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Strawberry tree honey in combination with 5-fluorouracil enhances chemosensitivity in human colon adenocarcinoma cells

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ABSTRACT

Colorectal cancer remains a challenging health burden worldwide. This study aimed to assess the potentiality of Strawberry tree honey (STH), a polyphenol-enriched food, to increase the effectiveness of 5-Fluorouracil (5-FU) in adenocarcinoma (HCT-116) and metastatic (LoVo) colon cancer cell lines. The combined treatment reduced cell viability and caused oxidative stress, by increasing oxidative biomarkers and decreasing antioxidant defence, in a more potent way compared to 5-FU alone. The expression of endoplasmic reticulum (ATF-6, XBP-1) and MAPK (p-p38 MAPK, p-ERK1/2) markers were also elevated after the combined treatment, enhancing the cell cycle arrest through the modulation of regulatory genes (i.e., cyclins and CDKs). Apoptotic gene (i.e., caspases) expressions were also increased after the combined treatment, while those of proliferation (i.e., EGFR), cell migration, invasion (i.e., matrix metallopeptidase) and epithelial–mesenchymal transition (N-cadherin, β-catenin) were suppressed. Finally, the combined treatment led cell metabolism towards a quiescent stage, by reducing mitochondrial respiration and glycolysis. In conclusion, this work represents an initial step to highlight the possibility to use STH in combination with 5-FU in the treatment of colon cancer, even if further *in vitro* an *in vivo* studies are strongly needed to confirm the possible chemo-sensitizing effects of STH.

1. Introduction

Worldwide, colorectal cancer (CRC) is the third most deadly diseases due to the lack of efficient treatment, unfavorable side effects and therapeutic resistance. Depending on the patient condition and the cancer stage, chemotherapeutics are selected alone or as adjuvants (Schmoll et al., 2012), but with them more than 50% patients will persistent the disease (Jemal et al., 2009). 5-Fluorouracil (5-FU) is the most commonly used anti-cancer agent, but its application reduces less than 50% of patient's responses at an early stage of CRC; additionally, its activity increases when it is combined with other anti-cancer drugs, but with adverse toxicity and highly therapeutic resistance in later stage (González-Vallinas et al., 2013). In order to increase the effectiveness of 5-FU and overcome chemo resistance and toxicity effects, a new approach is necessary based on the application of nutritionally rich foods or isolated bioactive compounds. In several studies, the synergistic

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and additive effects of phenolic compounds on different cancer models have already been examined (Liu, 2004); from this point of view, it could be helpful to use the whole food products and not only isolated compounds because of the intake facility and complex mixture of nutritive and non-nutritive bioactive compounds.

Diet has a significant impact on CRC, since there is an inverse relationship between polyphenol-rich food and colon cancer incidence (Núñez-Sánchez et al., 2015). Recently, there has been rising attention on the search and application of natural food or isolated compounds due to their advantage of effective pharmacological activities and safety (Núñez-Sánchez et al., 2015). Targeting different cancer models, natural products may affect several oncogenic pathways related to proliferation, inflammation, cell cycle, apoptosis, invasion, angiogenesis and metastasis (Lee et al., 2011; Cianciosi et al., 2020; Battino et al., 2021). Strawberry tree (*Arbutus unedo* L.) honey (STH) is a typical product from the Mediterranean area; however, to date no study has evaluated the chemo-sensitizing effects of STH in colon cancer cells. STH contains several phenolic acids and flavonols, including gallic acid, 4-hydroxybenzoic acid, protocatechuic acid, caffeic acid, vanillic acid, kaempferol, quercetin, luteolin and myricetin (Afrin et al., 2019b). Additionally, the antioxidant capacity of STH is higher compared to the well-known Manuka honey (Afrin et al., 2017). STH induces anti-cancer effects in colon cancer cells by decreasing cell proliferation, arresting cell cycle, activating apoptosis, increasing ROS, altering metabolic phenotype and suppressing metastasis ability, whereas in healthy cells it didn't exert any toxic effects (Afrin et al., 2019a, 2019b). The current work aimed to investigate the chemo-sensitizing effects of STH in combination with 5-FU on human colon HCT-116 and LoVo cancer cell lines.

