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Abstract

The development of chemo-sensitizers is urgently needed to overcome 5-fluorouracil (5-FU) therapeutic resistance and adverse toxicity in colorectal cancer. This work aims to evaluate the synergic effects of 5-FU and Manuka honey (MH), a rich source of bioactive compounds, in enhancing the anticancer effects of this drug on human colon cancer HCT-116 and LoVo cells. Compared to 5-FU alone, MH synergistically enhanced the chemotherapeutic effects of 5-FU, by reducing cell proliferation through the suppression of EGFR, HER2, p-Akt and p-mTOR expression, and promoting apoptosis by the modulation pro-apoptotic (p53, Bax, Cyto c, FasL caspase-3, -8, -9 and cleave-PARP) and anti-apoptotic (Bcl-2) markers. The activations of p-p38MAPK and p-Erk1/2 pathways and ROS production were also involved in this process. Downregulation of transcription factor (NF-κB and Nrf2) and antioxidant enzyme activity (SOD, catalase, glutathione peroxidase and glutathione reductase) and expression (SOD, catalase and HO-1) were more evident after the combined treatment, leading to more cell death by oxidative stress. Moreover, additive effects were also observed by increasing lipid and protein oxidation and arresting cell cycle. All the parameters of mitochondrial respiration and glycolysis function decreased and both cells entered the quiescent stage after the combined treatments. MH also influenced the anti-metastasis effects of 5-FU by decreasing migration ability, suppressing the expression of MMP-2, MMP-9 and increasing N-cadherin and E-cadherin. In conclusion, MH could be a useful preventive or adjuvant agent in the treatment of colorectal cancer with 5-FU.

Keywords: Colon cancer, 5-Fluorouracil, Manuka honey, Synergistic effect, Reactive oxygen species, Apoptosis.

Highlights

- Manuka honey enhances 5-FU-induced oxidative stress in colon cancer cells.
- Manuka honey enhances 5-FU-induced apoptosis, regulating intrinsic and extrinsic pathway.
- Manuka honey and 5-FU combination affects mitochondrial respiration and glycolysis.
- Manuka honey enhances 5-FU-induced anti-metastatic effects, controlling MMP-2, MMP-9, and EMT markers expression.

1. Introduction

Colorectal cancer (CRC) is the third most important cause of mortality and morbidity all over the world [1]. It is one of the most recurrent human tumors and prognosis for these tumors remains elusive for about 50% of individuals affected. Overall chemotherapy raises the survival rate in CRC patients but the cure rate is related to the cancer stages. In the early stages of CRC the cure rate is approximately 90%, while in the advanced stage, such as the metastasis one, the cure rate falls to a dismal range below 10% [2]. Conventional therapies are not suitable for distant metastasis in CRC patients and sustain a poor survival rate almost five years [2].

During the last four decades, 5-Fluorouracil (5-FU) has represented the first choice for CRC therapy due to a better understanding of its mechanism of action [3]. 5-FU metabolites are incorporated into DNA and RNA, acting as a thymidylate synthase inhibitor which suppresses cancer cell progression by blocking cell proliferation and activating apoptosis [3]. However, successes achieved by the chemotherapeutic effectiveness of 5-FU are inadequate in patients with colorectal cancer, mostly due to assimilated progressive resistance of CRC cells to 5- FU and toxicity to surrounding normal cells [4]. Moreover, only 10 to 15% of patients with an early stage cancer actively response to 5-FU alone and the preventive effects increases up to 50% when it is combined with other chemopreventive agents [5]. Activation of several survival and proliferative signaling pathways, including nuclear related factor 2 (Nrf2), nuclear factor kappa B (NF-κB), epidermal growth factor receptor(EGFR), mitogen-activated protein kinase (MAPK), rat sarcoma virus oncogene, rapidly accelerated fibrosarcoma, protein kinase B (Akt) and so on, leads to increase tumor growth and chemoresistance [6-8]. The complication and affordability issue, together with high drug resistance rate, has made CRC treatment challenging all over the world, thus the urgent research initiative should be taken. Therefore, downregulation or

alteration of these molecular targets with chemopreventives is an efficient mechanism to control the chemoresistance by combining 5-FU with other natural compounds.

Manuka honey (MH) is increasingly treasured for its diverse polyphenol and antioxidant properties, since it is already known for its wound healing and anti-microbial effects [9-11]. The chemopreventive role of this honey on human colon cancer cells has already been evaluated in our previous reports focusing on different molecular aspects such as anti-proliferation, cell cycle obstruction, apoptosis activation, induction of oxidative stress, disruption of cell metabolism and anti-metastatic observation [10, 12, 13]. Regarding the above observation, in this study we aim to investigate whether MH can chemosensitize colon adenocarcinoma (HCT-116) and metastatic (LoVo) cells to 5-FU, inducing a synergistic or additive cytotoxicity and elucidate the possible targets regulating the synergistic effects.

2. Materials and methods

2.1. Chemicals and honey samples

McCoy's 5A and F-12 K media and other reagents for cell culture were purchased from Carlo Erba Reagents (Milan, Italy). The EvaGreen 2X qPCRMaster Mix kit and the primers (Supplementary Table 1) for real time PCR were obtained from Applied Biological Materials Inc. (Canada) and Sigma-Aldrich (Milan, Italy). The primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Sigma-Aldrich (Milan, Italy). The following antibodies were used: Nrf2, superoxide dismutase (SOD), catalase, heme oxygenase 1 (HO-1), NF-κB, phosphorylated inhibitor of kappa B (p-IκBα), p53, caspase-3, cleaved-poly (ADP-ribose) polymerase (c-PARP), EGFR, human epidermal growth factor receptor 2 (HER2), p-Akt, p-mammalian target of rapamycin (p-mTOR), p-p38MAPK, p-

extracellular-signal-regulated kinase 1/2 (p-Erk1/2), matrix metalloproteinases 2 (MMP-2), MMP-9, E-cadherin, N-cadherin, β-catenin and glyceraldehyde-3-phosphate dehydrogenase (GADPH). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy). MH samples inventing from New Zealand were imported to Italy by EfitSrl and kept at 4°C until analysis.

2.2. Cell culture

Human colon adenocarcinoma (HCT-116) and Dukes' type C, grade IV, colon metastasis (LoVo) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Media were prepared with 10% of heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 µg /mL streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere (95% air, 5% CO_2) and cells were used among the 6^{th} and10^{th} passages for the subsequent experiments.

2.3. MTT assay and determination of combination index

Cytotoxic or proliferative effect of 5-FU and/or MH was determined by MTT assay as previously reported [10]. The percentage of viable cells was expressed as Abs_{590nn} of treated cells/Abs_{590nn} of untreated cells x100. To assess whether 5-FU and MH act in a synergistic or additive way, the combination index (CI) was determined [14]. CI was calculated according to the equation CI= (Dose of 5-FU) / (IC_{50} of 5-FU) + (Dose of MH) / (IC_{50} of MH) for 5-FU and MH combination tested. In this equation, the sum of 5-FU and MH reveals IC_{50} of cell survival. CI value < 1 indicate synergism and 1 to 1.2 indicate additive effects.

2.4. Determination of intracellular ROS levels

The reactive oxygen species (ROS) generation was determined by the CellROX® Oxidative Stress kit (Invitrogen™, Life Techonoliges, Milan, Italy), as previously described by Afrin *et al* [13]. Cells were treated for 48h, trypsinized, centrifuged and CellROX® Orange reagent was added (1 µL/0.5 mL). After incubation at 37°C for 30 min, the cells were centrifuged to remove excess dye and medium, re-suspended with PBS and analyzed by Tali® Image-Based cytometer (Invitrogen™, Life Techonoliges, Milan, Italy).

2.5. Determination of TBARS, protein carbonyl content and antioxidant enzyme activities

Cells were treated for 48h and the cellular lysates were prepared by RIPA buffer (Sigma-Aldrich, Milan, Italy). Thiobarbituric acid-reactive substance (TBARS) and protein carbonyl content were measured as biomarkers of lipid and protein oxidative damage, respectively, as previously described [15]. The antioxidant enzymes SOD, catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) were assessed by spectrophotometric methods as previously reported by Giampieri *et al* [15].

2.6. Determination of apoptotic cells

Tali™ Apoptosis Assay Kit–Annexin V Alexa Fluor® 488 (Invitrogen™, Life Techonoliges, Milan, Italy) kit were used for determination of apoptosis cells according to the manufacturer's instructions [10].

2.7. Cell cycle analysis

Cell cycle analysis was performed by the Tali® Cell Cycle Kit (Invitrogen™, Life Techonoliges, Milan, Italy), as previously described [10]. After the treatment, cells were fixed with 70% cold ethanol at -20°C overnight. The fixed cells were re-suspended in 100 µL PBS containing 20 µg/mL propidium iodide (Invitrogen™, Life Techonoliges, Milan, Italy), 0.1% Triton® X-100 and 0.2 mg/mL RNase A (Invitrogen) and analyzed by Tali™ Image-Based Cytometer.

2.8. Western blot analysis

Total proteins isolated from cells after indicated treatments were subjected to western blotting as previously described [10]. Proteins from cell supernatants were separated on 8 or 10% polyacrylamide gel and then transferred into a nitrocellulose membrane, incubated with primary antibody overnight at 4°C. After incubation the membrane was again incubated for another 1h with secondary antibody and the immunolabeled proteins were identified by using a chemiluminescence method (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany).

2.9. Real-time PCR analysis

Total RNA content of cells was isolated using a PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from 100 ng RNA as previously described [10]. Real-time PCR was performed using EvaGreen 2X qPCR Master Mix (Applied Biological Materials Inc. Canada) of forward and reverse primers and $2^{-\Delta\Delta ct}$ method was used for calculating the fold change values after normalizing the data.

2.10. Bioenergetic assay

Agilent Seahorse XF24 Analyzer (Seahorse Bioscience, North Billerica, MA, USA) was used to determine the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) after indicated treatments [13]. The energy phenotype of each cell was determined by using basal OCR and ECAR.

2.11. Colony formation and wounding assay

Colony formation and wounding assay were performed according to the method described previously with slight modifications [13, 16]. The colonies and scratch cells were fixed with methanol after the indicated treatments and stained with 0.2% methylene blue stain. For the colony formation assay, the plating efficiency was considered by the ability of a single cell to survive and grow into a colony, while for the wounding assay, the wound areas were analyzed by NIH Image J software.

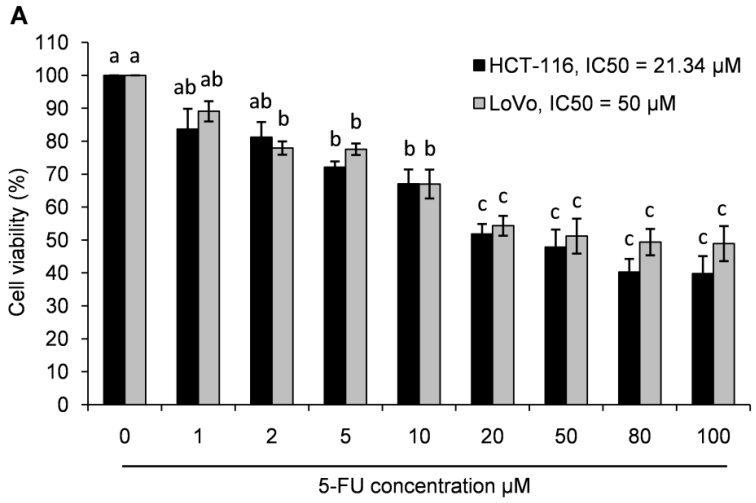
2.12. Statistical analysis

The error bars represent the mean values ± standard deviation of the experiments (n=3). Data analysis was carried out using STATISTICA software (Statsoft Inc., Tulsa, OK, USA) and the differences are symbolized by letters that were obtained by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test ($p < 0.05$).

