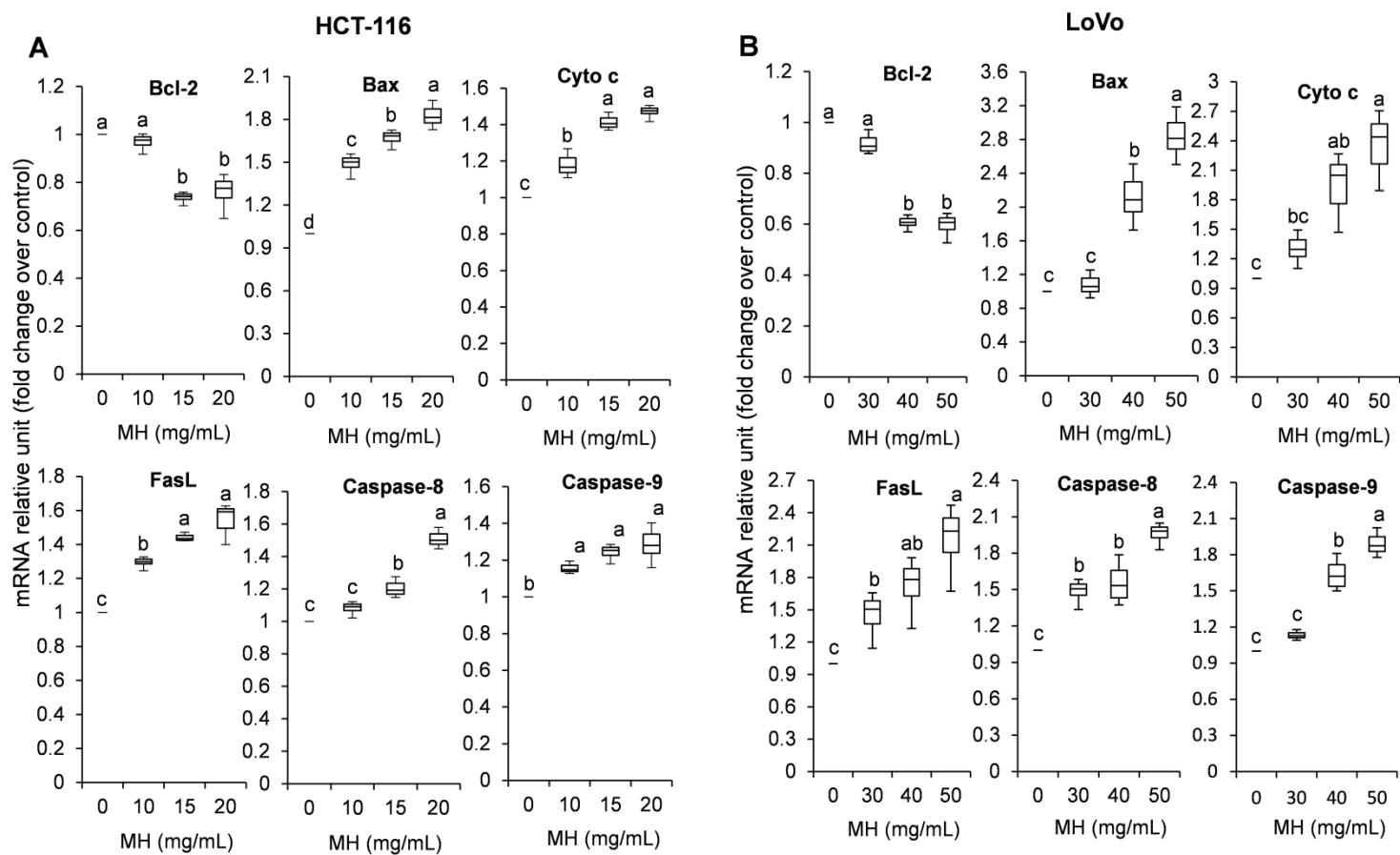


Fig. 3 MH induces intrinsic and extrinsic apoptosis on HCT 116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. mRNA expression of intrinsic and extrinsic apoptotic markers Bcl-2, Bax, Cyto c, FasL, caspase-8 and 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 ($p < 0.05$).