2. Materials and methods

2.1. Reagents, chemicals and antibodies

Reagents and chemicals used for the experimental purposed were purchased from Sigma-Aldrich (Milan, Italy), while all the media and other reagents for cell culture were obtained from Carlo Erba Reagents (Milan, Italy). STH samples, a kind gift of Prof. Gavino Sanna (Department of Chemistry and Pharmacy at the University of Sassari, Italy) were preserved at 4 °C until used. All the primary antibodies (i.e., activating transcription factor 6 (ATF6), X-box-binding protein 1 (XBP1), nuclear factor erythroid 2-related factor 2 (Nrf2), phosphorylated inhibitor of kappa B (p-IκBα), nuclear factor κB (NF-κB), heme oxygenase 1 (HO-1), catalase (CAT), superoxide dismutase (SOD), p53, caspase-3, cleaved-poly (ADP-ribose) polymerase (c-PARP), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), p-mammalian target of rapamycin (p-mTOR), phosphorylated protein kinase B (p-Akt), p-mitogen activated protein kinase (p-p38 MAPK), p-extracellular-signal regulated kinase 1/2 (p-Erk1/2), matrix metalloproteinases 2 (MMP-2), MMP-9, N-cadherin, β-catenin, E-cadherin, and glyceraldehyde-3-phosphate dehydrogenase (GADPH)) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Human colon carcinoma cell lines HCT-116 (organ: colon, disease: colorectal adenocarcinoma) and LoVo (organ: colon, disease: Dukes' type C, grade IV, colon metastasis) were obtained from American Type Culture Collection (ATCC).

2.2. Cell culture and proliferation assay

HCT-116 cell line was grown in McCoy's 5 A medium supplemented with 10% FBS and 1% antibiotic, while LoVo cell line was grown in F–12 K medium supplemented with 10% FBS and 1% antibiotic. Adherent monolayer cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 under standard conditions. Cultured HCT-116 and LoVo cells were treated with different concentrations of STH and 5- FU for 48 h respectively in order to find the IC_{50} concentration by MTT assay (Afrin et al., 2019b). SHT was filtered with a MF-Millipore™ Membrane Filter (0.45 μm pore size) and dissolved into each specific medium. The synergistic or additive effect of STH on 5-FU was determined by the combination index (CI) as explained in our previous paper (Afrin et al., 2018). For the subsequent experiment cells were treated with (i) only media (CTL group), (ii) IC_{50} of STH, (iii) IC_{50} of 5-FU and (iv) IC_{50} of STH+5-FU combination.

2.3. Determination of intracellular ROS levels, TBARS and protein carbonyl content, and antioxidant enzyme activity

The Cell ROX® Oxidative Stress kit (Invitrogen™, Life Technologies, Milan, Italy) was used for determining the intracellular reactive oxygen species (ROS) generation by Tali® Image-Based cytometer (Invitrogen™, Life Technologies, Milan, Italy) after STH treatment, as explained in et al., 2019. Results are expressed as % compared to control cells.

Cellular lysates were obtained using RIPA buffer (Sigma-Aldrich, Milan, Italy) at the end of the treatment and were used for determining the levels of oxidative biomarkers and the activities of the antioxidant enzymes. Thiobarbituric acid-reactive substance (TBARS), protein carbonyl content and antioxidant enzymes CAT, SOD, glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferases (GST) were measured through spectrophotometric methods as earlier reported by our group (Giampieri et al., 2017). The results of TBAR and carbonyl group were measured as mmol/mg of protein and expressed as % compared to control cells, while SOD-1, CAT, GPx, GR and GST activities were measured as unit/mg protein and expressed as % compared to control cells.

2.4. Cell cycle analysis

The Tali® Cell Cycle Kit (Invitrogen™, Life Technologies, Milan, Italy) was used for analyzing the cell cycle assay after the exposure of different treatment groups (Afrin et al., 2019b). The cellular pellets were fixed with 70% ethanol and reading by Tali™ Image-Based Cytometer after re-suspending with 100 μL PBS containing 20 μg/mL propidium iodide (Invitrogen™, Life Technologies, Milan, Italy), 0.2 mg/mL RNase A and 0.1% Triton® X-100 (Invitrogen). Results are expressed as fold change compared to control cells.