3. Results

3.1 MH enhances the 5-FU-induced cytotoxicity on HCT-116 and LoVo cells

The cytotoxic effect of 5-FU (1-100 μM) alone or in combination with MH (5-15 mg/mL and 20-30 mg/mL) on human colon cancer HCT-116 and LoVo cells was determined by the MTT assay after 48 h of incubation. As shown in Fig. 1A, the treatments of 5-FU inhibited the proliferation of the colon cancer cell lines in a dose-dependent manner. At the time points tested, the IC_{50} values differed depending on the colon cancer cell lines (HCT-116 cells were adenocarcinoma and LoVo cells were of a metastatic nature). In HCT-116 cells the value was 21.34 μM (Fig. 1A) while in LoVo cells it was 50 μM at 48 h, respectively (Fig. 1A). Treatment with MH alone provided IC_{50} values of 15.81 mg/mL for HCT-116 cells and 39.83 mg/mL for LoVo cells while similar concentrations did not induce any toxic effects in non-malignant cells as described earlier [10]. Next, we observed the effects of 5-FU in presence of MH by MTT assay after 48 h incubation. Interestingly, in the combined treatments, in the presence of MH, a significantly lesser ($p< 0.05$) concentration of 5-FU led to IC_{50} values compared to single doses of 5-FU. In the presence of MH (10.5 mg/mL), the IC_{50} values of 5-FU were 10.43 μM at 48 h in HCT-116 cells (Fig. 1A). At the same time, in LoVo cells the IC_{50} values of 5-FU were 20.11 μM in the presence of MH (20.34 mg/mL) at 48 h, (Fig. 1C). These results indicate that 5-FU and MH, when used in combination, induced similar effects at very lower concentrations compared to 5-FU alone. All the IC_{50} values were selected for further experiments after 48 h treatment.



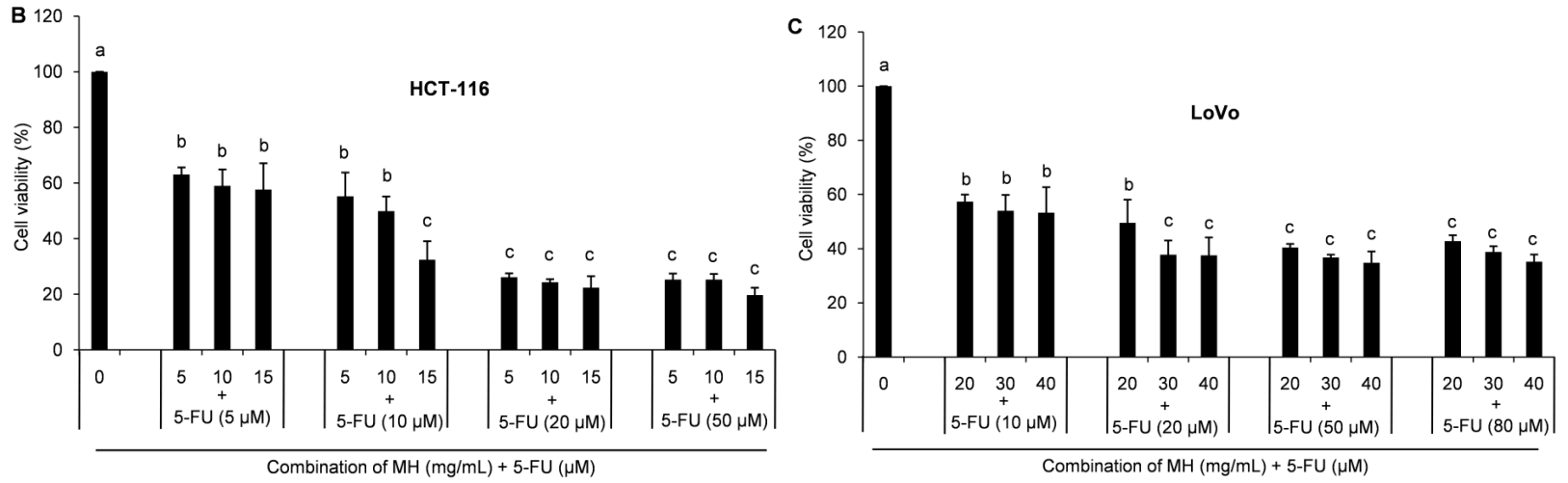


Fig. 1. Induction of cytotoxicity by 5-FU alone (A) or in combination of MH on HCT-116 (B) and LoVo (C) cell lines. After 24 h of cell seeding (5×10^3cells/ well), HCT-116 and LoVo cells were treated with different concentrations of 5-FU alone or in combination of MH for 48 h. Cell viability was measured by using MTT assay and results were expressed as a % of viable cells compared to non treated cells. All data are expressed as the mean ± standard deviation (SD) (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

3.2 MH increases the 5-FU-induced ROS production on HCT-116 and LoVo cells

Intracellular ROS levels were determined after treatment with 5-FU alone or in combination with MH in HCT-116 and in LoVo cells following 48 h treatments. In HCT-116 cells, ROS production increased by 25.33% after 5-FU treatment and 21.5% after MH treatment, compared to control (7%) (Fig. 2A). Similarly, in LoVo cells, treatment with 5-FU and MH increased ROS production by 36% and 34%, respectively compared to control (6%) (Fig. 2B). Furthermore, the combined treatment of 5-FU+MH increased ROS production in both cells by 28% and 40% (Fig. 2A and 2B). Taken together, MH enhanced the ROS production of 5-FU in HCT-116 and in

LoVo cells compared to 5-FU single doses and in LoVo cells the combination was more effective.

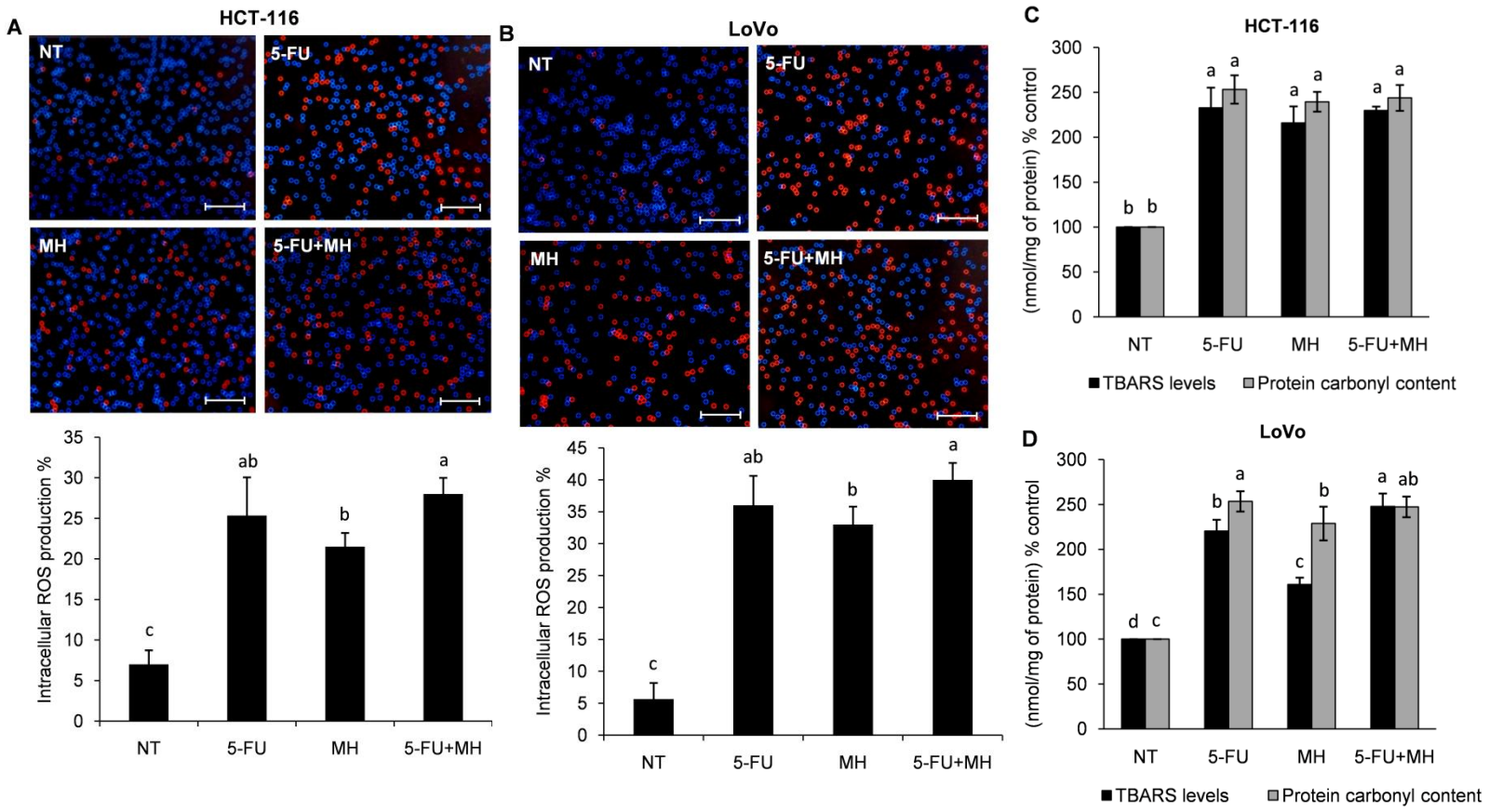


Fig. 2. ROS accumulation after treatment with 5-FU and MH alone or in combination. HCT-116 and LoVo cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT corresponds to no treatment. Intracellular ROS levels were determined by using the CellROX® Orange assay kit and the Tali™ Image-based Cytometer in (A) HCT-116 and (B) LoVo cells. Representative fluorescence images of cells show the effect of treatment: blue color corresponds to live cells and red color corresponds to ROS induced cells. Scale bars = 50 µm. Lipid and protein oxidative damage was evaluated by assessing the TBARS levels and protein carbonyl content in (C) HCT-116 and (D) LoVo cells. The results were measured as mmol per mg of protein and expressed as the percent of the control. All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

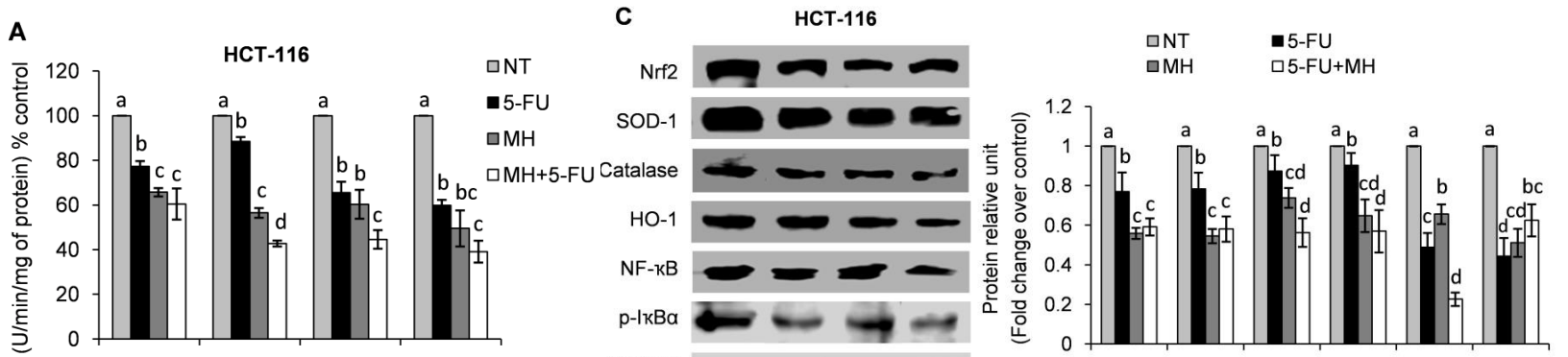
The biomarkers of lipid (TBARS level) and protein (carbonyl content) levels were determined after treatment with 5-FU and/ or MH in both colon cancer cells. In HCT-116 and LoVo cells, the levels of TBARS significantly ($p < 0.05$) increased by 232% and 220% after 5-FU treatment and by 216% and 162% after MH treatment (Fig. 2C). The combined treatment of 5-FU+MH increased TBARS levels by 230% for HCT-116 and by 248% for LoVo cells (Fig. 2C). Similarly, the level of protein carbonyl content was also increased after 5-FU (253% and 249%) and MH (239% and 232%) treatment in both cell lines (Fig. 2D). Furthermore, the combined treatment increased the levels of protein carbonyl content by 243% in HCT-116 cells and by 245% in LoVo cells respectively (Fig. 2D), which was less compared to 5-FU alone.