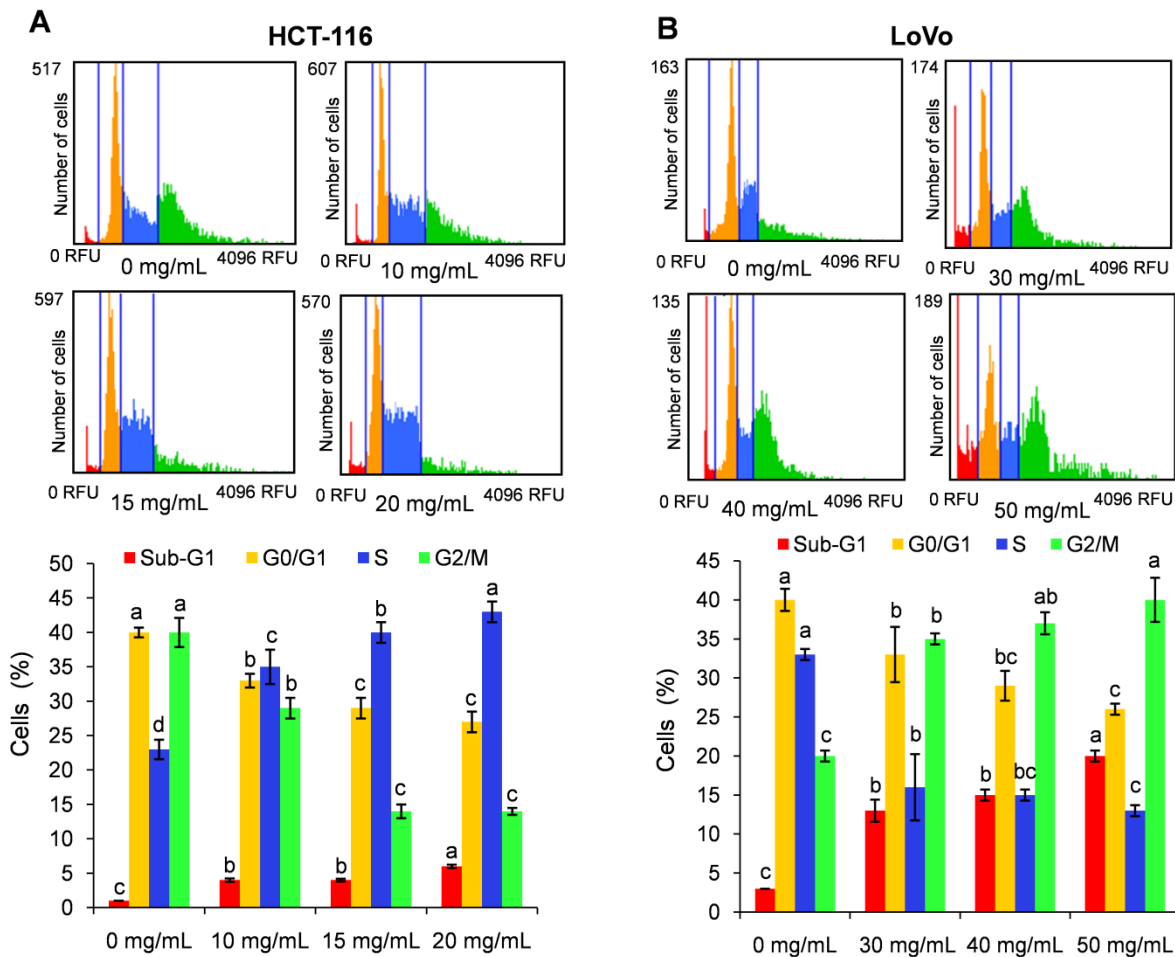


Fig. 4 Cell cycle alteration induced by MH in HCT-116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. The percentages of cells in each phase Sub-G1 (apoptotic cells), G0/G1, S and G2/M were calculated by the Tali[®] Cell Cycle Assay kit and Tali[™] Image-based Cytometer. Representative fluorescence images of (A) HCT-116 and (B) LoVo cells cycle show 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 superscript letters for each column indicated significant differences ($p < 0.05$).