2.5. Determination of apoptotic rate

Tali™ Apoptosis Assay Kit–Annexin V Alexa Fluor® 488 (Invitrogen™, Life Technologies, Milan, Italy) kit was used for the determination of apoptosis cells by Tali™ Image-Based Cytometer (Afrin et al., 2019b). Results are expressed as fold change compared to control cells.

2.6. RNA isolation and real-time PCR

PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) was used for isolating total RNA content according to the manufacturer's protocol. cDNA was synthesized following reverse transcription by using 5 × All-In-One RT MasterMix kit (Applied Biological Materials Inc. Canada). Real-time PCR was performed using EvaGreen 2× qPCRMaster Mix (Applied Biological Materials Inc. Canada) of forward and reverse primers (Supplementary Table 1) for p53, Caspase-3, 8, 9, c-PARP, Bcl-2, Bax, Cyt C, Fas L, Cyclin D1, E, CDK2, CDK4, p21waf1/cip 1, p27kip1, p-Rb genes; $2^{-\Delta\Delta ct}$ method was used for calculating the fold change values after normalizing the data (Afrin et al., 2019b).

2.7. Determination of energetic metabolism

The baseline metabolic phenotype of each cell was determined by using basal values of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) by using an Agilent Seahorse XF24 Analyzer

(Seahorse Bioscience, North Billerica, MA, USA), as previously described in our paper (Afrin et al., 2019a). OCR was expressed in picomoles/minute (pmole/min) per 3.0 × 10^4 cells and ECAR was stated in milli-pH/minute (mpH/min) per 3.0 × 10^4 cells.

2.8. In vitro scratch assay

In vitro scratch assay was carried out according to the previous method described by our group (Afrin et al., 2019a). At the end of the treatment, the scratch areas were analyzed by NIH Image J software.

2.9. Western blotting analysis

Cells proteins were isolated by using lysing buffer (120 mmol/L NaCl, 40 mmol/L Tris [pH 8], 0.1% NP40) containing protease inhibitors and measured by Bradford protein assay. Western blot analyses were performed as described in our paper (Afrin et al., 2019b). The blots were assessed through a chemiluminescence method (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany) and the band intensity was measured through image studio digits software 3.1 (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany).

2.10. Statistical analysis

Statistical analysis was carried out through the software STATISTICA (Statsoft Inc., Tulsa, OK, USA). All the results are expressed as mean ± SD of triplicate experiments. Significance differences between the means of groups were statistically performed by one-way analysis of variance (ANOVA) and by Tukey's honestly significant difference (HSD) post hoc test. The level of the significance for all test set at *P < 0.05, **P < 0.01 and ***P < 0.0001, compared to the control group.

3. Results

3.1. STH sensitizes colon cancer cells to 5-FU-treatment decreasing cell viability

The cytotoxic activity of STH and 5-FU was screened alone or in combination in HCT-116 cells and LoVo cells by MTT assay after 48 h incubation (Table 1).

In HCT-116 cells, 3–12 mg/mL concentrations were chosen for the STH (Afrin et al., 2019b) and 5–50 μM concentrations were selected for 5-FU treatments. In LoVo cells, the concentrations were higher because of its metastatic nature, being 10–40 mg/mL for STH and 10–80 μM for 5-FU. The IC_{50} values for the combined treatment were 9.48 mg/mL for STH and 21.34 μM for 5-FU in HCT-116 cells whereas in LoVo cells the IC_{50} values were 35.12 mg/mL for STH and 50.11 μM for 5-FU (Table 1). In order to investigate the synergistic (CI < 1) or antagonistic (CI > 1) effects of STH, various STH+5-FU combinations were used to find out the IC_{50} values of the combined treatment. We found that 6 mg/mL STH and 10 μM 5-FU induced 26% and 33% cytotoxicity in HCT-116 cells, while the combined treatment induced 50% toxicity, which is same than that obtained with 21.34 μM 5-FU (Table 1). Furthermore, in LoVo cells, 15 mg/mL STH and 20 μM 5-FU induced 19% and 44% cytotoxicity, the combined treatment induced 50% toxicity, which is the same that found with 50.11 μM 5-FU (Table 1). All the IC_{50} concentrations were selected for further experimental analysis after 48 h of incubation.