3.3 MH enhances 5-FU-induced oxidative stress by suppressing antioxidant enzyme activity and the expression of Nrf-2 and NF-κB family proteins

In the next step, we evaluated the effect of 5-FU and/or MH treatments on the activity and expression of the antioxidant enzymes in HCT-116 and LoVo cells. The activity of SOD and catalase was reduced by 77% and 88% in HCT-116 cells and by 71% and 69% in LoVo cells after 5-FU treatment compared to control (100%) (Fig. 3A and 3B). In contrast MH alone and the combined treatment significantly decreased the SOD and catalase activity by 65-60% and 56-42% in HCT-116 cells and by 63-57% and 68-49% in LoVo cells, which were more effective compared to 5-FU alone. Similarly, in HCT-116 and LoVo cells, the activity of GPx and GR was decreased by 65-63% and 59-57% after 5-FU treatment (Fig. 3A and 3B). After MH alone, all the activities decreased more compared to 5-FU, while the combined treatments were most effective: 5-FU+MH decreased the activity of GPx by 59-44% and GR by 46-33% respectively

(Fig. 3A and 3B). We observed the pro-oxidative effects of 5-FU in the presence of MH in HCT-116 and LoVo cells.

The expression of the transcription factor Nrf2 and other antioxidant enzymes, SOD, catalase and HO-1, were determined after treatment with 5-FU and/or MH in HCT-116 and LoVo cells following 48 h treatments. In HCT-116 cells, the expression of Nfr2, SOD, catalase and HO-1 decreased 0.76 fold, 0.78 fold, 0.87 fold and 0.90 fold after 5-FU treatment and 0.55 fold, 0.54 fold, 0.73 fold and 0.64 fold after MH treatment, respectively compared to control (Fig. 3C); the combined treatment decreased the expression 0.59 fold, 0.58 fold, 0.56 fold and 0.60 fold, respectively (Fig. 3C). In the case of LoVo cells, the expression of Nfr2, SOD, catalase and HO-1 decreased 0.89 fold, 0.80 fold, 0.83 fold and 0.91 fold after 5-FU treatment and 0.56 fold, 0.71 fold, 0.52 fold and 0.77 fold after MH treatment (Fig. 3D). The combination treatment was more profound in LoVo cells compared to HCT-116 cells, significantly ($p < 0.05$) decreasing the expression of Nfr2, SOD, catalase and HO-1 0.33 fold, 0.30 fold, 0.52 fold and 0.62 fold (Fig. 3D). Furthermore, the expression of NF-κB and p-IκBα decreased 0.48 fold and 0.44 fold after 5-FU treatment, 0.65 fold and 0.51 fold after MH treatment in HCT-116 cells (Fig. 3C). Interestingly, the expression of the same proteins decreased 0.22 fold and 0.60 fold in the presence of MH at lower concentrations of 5-FU (Fig. 3C). In LoVo cells the combination treatment (decreased 0.36 fold for NF-κB and 0.51 fold for p-IκBα) induced similar effects to 5-FU (decreased 0.36 fold for NF-κB and 0.62 fold for p-IκBα) alone at lower concentrations (Fig. 3D). Taken together, MH enhanced the pro-oxidative effects of 5-FU at lower concentrations by inducing cell death by oxidative stress.



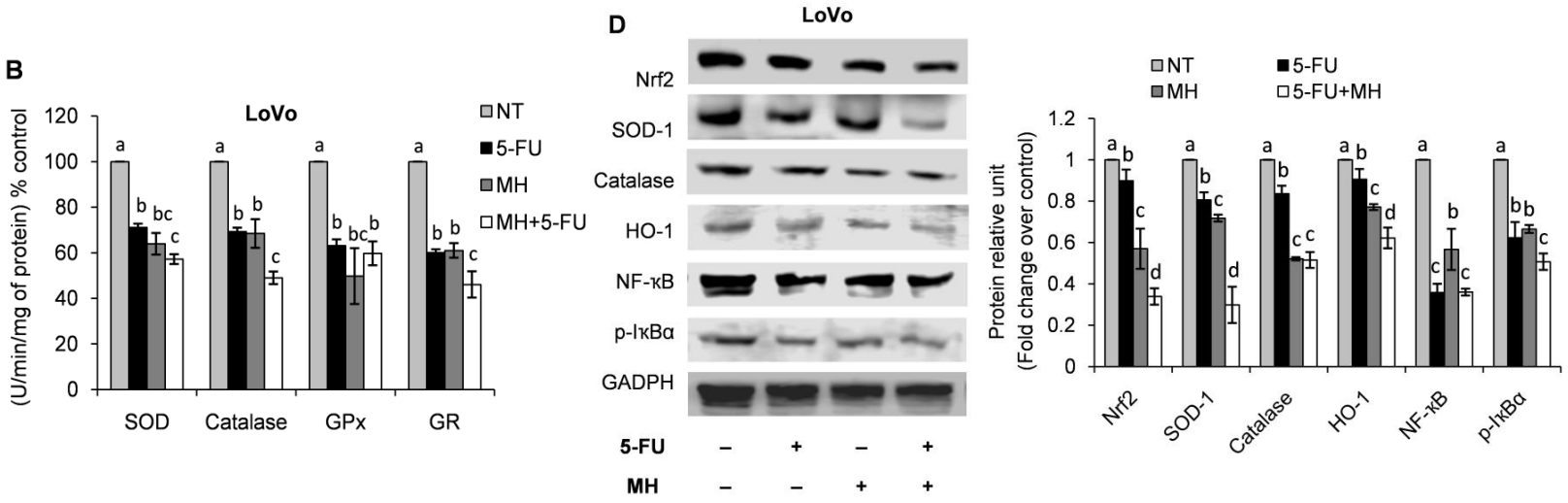


Fig. 3. Induction of oxidative stress after treatment with 5-FU and MH alone or in combination. HCT-116 and LoVo cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT corresponds to no treatment. SOD, Catalase, GR and GPx activities were expressed as unit per mg protein and the results were expressed as the percent of the control in (A) HCT-116 and (B) LoVo cells. The protein expression of Nrf2, SOD-1, Catalase, HO-1, NF-κB and p-IκBα was analyzed by western blotting in (C) HCT-116 and (D) LoVo cells. GADPH was utilized as a loading control. All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (*p*-value < 0.05) between each treatment.

3.4 MH enhances the 5-FU-induced apoptosis on HCT-116 and LoVo cells

In both cell lines, as shown in Fig. 4, the number of apoptosis cells significantly ($p < 0.05$) increased after 5-FU (5.52 fold and 5.02 fold) and MH (3.38 fold and 3.07 fold) treatments when compared to control (1.00 fold). In HCT-116 cells, combined treatment was slightly less effective (4.17 fold) than 5-FU alone (Fig. 4A). At the same time, in LoVo cells after combined treatment the apoptotic effect was 3.98 fold (Fig. 4B). The number of live and dead cells decreased in each treatment and a similar trend was also observed when combined treatment was

used in both cell lines (Fig. 4). At the same time, in non-cancer cells, MH also had the ability to decrease stress inducing apoptotic effects [9].

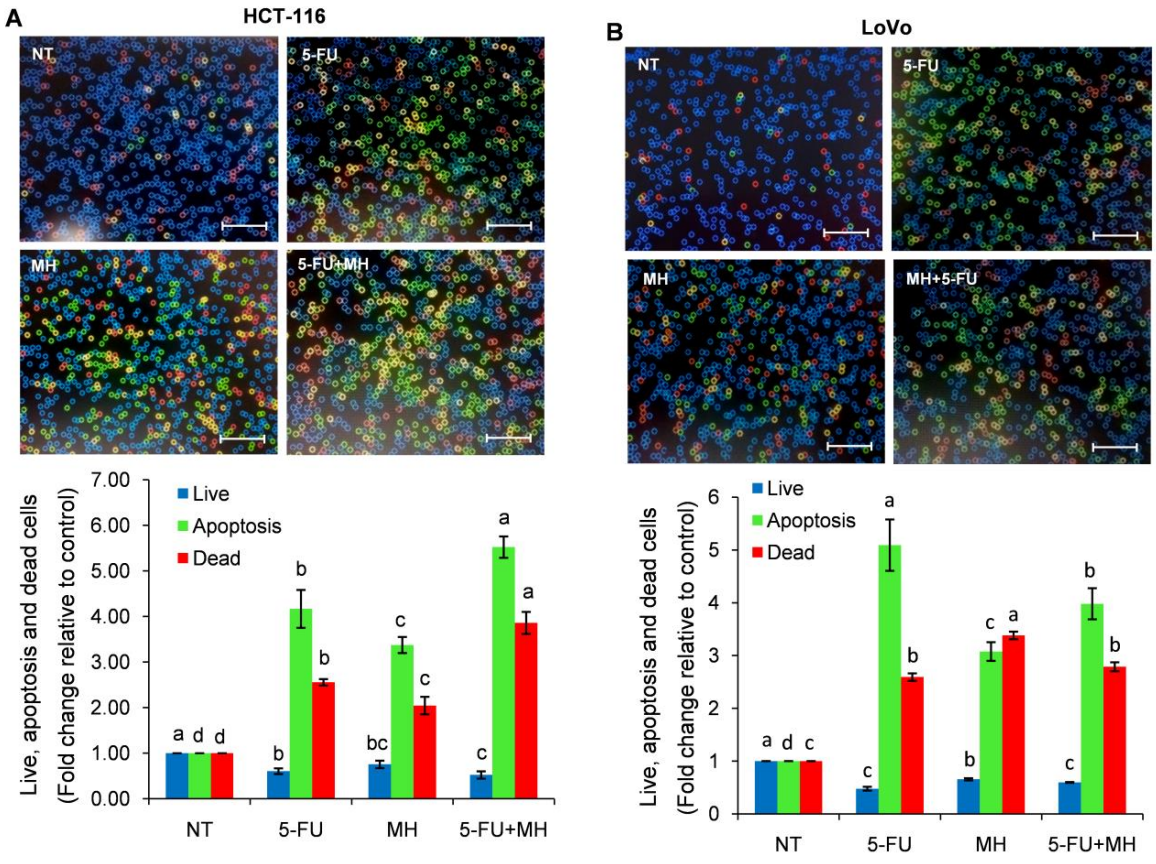


Fig. 4. Apoptosis induction after the treatment with 5-FU and MH alone or in combination. HCT-116 and LoVo cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT corresponds to no treatment. Annexin V Alexa Fluor® 488 and PI staining was used for the determination of apoptotic effect 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 treatment: blue colour corresponds to live cells, green colour corresponds to apoptotic cells and red and yellow colour corresponds to dead cells. Scale bars = 50 µm. All data are indicated as the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

3.5 Cell cycle distributions after the treatment of 5-FU and/or MH

Cell cycle was evaluated by 5-FU alone or in a combination with MH at doses corresponding to their IC_{50} values after 48 h in HCT-116 and LoVo cells. As shown in Fig. 5A, accumulation of HCT-116 cells in S phase significantly ($p < 0.05$*)* increased to 53% after 5-FU treatment and to 40% after MH treatment compared to control (23%), while the combined treatment of 5-FU+MH induced a 50% accumulation of HCT-116 cells at S phase, respectively (Fig. 5A). The percentage of HCT-116 cells in the G0/G1 and G2/M phase significantly ($p < 0.05$) decreased in each treatment compared to control cells (Fig. 5A). In LoVo cells, 5-FU treatments accumulated 43% of cells at the G2/M phase at IC_{50} doses compared with control (20%) (Fig. 5B). On the other hand, MH induced a 37% accumulation and the combination induced a 40% accumulation of LoVo cells in G2/M phase, with almost similar effects to 5-FU (Fig. 5B). Furthermore, the percentage of cells at G0/G1 and S phase significantly ($p < 0.05$) decreased after all treatments compared with control (Fig. 5B). The accumulation of cells at Sub-G1 phase indicated apoptotic effects. All treatments increased the cell percentage at Sub-G1 phase compared to control but in HCT-116 cells the combination induced similar effects like 5-FU alone (Fig. 5B), while in LoVo cells it was slightly lower compared to 5-FU (Fig. 5B). The results suggest that the 5-FU+MH induced an equal or slightly less pronounced effect for arresting the cell cycle compared to 5-FU treatment in both cell lines.