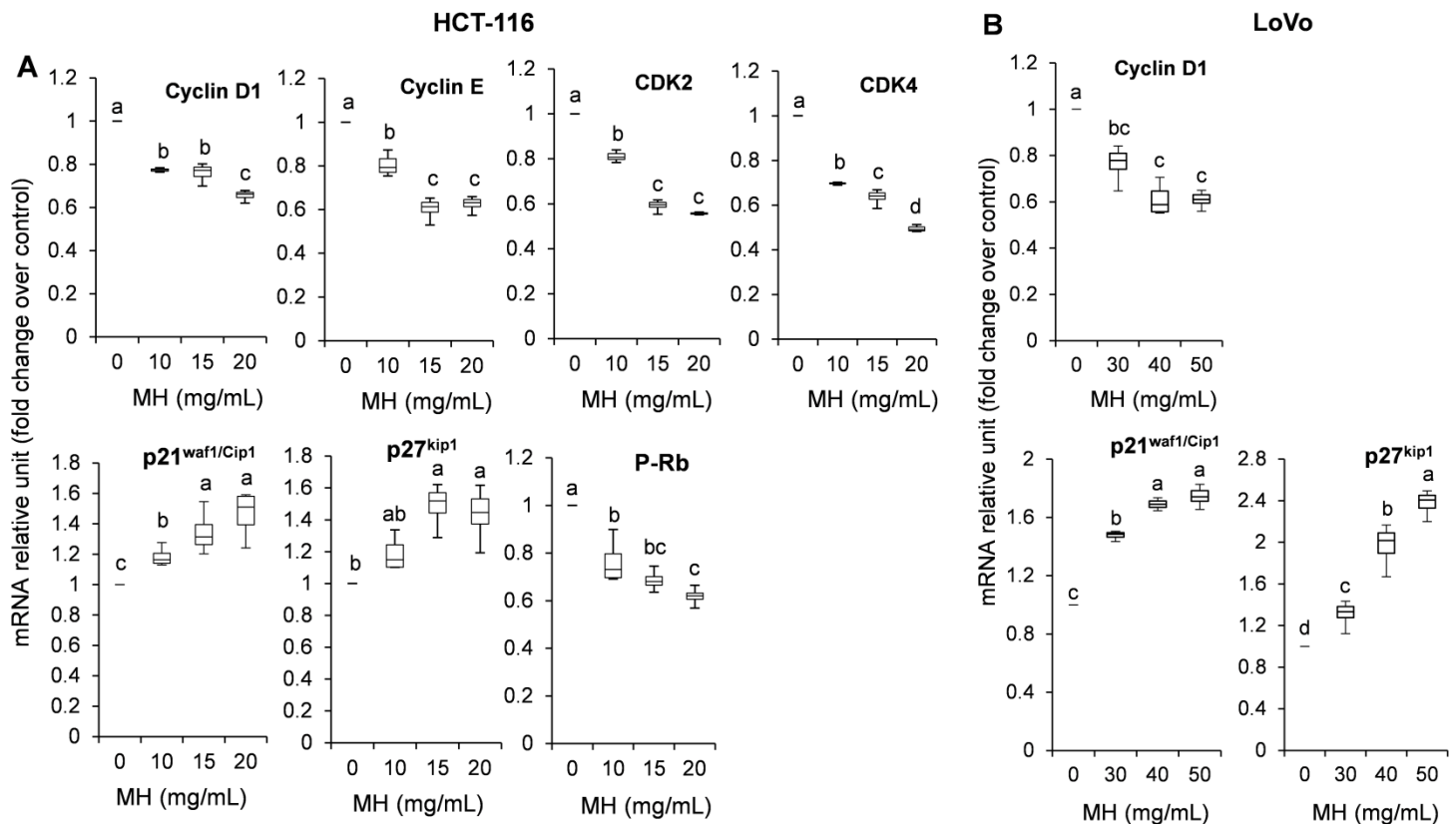


Fig. 5 MH induces alteration of cell cycle regulatory mRNA expression in HCT-116 and Lovo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. Cell cycle regulatory proteins (cyclin D1 and cyclin E), cyclin dependent kinases (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 ($p < 0.05$).