3.2. STH potentiates 5-FU-induced oxidative stress in colon cancer cells

Tali® Image-Based cytometer was used for measuring intracellular ROS production after the treatment with STH and/or 5-FU in HCT-116 and LoVo cells. IC_{50} doses significantly increased the ROS levels in both colon cancer cells (Fig. 1).

5-FU induced more ROS compared to STH, while the combined treatment was more effective in HCT-116 cells (Fig. 1A). As shown in Fig. 1B, the STH treatment induced more ROS production in LoVo cells compared to the treatment of 5-FU and 5-FU + STH.

The main oxidative biomarkers of biomolecules, lipid (TBARS) and protein (protein carbonyl content, PCC) were further determined by the spectrophotometer method. In HCT-116 cells, STH significantly increased the production of TBARS and PCC up to 199% and 164%, while 5-FU enhanced more percentage of TBARS (228%) and PCC (253%), but the STH+5-FU treatment was slightly less effective compared to 5-FU alone (Fig. 1C). A similar trend was observed in LoVo cells: for example, STH and 5-FU treatment increased the levels of TBARS (212% and 278%) and PCC (211% and 280%), while the combined treatment was less effective, respectively compared to 5-FU

Table 1
Cytotoxic activities of 5-FU alone or 48 h treated with STH in HCT-116 and LoVo cell lines.

Treatment for HCT-116	Cell viability (%) HCT-116	IC_{50} values for HCT-116	Treatment for LoVo	Cell viability (%) LoVo	IC_{50} values for LoVo
STH (3 mg/mL)	81.78 ± 2.13	9.48 mg/mL	STH (10 mg/mL)	88.51 ± 1.59	35.12 mg/mL
STH (6 mg/mL)	73.72 ± 2.65		STH (20 mg/mL)	78.09 ± 3.15	
STH (9 mg/mL)	66.21 ± 5.62		STH (30 mg/mL)	60.88 ± 2.78	
STH (12 mg/mL)	40.31 ± 4.00		STH (40 mg/mL)	39.22 ± 0.62	
5-FU (5 μM)	78.12 ± 1.74	21.34 μM	5-FU (10 μM)	67.01 ± 6.80	50.11 μM
5-FU (10 μM)	67.04 ± 4.38		5-FU (20 μM)	56.54 ± 3.08	
5-FU (20 μM)	50.84 ± 3.02		5-FU (50 μM)	51.18 ± 2.01	
5-FU (50 μM)	47.89 ± 5.30		5-FU (80 μM)	48.91 ± 2.36	
CI: STH + 5-FU			**CI: STH + 5-FU**		
3 mg/mL+5 μM	67.77 ± 5.51		10 mg/mL+10 μM	63.93 ± 3.92	
6 mg/mL+5 μM	63.75 ± 2.80		20 mg/mL+10 μM	62.61 ± 5.62	
9 mg/mL+5 μM	57.13 ± 3.21		30 mg/mL+10 μM	59.58 ± 4.19	
3 mg/mL+10 μM	65.58 ± 5.84	6.12 mg/mL + 10 μM	10 mg/mL+20 μM	53.94 ± 4.19	14.81 mg/mL + 20 μM
6 mg/mL+10 μM	51.52 ± 6.33		20 mg/mL+20 μM	48.80 ± 4.97	
9 mg/mL+10 μM	25.22 ± 1.77		30 mg/mL+20 μM	45.76 ± 3.99	
3 mg/mL+20 μM	37.82 ± 1.90		10 mg/mL+50 μM	50.83 ± 5.25	
6 mg/mL+20 μM	26.18 ± 3.13		20 mg/mL+50 μM	47.42 ± 4.60	
9 mg/mL+20 μM	16.69 ± 0.78		30 mg/mL+50 μM	45.03 ± 3.62	
3 mg/mL+50 μM	29.17 ± 1.17		10 mg/mL+80 μM	42.78 ± 5.80	
6 mg/mL+50 μM	16.00 ± 2.89		20 mg/mL+80 μM	38.83 ± 3.88	
9 mg/mL+50 μM	9.90 ± 0.59		30 mg/mL+80 μM	35.20 ± 3.31	

The IC_{50} value indicates a concentration of each treatment which caused 50% reduction in cell viability based on MTT assay. Each value is expressed as mean ± standard deviation (SD) of three replicates of three independent experiments.