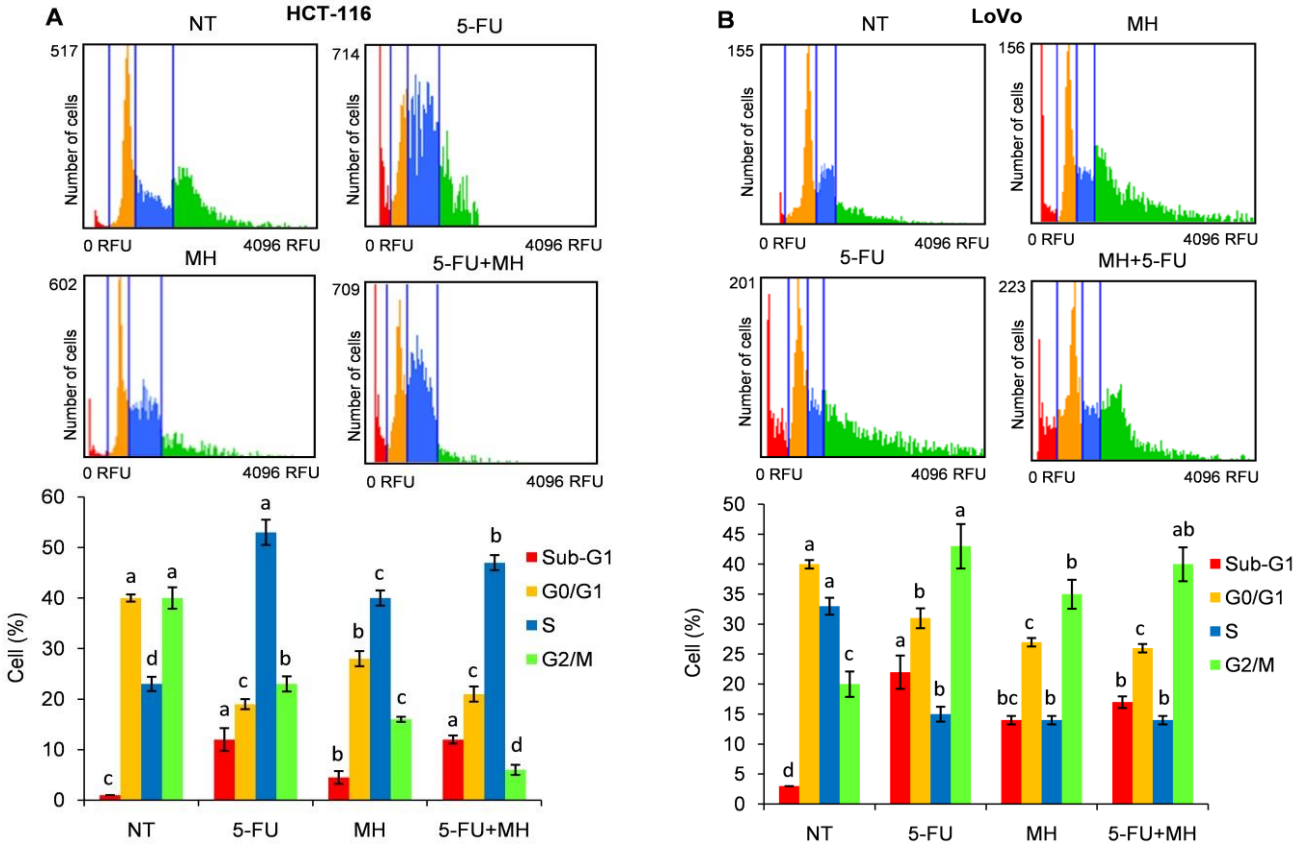


Fig. 5. Cell cycle distributions after the treatment with 5-FU and MH alone or in combination. HCT-116 and LoVo cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT corresponds to no treatment. The percentages of (A) HCT-116 and (B) LoVo cells in each phase Sub-G1 (apoptotic cells), G0/G1, S and G2/M were calculated by the Tali® Cell Cycle Assay kit and Tali™ Image-based Cytometer. Representative fluorescence image shows the effect of treatment: red colour corresponds to Sub-G1 phase, yellow colour corresponds to G0/G1 phase, blue colour corresponds to S phase and green yellow colour corresponds to G2/M phase. All data shown were the mean ± SD (n=3). Different letters indicate significant differences (*p*-value < 0.05) between each treatment.

3.6 Alteration of apoptosis and cell cycle related gene expression after the treatment of 5-FU and/or MH

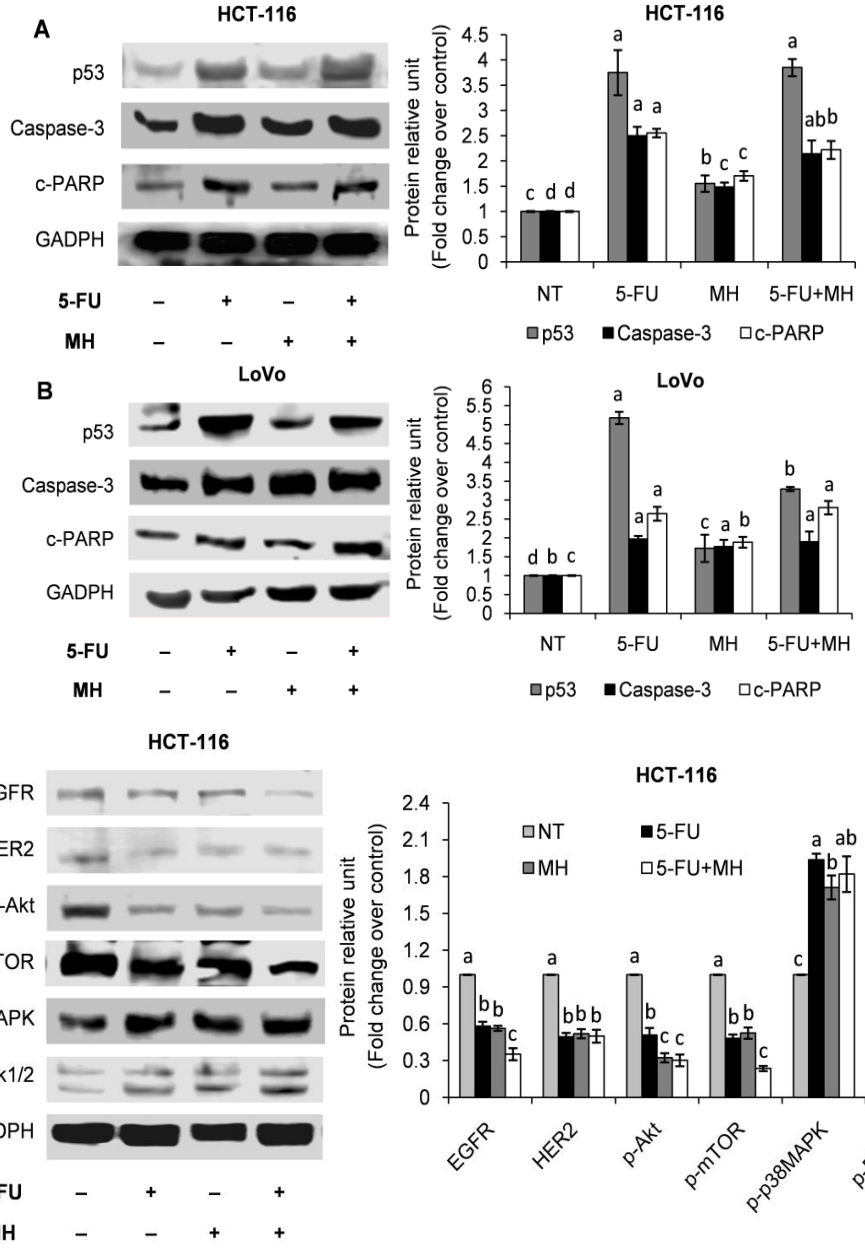
We quantified the mRNA expression of several apoptotic markers to highlight if apoptotic induction was due to the activation of intrinsic or extrinsic apoptotic pathway. All the treatments significantly ($p < 0.05$) increased the expression of p53, Bcl-2 associated X protein (Bax), cytochrome c (Cyto c), fatty acid synthetase ligand (FasL), caspase-3, -8, -9 and c-PARP, while the expression of B-cell lymphoma-2 (Bcl-2) was reduced in HCT-116 and LoVo cells (Table 1). In HCT-116 cells, the combined treatment induced similar effects in the expression of all the apoptotic mRANs, with the exception of Cyto c that was more highly expressed compared to a single dose of 5-FU (Table 1). Furthermore, in LoVo cells, the combined treatment induced higher effects in the expression of Bax, Cyto c, FasL, caspase-8 and caspase-9, and induced similar effects in the expression of p53, caspase-3 and c-PARP compared to a single dose of 5-FU (Table 1).

Table 1. Differential expression of mRNA in HCT-116 and LoVo cells after MH treatment.