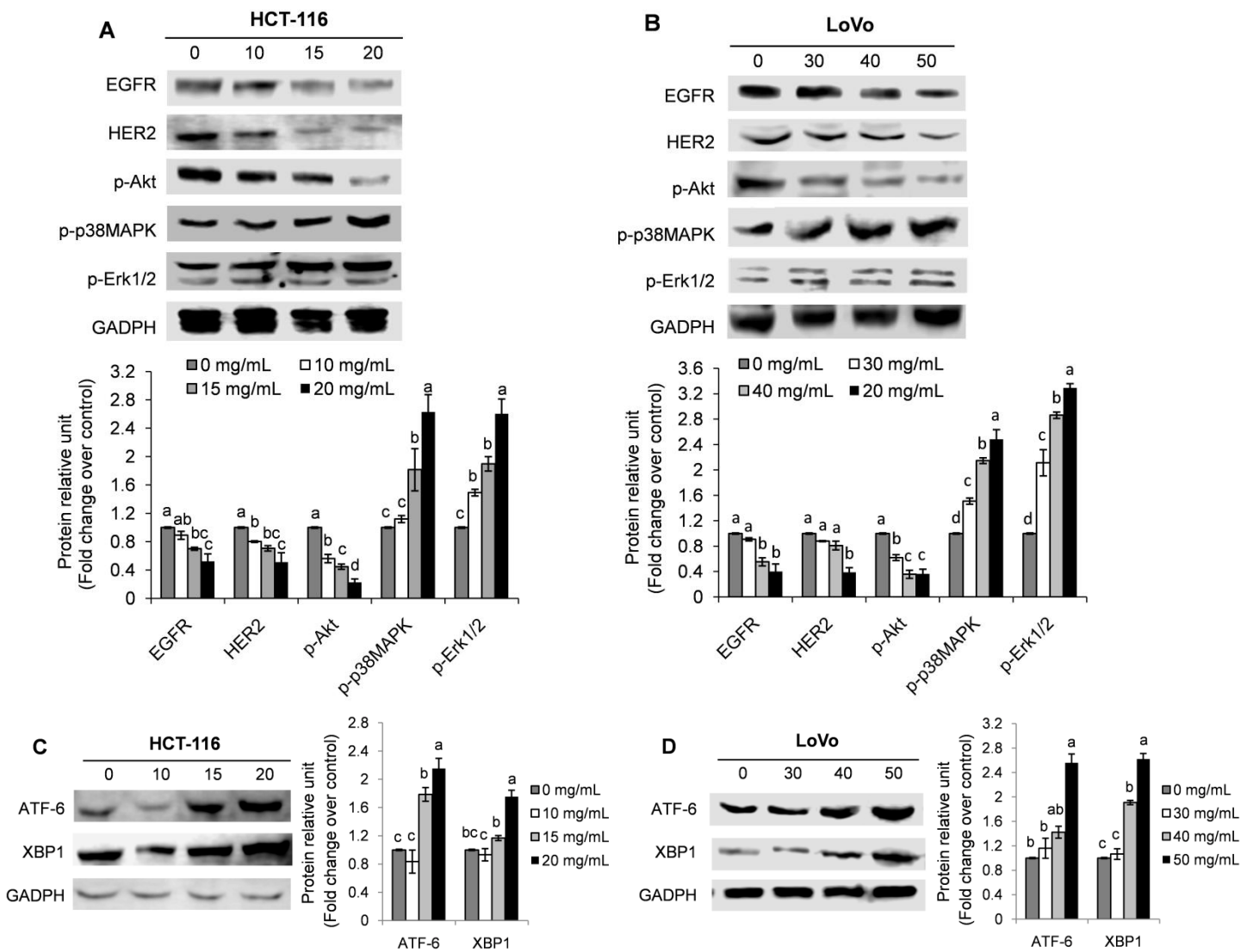
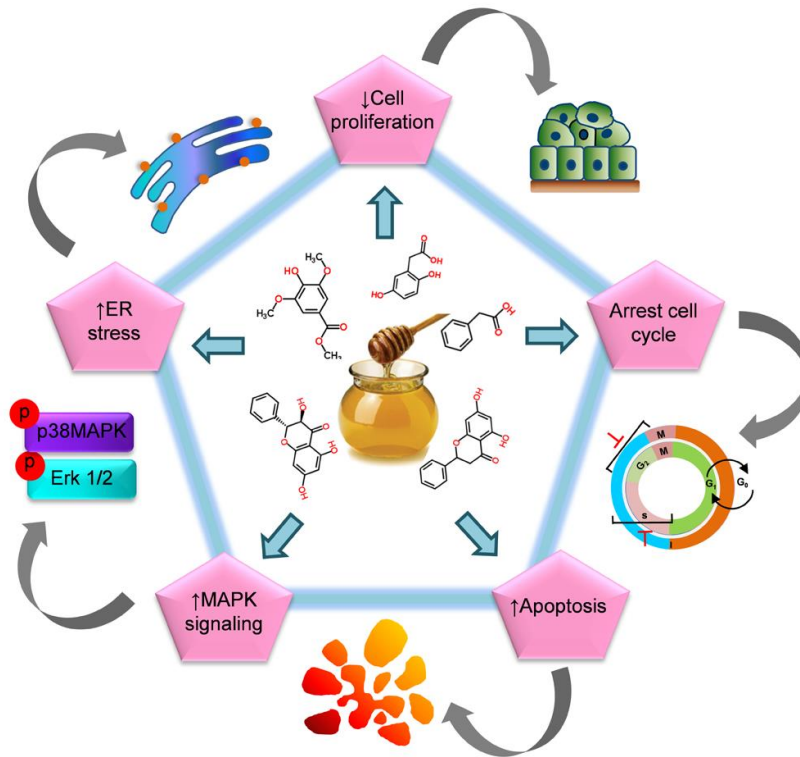