Gene symbol	HCT-116				LoVo			
Apoptosis related mRNA	Expression fold				Expression fold			
	NT	5-FU	MH	5-FU+MH	NT	5-FU	MH	5-FU+MH
p53	1.00±0.00^{b}	2.18±0.32^{a}	1.60±0.15^{ab}	2.20±0.30^{a}	1.00±0.09^{c}	3.69±0.35^{a}	1.84±0.17^{b}	3.13±0.12^{a}
Bcl-2	1.00±0.00^{a}	0.58±0.05^{b}	0.67±0.10^{b}	0.59±0.16^{b}	1.00±0.28^{a}	0.62±0.03^{bc}	0.65±0.08^{b}	0.46±0.04^{c}
Bax	1.00±0.00^{c}	1.99±0.08^{a}	1.58±0.05^{b}	1.67±0.12^{b}	1.00±0.10^{c}	2.93±0.02^{ab}	2.43±0.02^{b}	3.69±0.42^{a}
Cyto C	1.00±0.00^{c}	1.77±0.04^{ab}	1.51±0.33^{bc}	2.17±0.08^{a}	1.00±0.22^{c}	3.50±0.59^{ab}	2.53±0.47^{b}	4.09±0.21^{a}
FasL	1.00±0.00^{b}	1.63±0.03^{a}	1.48±0.32^{a}	1.58±0.16^{a}	1.00±0.18^{d}	3.02±0.04^{b}	1.70±0.32^{c}	3.58±0.18^{a}
Caspase-3	1.00±0.00^{c}	2.33±0.06^{a}	1.59±0.19^{b}	2.35±0.26^{a}	1.00±0.11^{c}	1.80±0.07^{a}	1.33±0.12^{b}	1.72±0.29^{ab}
Caspase-8	1.00±0.00^{b}	1.60±0.13^{a}	1.42±0.05^{a}	1.48±0.04^{a}	1.00±0.26^{c}	2.49±0.50^{ab}	1.56±0.14^{bc}	3.19±0.38^{a}
Caspase-9	1.00±0.00^{c}	1.63±0.06^{a}	1.26±0.11^{bc}	1.31±0.18^{ab}	1.00±0.07^{c}	2.62±0.07^{ab}	1.81±0.02^{bc}	3.35±0.64^{a}
c-PARP	1.00±0.00^{c}	2.38±0.13^{a}	1.79±0.08^{b}	2.28±0.22^{a}	1.00±0.05^{c}	2.03±0.29^{a}	1.56±0.12^{b}	2.05±0.07^{a}
Cell cycle related mRNA								
Cyclin D1	1.00±0.00^{a}	0.63±0.12^{b}	0.72±0.08^{b}	0.57±0.06^{b}	1.00±0.25^{a}	0.58±0.03^{c}	0.70±0.02^{b}	0.47±0.05^{d}
Cyclin E	1.00±0.00^{a}	0.58±0.09^{bc}	0.74±0.13^{b}	0.44±0.06^{c}	nc	nc	nc	nc
CDK2	1.00±0.00^{a}	0.56±0.12^{b}	0.64±0.06^{b}	0.54±0.14^{b}	nc	nc	nc	nc
CDK4	1.00±0.00^{a}	0.46±0.11^{b}	0.50±0.03^{b}	0.49±0.13^{b}	nc	nc	nc	nc
p21waf1/cip1	1.00±0.00^{b}	1.75±0.18^{a}	1.33±0.22^{ab}	1.65±0.23^{a}	1.00±0.06^{b}	1.72±0.05^{a}	1.61±0.11^{a}	1.72±0.01^{a}
p27kip1	1.00±0.00^{b}	1.81±0.33^{a}	1.43±0.27^{ab}	1.68±0.19^{a}	1.00±0.09^{c}	2.43±0.24^{a}	1.88±0.17^{b}	2.12±0.19^{ab}
p-Rb	1.00±0.00^{a}	0.62±0.03^{b}	0.71±0.07^{b}	0.67±0.09^{b}	1.00±0.34^{a}	0.53±0.06^{b}	0.66±0.09^{b}	0.48±0.08^{b}

Effect of 5-FU, MH or 5-FU+MH on the mRNA expression in HCT-116 and LoVo cells after 48h treatment. GADPH was amplified under same RT-PCR conditions for normalize quantitative data. Results are expressed as a fold change in comparison with not treatment. All values are mean ± SD (n=3). NT, not treatment, 5-FU, 5-fluorouracil, MH, Manuka honey, nc, no change. Different letters indicate significant differences (p-value < 0.05) between each treatment.

The apoptotic effects were also confirmed by further evaluating the protein expression of p53, caspase-3 and c-PARP by Western Blot (Fig. 6). In HCT-116 cells, 5-FU significantly ($p < 0.05$) increased the expression of p53 (3.75 fold), caspase-3 (2.5 fold) and c-PARP (2.55 fold) compared to control, while the combination increased 3.8 fold for p53, 2.3 fold for caspase-3 and 2.27 fold for c-PARP, respectively (Fig. 6A). On the other hand, the expression of all these three proteins significantly ($p < 0.05$) increased 5.17 fold, 1.97 fold and 2.63 fold after 5-FU treatment and the effects were slightly less pronounced when it was combined with MH compared with 5-FU alone (Fig. 6B) in LoVo cells. Overall these results suggest that when 5-FU was combined with MH it induced similar or slightly lesser effects at half the concentration than 5-FU alone.



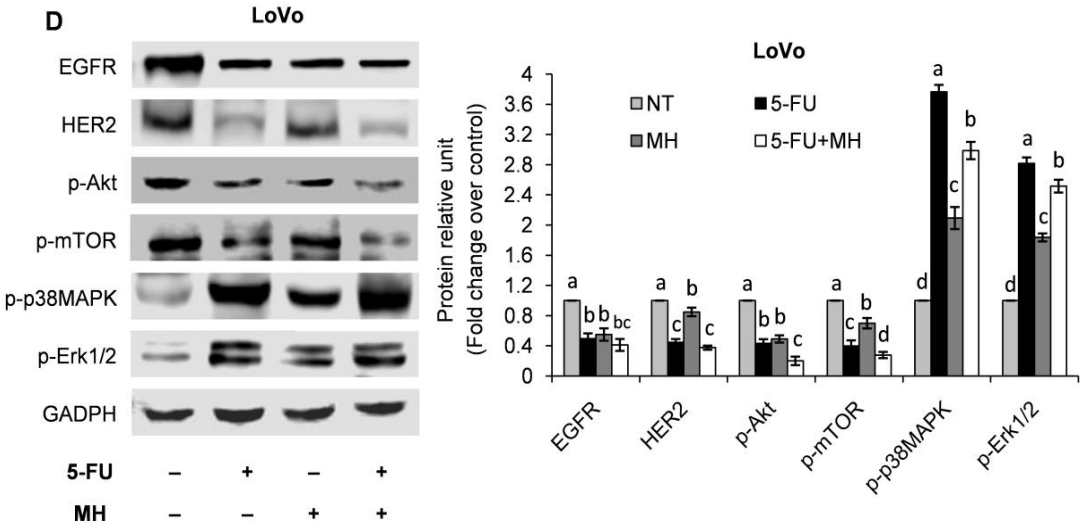


Fig. 6. Alteration of apoptotic markers and EGFR signaling protein expression after the treatment with 5-FU and MH alone or in combination. HCT-116 and LoVo cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT corresponds to no treatment. Protein expression of apoptotic markers p53, Caspase-3 and cleaved PARP (c-PARP) were determined by western blotting in (A) HCT-116 and (B) LoVo cells after the treatments. Similarly, the protein expression of EGFR, HER2, p-Akt, p-mTOR, p-p38MAPK and p-Erk1/2 were determined in (C) HCT-116 and (D) LoVo cells. GADPH was used as a loading control. All data shown were the mean ± SD (n=3). Different letters indicate significant differences (*p*-value < 0.05) between each treatment.

The mRNA expression of cell cycle regulatory proteins (cyclin D1 and cyclin E), cyclin-dependent kinases (CDK2 and CDK4), cyclin-dependent kinase inhibitor (p21waf1/Cip1 andp27kip1) and p-retinoblastoma (p-Rb) were quantified in both colon cancer cells after the indicated treatments (Table 1). We found that 5-FU treatment decreased the expression of cyclin D1, cyclin E, CDK2, CDK4 and p-Rb, while p21waf1/Cip1 and p27kip1 expression was elevated in HCT-116 cells compared to control. In the presence of MH, lower concentration of 5-FU largely decreased the expression of cyclin D1 and cyclin E, and expressed similar effects on the expression of CDK2, CDK4 and p-Rb compared to 5-FU alone (Table 1). In LoVo cells, we

only observed the changes on the expression of cyclin D1, p21waf1/Cip1, p27kip1 and p-Rb, while cyclin D1 was more highly expressed after the combined treatment and p27kip1 and p-Rb induced similar effects to 5-FU alone (Table 1).

3.7 Effect of 5-FU and/or MH on EGFR signalling pathway

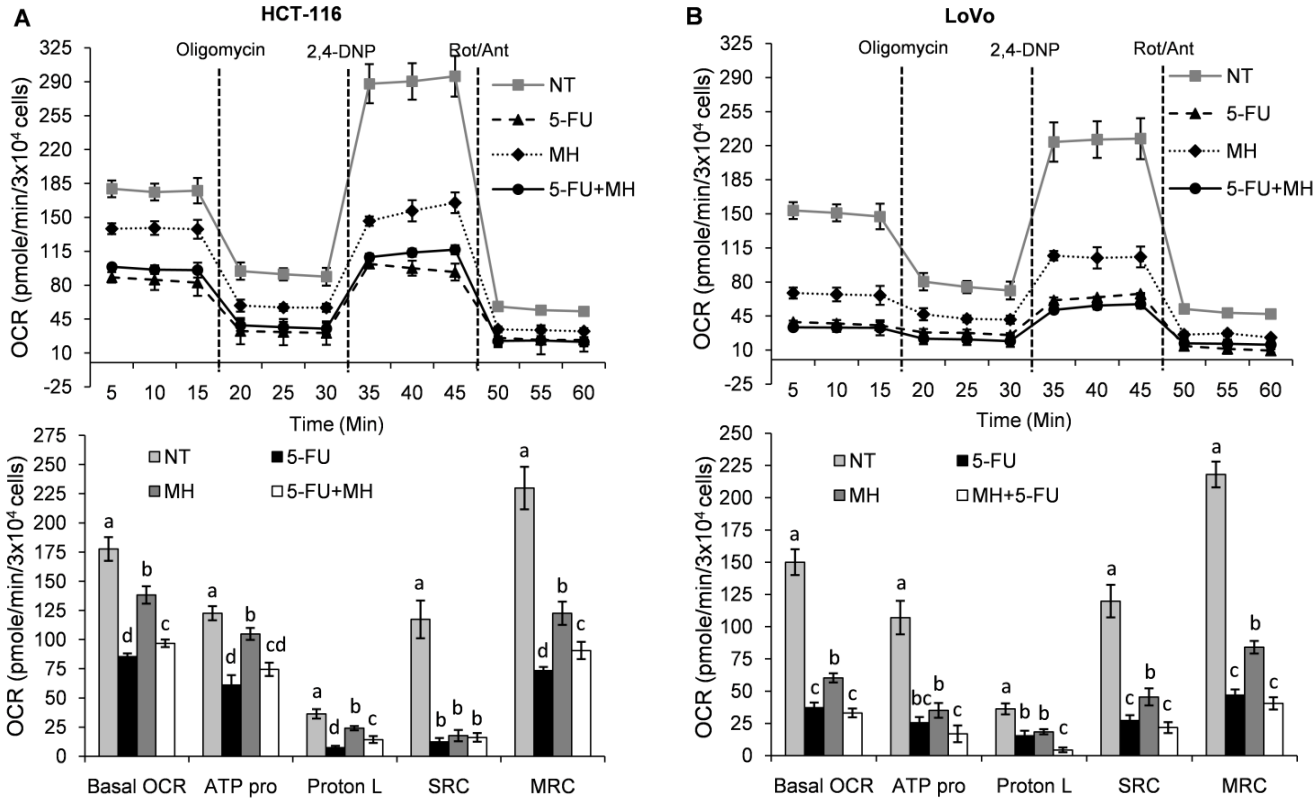
The effect of 5-FU alone or combined with MH on the cell surviving signaling EGFR in HCT-116 and LoVo cells was evaluated (Fig. 6). The expression of EGFR, HER2, p-Akt and p-mTOR decreased 0.58 fold, 0.49 fold, 0.51 fold and 0.48 fold after 5-FU treatment, while the combined treatments decreased 0.35 fold, 0.50 fold, 0.30 fold and 0.24 fold in HCT-116 cells (Fig. 6C). In LoVo cells, the expression of these proteins was suppressed 0.49 fold, 0.44 fold, 0.43 fold and 0.39 fold after 5-FU treatment, while with combined treatments it was suppressed 0.41 fold, 0.37 fold, 0.21 fold and 0.27 fold (Fig. 6D). In both cell lines, the combination of 5-FU+MH induced more profound effects at lower concentrations.

As shown in Fig. 6, 5-FU treatment increased the activation status of major MAPK signaling compounds (p-p38MAPK and p-Erk1/2) 1.93 fold and 1.87 fold in HCT-116 cells (Fig. 6C) and 3.77 fold and 2.81 fold in LoVo cells (Fig. 6D). Regarding the combined treatment, in HCT-116 cells it induced a similar effect (Fig. 6C) and in LoVo cells it induced a slightly less pronounced effect (Fig. 6D) compared to 5-FU alone.

3.8 Alteration of metabolic phenotype after the treatment of 5-FU and/or MH

Our next step was to investigate the effect of 5-FU alone or in combination with MH on metabolic phenotype, mainly mitochondrial respiration and glycolysis on each CRC cell lines. As shown in Fig. 7A, 5-FU treatment significantly ($p < 0.05$) reduced the basal OCR, the ATP-

linked respiration, the proton leak, the spare respiration capacity (SRC) and the maximal respiration capacity (MRC), up to 85.33 ± 2.82, 61.00 ± 6.30, 7.33 ± 1.60, 12.33 ± 3.29 and 73.50 ± 3.06 pmol/min per 3 $\times 10^4$ cells, respectively compared to the control, in HCT-116 cells. In addition, 5-FU+MH decreased the basal OCR (96.83 ± 3.23), the ATP-linked respiration (74.50 ± 1.71), the proton leak (14.33 ± 0.94), the SRC (16.71 ± 0.71) and the MRC (90.00 ± 1.41) but induced slightly less pronounced effects compared to 5-FU (Fig. 7A). Furthermore, in LoVo cells the basal OCR, the ATP-linked respiration, the proton leak, the SRC and MRC decreased to 37.15 ± 0.97, 25.60 ± 4.29, 15.43 ± 3.85, 27.22 ± 4.09 and 46.90 ± 4.37 pmol/min per 3 $\times 10^4$ cells after 5-FU treatment, while the presence of MH, 5-FU decreased all these parameters to 33.03 ± 1.43, 16.48 ± 0.48, 4.39 ± 1.86, 21.68 ± 3.23 and 40.45 ± 2.75 pmol/min per 3 $\times 10^4$ cells by inducing synergistic effects (Fig. 7B).



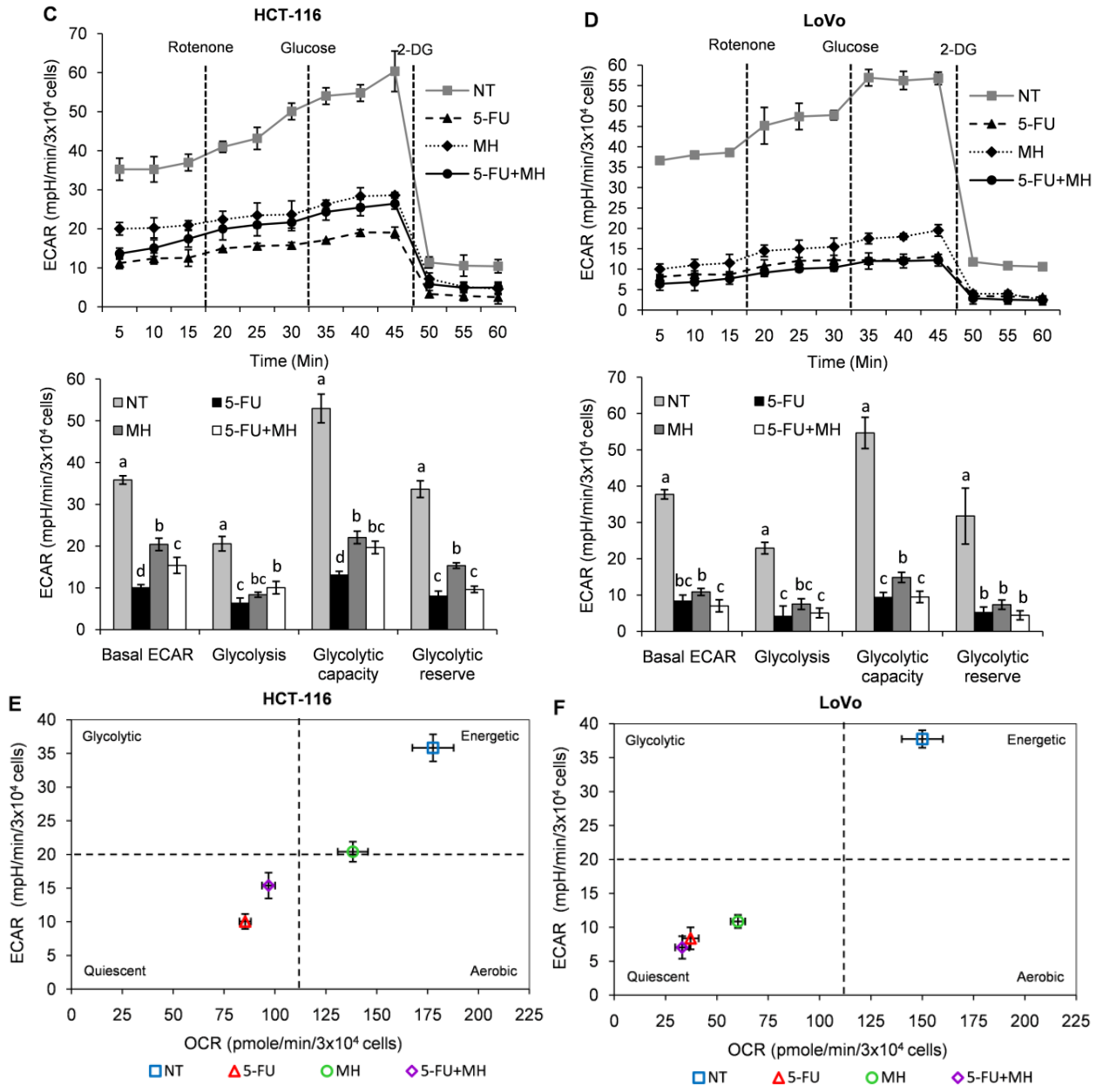


Fig. 7. Effect of 5-FU and MH alone or in combination on bioenergetic phenotype. HCT-116 and LoVo cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT corresponds to no treatment. OCR was determined by using the Seahorse XF-24 Extracellular Flux Analyzer after the following sequential injections: oligomycin (3µg/mL), 2,4-DNP (300 µM), and rotenone/antimycin (1µM/10µM) in (A) HCT-116 and (B) LoVo cells. Basal OCR, ATP production (ATP pro), proton leak (proton L), spare respiration capacity (SRC) and maximal respiration capacity (MRC) were calculated from the XF cell Mito stress test profile in both cell lines. ECAR was determined by using the Seahorse XF-24 Extracellular Flux Analyzer after the following sequential injections: rotenone (1 µM), glucose (30 mM), and 2-DG (100 mM) in (C)

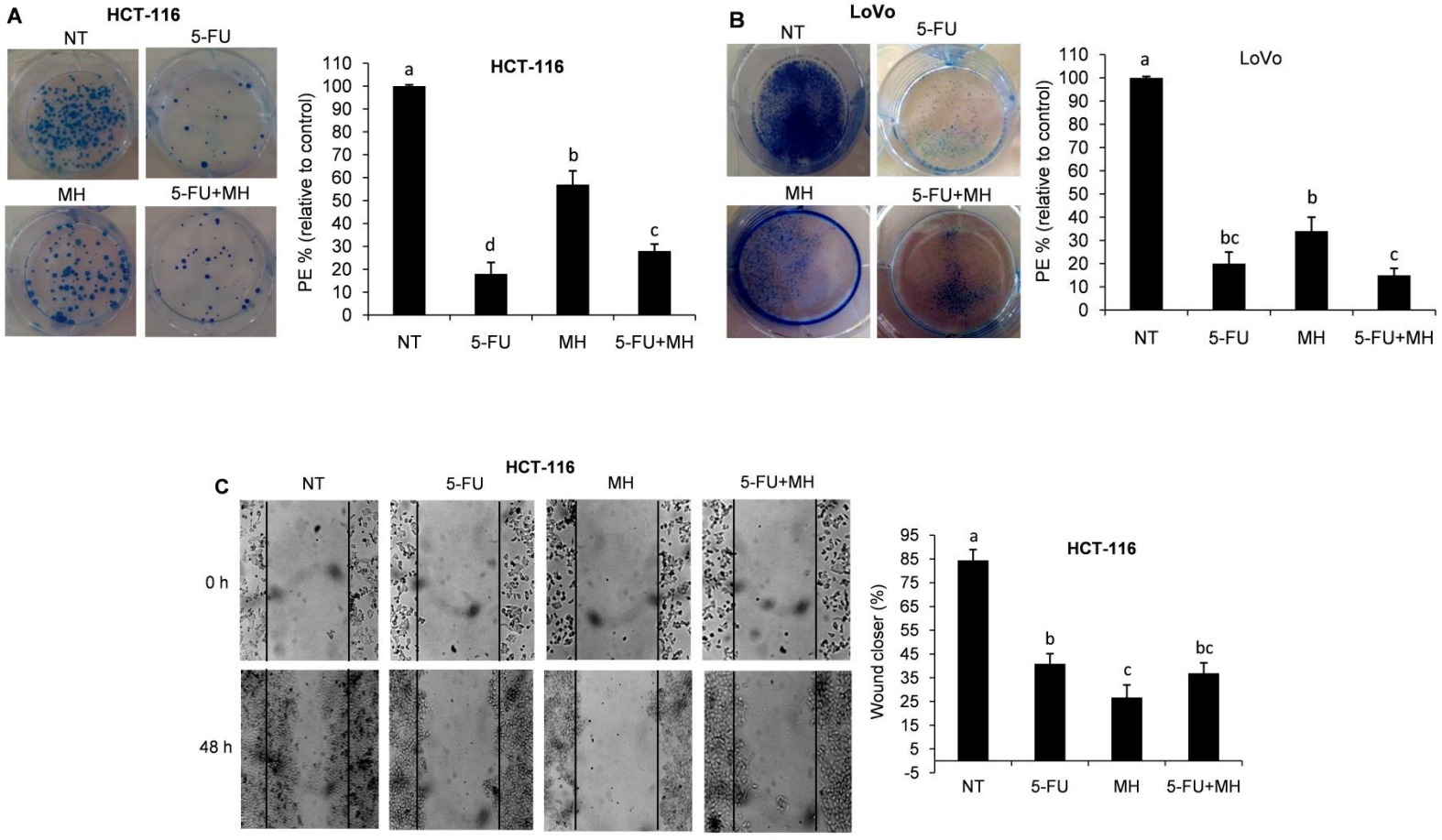
HCT-116 and (D) LoVo cells. Basal ECAR, glycolysis, glycolytic capacity and glycolytic reserves were calculated from the XF glycolysis stress test profile. The ratio of OCR: ECAR showed the metabolic phenotype of (E) HCT-116 and (F) LoVo cells. All data shown were the mean ± SD (n=3). Different letters indicate significant differences (p-value < 0.05) between each treatment.

Regarding glycolysis function, we observed that 5-FU and/or MH reduced the basal values to 10.00 ± 0.72 and 15.37 ± 1.93 mpH/ min per 3×10^4 respectively in HCT-116 cells compared to control (Fig. 7C). Furthermore, the activity of glycolysis, glycolytic capacity and glycolytic reserve decreased to 6.00 ± 1.00, 13.07 ± 0.87 and 8.00 ± 1.00 mpH/ min per 3×10^4 after 5-FU treatment. In the presence of MH, 5-FU reduced the activity of glycolysis to 10.04 ± 1.50, glycolytic capacity to 19.67 ± 1.00, and glycolytic reserve to 9.62 ± 0.76 mpH/ min per 3×10^4, respectively compared to control (Fig. 7C). Furthermore, in LoVo cells, the basal ECAR was 8.53 ± 0.32 mpH/ min per 3×10^4 after 5-FU, while the effect of combined treatment was 7.02 ± 0.67 mpH/ min per 3×10^4 (Fig. 7D). Moreover, the activity of glycolysis was reduced to 4.12 ± 0.87 after 5-FU and 5.06 ± 1.33 after 5-FU+MH treatment (Fig. 7D). Finally, the glycolytic capacity and glycolytic reserve decreased to 9.34 ± 0.38 and 5.23 ± 0.49 mpH/ min per 3×10^4 after 5-FU treatment and 9.48 ± 1.58 and 4.48 ± 0.25 mpH/ min per 3×10^4 after 5-FU+MH treatment compared to the control (Fig. 7D).