Fig. 6 Effect of MH on EGFR, MAPK and endoplasmic reticulum stress signaling in HCT-116 and LoVo cells. Both cells were exposed with different concentrations of MH for 48 h, while 0 concentrations correspond to untreated cells. (A) and (B), the protein involved on EGFR (EGFR, HER2 and p-Akt) and MAPK 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

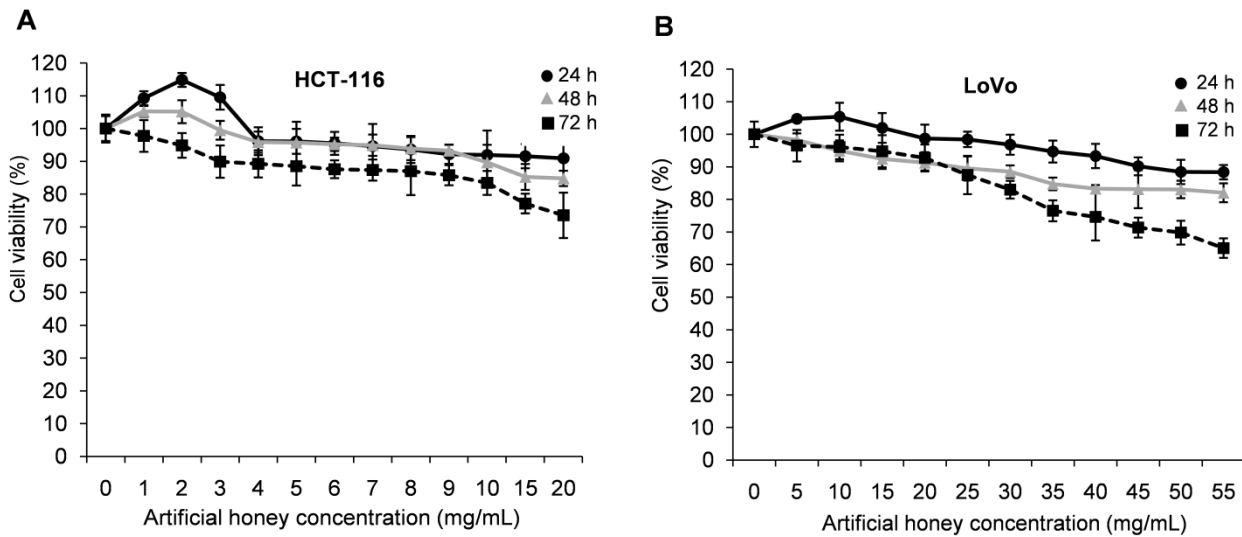
Manuka honey suppresses human colon cancer cells proliferation through promotion of apoptosis, arrest of cell cycle, activates MAPK signaling and induces endoplasmic reticulum stress.



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	GGTCAGCATTTCAGTAGC
p21 ^{waf1/cip1}	Forward	GCGATGGA ACTTCGACTTTGT
	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	TGTCCTACTCTACTTTCCAGTTTT
	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).