In Fig.7E and Fig.7F, we observed the metabolic phenotype of each colon cancer cells after the different treatments. Both cells are in an energetic state before the treatment but in the presence of 5-FU and/or MH cells were in a quiescent state which is metabolically inactive. Like in the mitochondrial respiration and glycolysis effects, in HCT-116 cells, 5-FU was more effective and in LoVo cells the combined treatment induced similar effects to 5-FU alone (Fig. 7E and 7F).

3.9 MH enhances the 5-FU-induced anti-colony formation and anti-migration ability

In HCT-116 and LoVo cells, chronic exposure to 5-FU significantly decreased colony formation 80% and 52% compared with control (10%). In the presence of MH with a lower concentration of 5-FU, the colony formation decreased by 72% in HCT-116 cells (Fig. 8A) and 83% in LoVo cells (Fig. 8B), respectively. Similarly, the migration ability of HCT-116 and LoVo cells decreased by 60% and 57% after 5-FU treatment, while in the presence of MH the inhibitory activity of 5-FU increased by 64% in HCT-116 cells (Fig. 8C) and 68% for LoVo compared to control (Fig. 8D). On the contrary, MH promoted the migration ability in non-cancer cells due to the enhancement of wound healing activities [9].



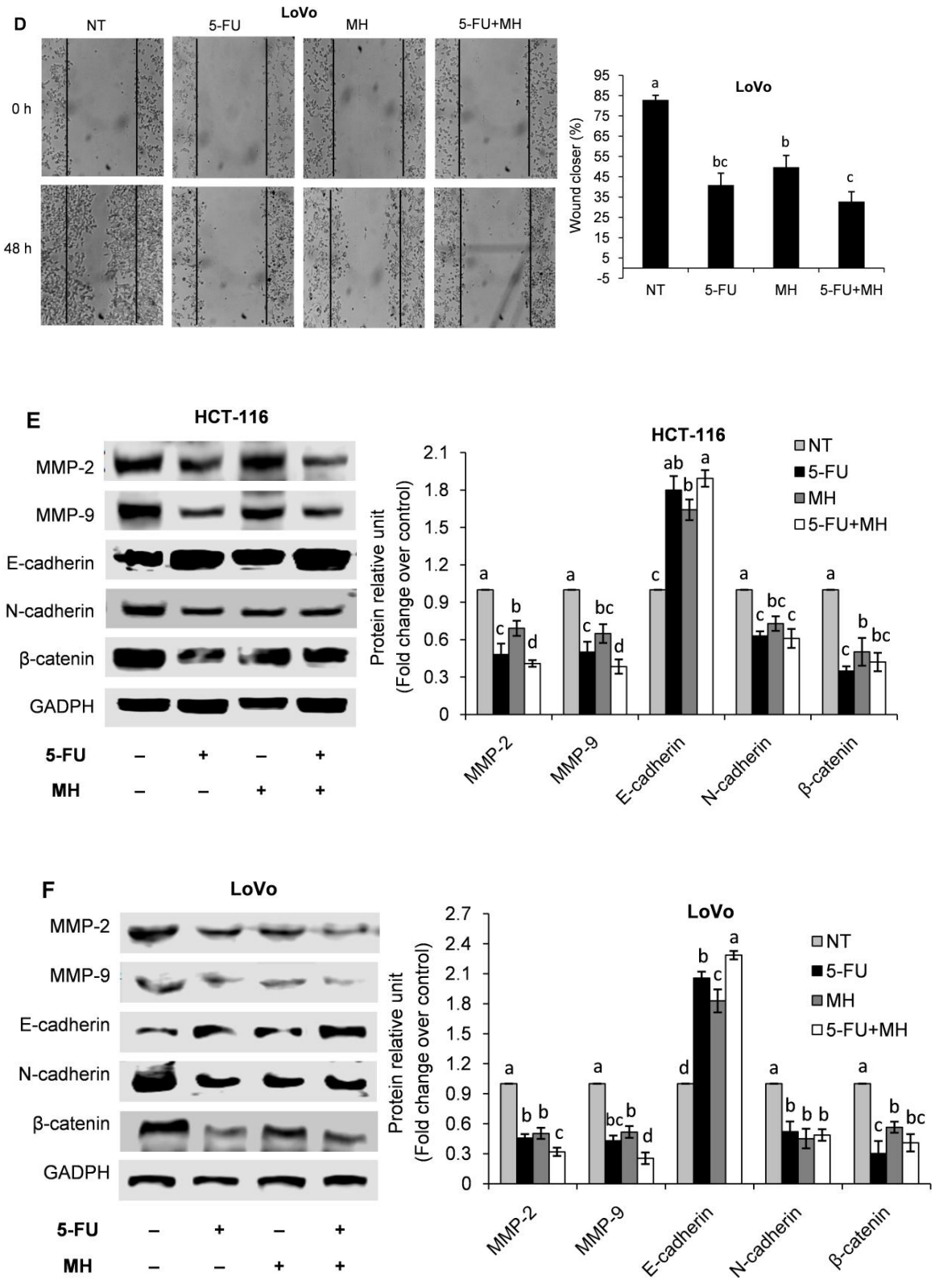


Fig. 8. Anti-metastatic effects of 5-FU and MH alone or in combination on HCT-116 and LoVo cells. Both cells were exposed to IC_{50} doses of 5-FU, MH and 5-FU+MH for 48 h. NT

corresponds to no treatment. The colony formation ability of (A) HCT-116 and (B) LoVo cells were analyzed after 12 days culture and the results were expressed as % of plating efficiency corresponds to not treated cells. The migration ability of (C) HCT-116 and (D) LoVo cells were analyzed by Image J software. The protein expression of invasion (MMP-2 and MMP-9) and EMT (E-cadherin, N-cadherin and β-catenin) markers were determined by western blotting analysis in (E) HCT-116 and (F) LoVo cells. GADPH was used as a loading control. All data shown were the mean ± SD (n=3). Different letters indicate significant differences (*p*-value < 0.05) between each treatment.

3.10 Effect of 5-FU and/or MH on invasion and EMT markers on HCT-116 and LoVo cells

Invasion ability after 5-FU or 5-FU+MH treatments was observed by the expression of MMP-2 and MMP-9 proteins in both CRC cell lines. In HCT-116 cells, the expression of MMP-2 and MMP-9 decreased 0.48 fold and 0.50 fold after 5-FU treatment, but in the presence of 5-FU+MH the expression decreased 0.40 fold and 0.38 fold (Fig. 8E). A similar trend was also observed in LoVo cells by reducing the expression of MMP-2 (0.45 fold) and MMP-9 (0.42 fold) after exposure to 5-FU but after the combined treatments the expression decreased 0.31 fold and 0.25 fold (Fig. 8F).

The expression of EMT related markers E-cadherin, N-cadherin and β-catenin was evaluated in colon cancer HCT-116 and LoVo cells after the treatment with 5-FU and/or MH. In HCT-116 cells, E-cadherin levels increased 1.70 fold after treatment with 5-FU, but the expression of the same proteins increased up to 1.89 fold when it was combined with MH (Fig. 8E). Furthermore, N-cadherin and β-catenin expression was suppressed 0.62 fold and 0.34 fold after treatment with 5-FU. Moreover, when 5-FU was combined with MH the expression was almost similar to 5-FU (Fig. 8E). In LoVo cells, 5-FU elevated the levels of E-cadherin 2.06 fold, while the combined treatment elevated the levels 2.29 fold (Fig. 8F). Expression levels of N-cadherin and β-catenin decreased 0.52 fold and 0.31 fold after 5-FU treatment, but when 5-FU was combined with MH,

the expression of N-cadherin decreased 0.48 fold and the expression of β-catenin decreased 0.39 fold, respectively compared to control (Fig. 8F).

4. Discussion

Chemotherapy extends the overall survival rate in CRC patients. 5-FU stands as the first choice for treatment of CRC, but unfortunately nearly 15% of CRC patients respond to this monotherapy drug, while the response rate is enhanced by 50% when it is combined with other anticancer agents [5]. Therefore, the development of new approaches to increase the effectiveness of 5-FU combining with polyphenols or polyphenol containing foods is vitally necessary to overcome cancer cell resistance and reduce the severity of adverse toxicity. The complex mixtures of polyphenols present in whole foods, which are easy to consume, are more efficient compared to purified molecules in cancer prevention by acting both synergistic and additive effects [17]. For this purpose, it is necessary to examine the anticancer effects not only using isolated fractions or single compounds but also whole foods that contain all the bioactive constitutes.

There is rising interest in honeys, and in particular MH, due to their diverse bioactive compounds [9-11]. In our previous report, we showed that MH is endowed with high levels of antioxidant compounds, most of which are flavonols such as quercetin (11.81%), luteolin (8.30%) and kaempferol (3.70%) and phenolic acids such as gallic acid (36.57%) and syringic acid (32.55%) of the total phenolic content [10]. In addition, other phenolic compounds such as apigenin, isorhammentin, 4-hydroxybenzoic acid and caffeic acid were also present in MH in low amounts [10]. These representative phenolic compounds are well known to exert chemoprevention and thought to be involved in various mechanisms [18-23].

5-FU induces cytotoxic effect by increasing ROS generation, promoting DNA damage, obstructing cell cycle progression and activating apoptosis in cancer cells [3]. A number of investigations has demonstrated that honey increases the anti-cancer effects of chemotherapy and reduces the cytotoxic side effects in several *in vit*ro and *in vivo* cancer models [24-26]. In a rat model of melanoma cancer, MH decreased the toxic effects of paclitaxel and increased the anti-tumor activity of the drug [24]. In colon cancer HCT-116 cells, 5-FU treatment inhibited cellular proliferation, but the inhibitory effects significantly increased even at low concentrations of 5-FU when it was combined with Gelam honey [25]. Similarly, Tualang honey increased the anticancer activity of Tamoxifen drug in a breast cancer MCF-7 cells at a lower concentration [26]. In the present work, in HCT-116 and LoVo cells the IC_{50} value of 5-FU decreased 2 fold when it was combined with MH, exerting similar effects on cytotoxicity compared to a single dose of 5-FU (Fig. 1).

Current research has supported the therapeutic importance of ROS generation to effectively induce cancer cells death by damaging lipids, proteins and DNA [27]. MH exerts oxidative stress inducing CRC cells death by ROS over production and concomitantly disrupts the antioxidant defense system [13]. Here, we found that the intracellular ROS level increased more after the combined treatment compared to a single dose of 5-FU in both colon cancer cells (Fig. 2). Previous investigations by other researchers also confirmed that natural compounds have the ability to enhance ROS production ability of 5-FU compared to a single agent in different types of cancer cells [19, 28-31]. On the other hand, in order to confirm the oxidative stress-induced cell death, we also observed the effects of this combined treatment on the oxidative biomarkers of lipids and proteins, highlighting that the combined treatment was more effective in LoVo cells compared to HCT-116 cells, by inducing more percentage of TBARS and protein carbonyl

content (Fig. 2). In a previous report, we have shown that MH protected healthy cells from oxidative stress by suppressing ROS generation and improving lipid, protein and DNA damage [9].

The activities of antioxidant enzymes, such as SOD, catalase, GPx and GR, distinctly decreased with the combined treatment compared to 5-FU alone in both cell lines (Fig. 3). In a previous report Fan *et al.* found that selenium enhanced 5-FU induced human melanoma cancer cell death by triggering oxidative stress through the increase of ROS mediated DNA damage and by inhibiting antioxidant enzyme (SOD, catalase, GPX and GR) activities [30]. These results confirm that MH increase ROS generation within the cells, which disrupt the cellular defense system, thus sensitizing the cancer cells to death induced by 5-FU provoked oxidative stress by activating apoptosis or altering cell death signaling.

There is an increasing amount of literature highlighting that the upregulation of Nrf2 and HO-1 expression by epigenetic modification is associated with resistance of 5-FU in CRC [32-34]. Natural compounds have the ability to enhance the sensitivity of Nrf2 sensitize tumor cells to chemotherapeutic drugs both in *in vitro* and *in vivo* models [35, 36]. In our present work, we evaluated the expression of Nrf2 and HO-1, in addition with SOD and catalase in HCT-116 and LoVo cells after the diverse treatments. All the treatments suppressed the expression of all the enzymes, while the combined treatment was most effective compared to 5-FU alone in both cell lines (Fig. 3). Furthermore, some investigations observed the synergistic effects of natural compounds and chemotherapeutic drugs in the Nrf2 pathway. For example, chrysin and apigenin enhanced the therapeutic efficacy of the anticancer drug doxorubicin by suppressing mRNA and protein levels of Nrf2 in hepatocellular carcinoma cells [20, 37]. Similarly, nobiletin increased the anticancer effects of several chemotherapeutic drugs by inhibiting the expression of Akt and

Nrf2 in multidrug resistant ovarian cancer cells [38]. Interestingly, MH increased SOD and catalase activities as well as the expressions of Nfr2, SOD and catalase in non-cancer cells protecting oxidative stress induced cell damage [9].

NF-κB is the important signaling pathway responsible for chemoresistance that the suppression of this pathway increases the therapeutic efficacy of 5-FU [6]. We previously evaluated that MH significantly suppressed NF-κB and p-IκBα expression in HCT-116 and LoVo cells [13], though co-treatment with 5-FU and MH was more effective than 5-FU alone (Fig.3). In a previous report, oral administration of crude honey decreased the cytotoxicity of the chemotherapeutic drug cisplatin by suppressing NF-κB activation [39]. Furthermore, curcumin enhanced the 5-FU chemotherapeutic activity, by inhibiting the expression NF-κB in chemoresistant CRC cells [40].

Induction of apoptosis is one of the most effective methods for cancer therapy [27]. According to the flow cytometry analysis, the combination treatment of 5-FU+MH significantly increased the apoptotic effect but more profound effects were observed in HCT-116 cells compared to LoVo cells (Fig.4). These findings were similar to those reported by Kao *et al.* who observed that ellagic acid synergistically enhanced the chemosensitivity of 5-FU by inducting apoptosis in colon cancer HT-29, Colo 320DM and SW-480 cells but not in LoVo cells [21].

The expression of key apoptotic markers p53, caspase-3, -8, -9, c-PARP, Bax, Cyto c and Fas L significantly increased and Bcl-2 decreased after co-treatment with 5-FU and MH in both cancer cells (Table 1 and Fig. 6), demonstrating the involvement of both intrinsic and extrinsic apoptotic pathways. MH increased the number of caspase-3 positive cells in a tumor bearing mouse model after combining with paclitaxel [24]. In other studies, quercetin and gypenosides augmented 5-FU induced apoptotic effects by increasing the expression of caspase-3, -9, c-PARP and p53 in

CRC cells [22, 28]. The combination of curcumin with 5-FU also induced more apoptotic effects by increasing the expression of caspase-3, -8, -9, Bax and PARP compared to a single agent in colon cancer HCT-116 cells [40].

5-FU can incorporate the cell cycle DNA and RNA by mainly targeting at S phase [3]. In our previous reports, MH arrested the cell cycle at S phase on HCT-116 cells and G2/M phase on LoVo cells [10]. The arresting ability of 5-FU increased when it was combined with other natural compounds at very low concentrations [23]. In colon cancer Caco-2, HT-29 and SW-480 cells, urolithin A increased the treatment efficacy of 5-FU in a dose dependent manner and the combined treatment arrested the cell cycle at S and G2/M phase, slightly increasing the apoptotic effects [23]. Furthermore, the combination of curcumin and 5-FU arrested the cell cycle at S phase and increased apoptosis in CRC cells compared to treatment with the single agents [40]. In agreement with previous studies, we also observed similar effects in the present work. Indeed, cell cycle analysis showed that 5-FU treatment arrested the cell cycle at S-phase in HCT-116 cells and G2/M phase in LoVo cells and also induced apoptosis by increasing cells at the Sub-G1 phase (Fig. 5). The combined data indicate that 5-FU+MH induced almost similar or slightly less pronounced effects than 5-FU alone at IC_{50} doses (Fig. 5). Real time PCR analysis indicated that the 5-FU+MH treatment notably increased p21 and p27 levels, while cyclin D1, cyclin E, CDK2, CDK4 and p-Rb levels decreased in both cells (Table 1). Co-treatment of gypenosides and 5-FU augmented the cell cycle at G0/G1 and S phase by decreasing cyclin E and CDK2 expression in SW-480 colon cancer cells [28].

The EGFR and its downstream pathways are also involved in the 5-FU chemoresistance [7]. Western blotting results indicate that 5-FU treatment suppressed the expression of EGFR, HER2, p-Akt and p-mTOR but in the presence of MH it induced more profound effects in both cell lines

(Fig. 6). A similar effect was also observed when fisetin and curcumin enhanced the treatment efficacy of 5-FU and other chemotherapeutic drugs by suppressing the expression of p-EGFR, p-HER2, p-IGR-1R, p-PI3K, p-Akt and p-mTOR in genetically modified *in vitro* and *in vivo* colon cancer models [41, 42]. It has been previously reported that chemotherapeutic drugs induced DNA damage and consequently apoptosis activating the MAPK pathway [43]. Similarly, in our present work, the co-treatment with 5-FU and MH activated p-p38MAPK and p-Erk1/2 expression in inducing colon cancer cell death by apoptosis and the effects were similar or slightly less pronounced compared to 5-FU alone (Fig.6). Similar effects were observed when the combination of resveratrol and 5-FU induced apoptosis by activating p-p38MAPK and p-JNK while the expression of p-Erk1/2 was unchanged in human colon cancer HCT-116 cells [44]. Furthermore, green tea polyphenols dramatically increased the expression of p-p38MAPK and p-Erk1/2 in lung cancer cells when they were added together with celecoxib [45]. In another study, the combination of two plant-derived drugs triptolide and hydroxycamptothecin enhanced the expression of p-p38MAPK and p-Erk1/2 which increased apoptosis in lung adenocarcinoma cells [46].

Some evidence has been found reporting that the dysregulated metabolism is associated with chemoresistance [47]. In order to overcome therapeutic resistance and improve therapeutic efficacy there is an urgent need to use combination chemotherapeutic agents with targeted compounds able to disrupt dysregulated cancer cell metabolism. Higher aerobic glycolysis has been observed in drug resistant cancer cells able to produce ATP more quickly so as to survive under stress conditions [48]. 5-FU resistance colon cancer cells can become addicted to oxidative phosphorylation (OXPHOS) in order to survive [49] and maintain SRC and MRC by upregulating OXPHOS for ATP use under stress conditions or during enhanced energy demand

for proliferation [50, 51]. To the best of our knowledge, this is the first time that the effects of 5-FU in the presence of MH in colon cancer cell metabolism, targeting mitochondrial respiration and glycolysis function, have been evaluated. All the parameters of mitochondrial respiration, such as basal OCR, ATP production, proton link, SRC and MRC, decreased after treatment with 5-FU alone or in the presence of MH, with similar effects in both cells (Fig. 7). In contrast, MH increased the mitochondrial respiration in healthy cell lines by controlling oxidative stress [9].

Furthermore, we observed the ECAR values as an indicator of glycolysis, glycolytic capacity and glycolytic reserve. Here we also evaluated trends similar to mitochondrial respiration. In HCT-116 cells, the combination of 5-FU+MH was slightly less effective compared to 5-FU treatment but in LoVo cells the combination was more effective in reducing the glycolysis function (Fig. 7).

The anti-metastatic effects of 5-FU in the combination with MH were observed in HCT-116 and LoVo cells depending on different aspects. Most of the natural compounds increased the colony inhibiting ability of the chemotherapeutic drug compared to a single dose in the different types of *in vitro* cancer models [40, 52, 53]. However, in our results, the combined treatment significantly reduced the colony formation ability, more in LoVo cells than in HCT-116 cells (Fig. 8).The anti-migration effects of 5-FU increased when it was combined with MH in both cell lines at a lower concentration than using 5-FU alone (Fig. 8). These results were similar to previous studies, which observed the combination of polyphenols with 5-FU in colon cancer HCT-116 cells [40, 44]. Additionally, the expression of MMP-2 and MMP-9 was suppressed more in both cell lines after 5-FU+MH treatments compared to 5-FU alone (Fig.8). To date, only one study showed that curcumin potentiates 5-FU in inhibiting the expression of MMP-9 in colon cancer HCT-116 cells [40]. The expression of EMT related markers, E-cadherin, N-cadherin and β-

catenin, were also modulated after the combination treatment in both cell lines (Fig. 8). The expression of N-cadherin induced almost similar effects after treatment with 5-FU or 5-FU+MH but the expression of β-catenin was slightly less suppressed after the combined treatment compared to 5-FU in both cells (Fig. 8). Additionally, E-cadherin expression significantly increased after the combined treatments in both cell lines (Fig. 8) and a similar trend was observed by Buhrmann *et al.* in HCT-116 cells for the co-treatment of curcumin and 5-FU [54].

5. Conclusion

The development of new approaches to increase the effectiveness of 5-FU combined with polyphenol containing foods is vitally necessary: (i) to improve the effectiveness of conventional chemotherapy, (ii) to overcome t cancer cell resistance, and (iii) to reduce the severity of adverse toxicity. In this study, we demonstrated that the combination of 5-FU and MH enhanced the anti-proliferative effects by inducing toxicity, up-regulating ROS and apoptotic markers, blocking cell cycle and suppressing EGFR, p-Akt and p-mTOR expressions, as well as by increasing p-p38MAPK and p-ERK1/2 levels. We also showed that this combined treatment significantly altered the metabolic phenotype by reducing both mitochondrial respiration and glycolysis function. Moreover, the anti-metastatic effects of 5-FU also increased when it was combined with MH by decreasing colony formation and migration ability, as well as altering the expression of MMP-2, MMP-9, E-cadherin, N-cadehrin and β-catenin proteins. These encouraging results increased the therapeutic efficacy of 5-FU combined with MH in colon cancer. The proposed mechanisms need to be validated in *in vivo* models to discover the possible role of MH as a chemotherapy adjuvant.

Conflict of interest

The authors declare no conflicts of interest.

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Abbreviation

Akt, protein kinase B; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; CDK, cyclin-dependent kinase; CRC, colorectal cancer; CI, combination index; Cyto c, cytochrome c; ECAR, extracellular acidification rate; EGFR, epidermal growth factor receptor; Erk1/2, extracellular-signal-regulated kinase 1/2; 5-FU, 5-Fluorouracil; FasL, fatty acid synthetase ligand; GPx, glutathione peroxidase; GR, glutathione reductase; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HER2, human epidermal growth factor receptor 2; HO-1, heme oxygenase 1; p-IκBα, phosphorylated inhibitor of kappa B; MH, Manuka honey; MAPK, mitogen-activated protein kinase; MMP-2, matrix metalloproteinases 2; mTOR, mammalian target of rapamycin; MRC, maximal respiration capacity; Nfr2, nuclear related factor 2; NF-κB, nuclear factor kappa B; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; c-PARP, cleaved-poly (ADP-ribose) polymerase; ROS, reactive oxygen species; Rb, retinoblastoma; SRC, spare respiration capacity; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substance.

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Graphical abstract