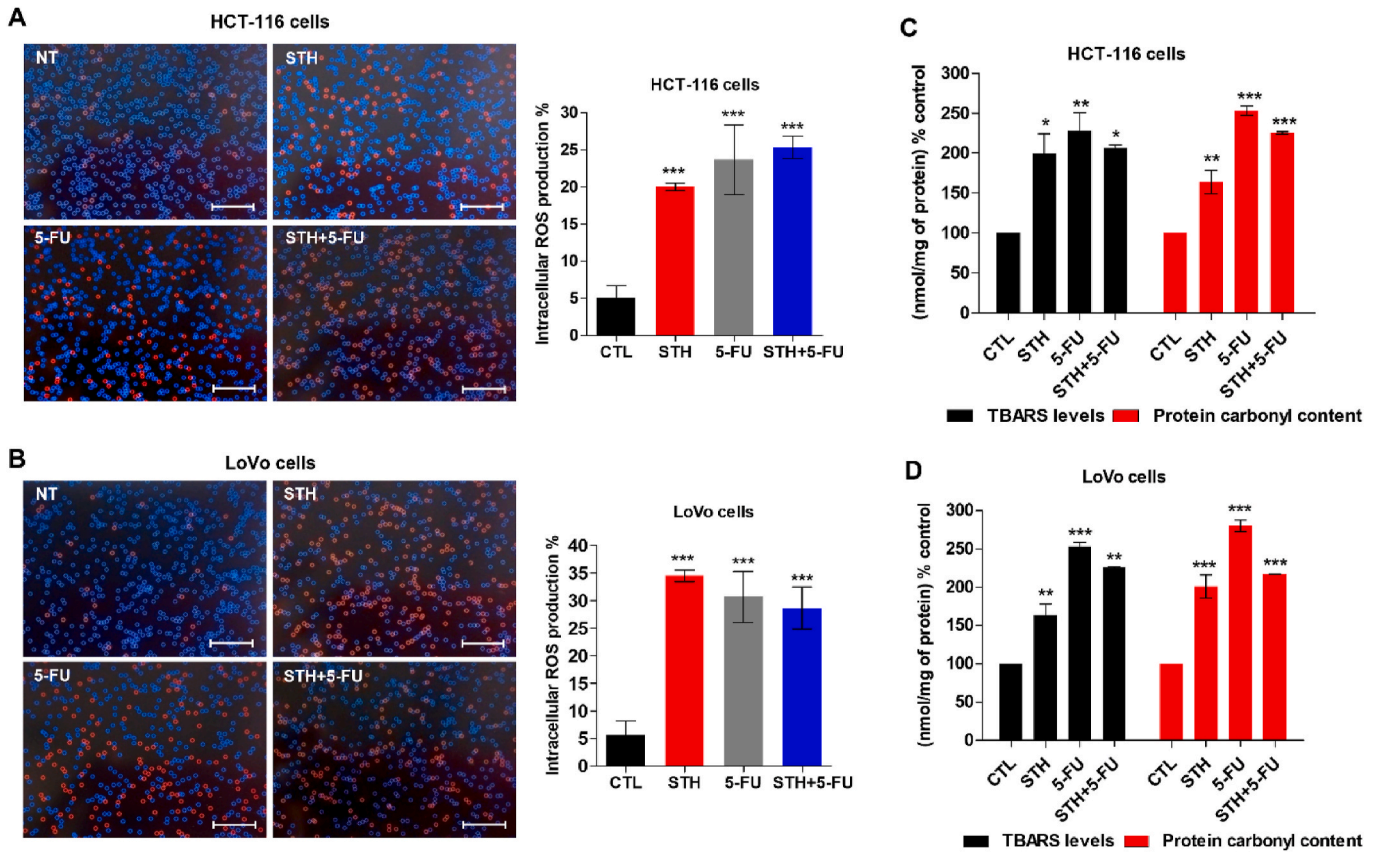


Fig. 1. STH sensitizes colon cancer cells to 5-FU induced oxidative stress. HCT-116 and LoVo cells were treated with STH alone or in combination with 5-FU. ROS generation in (A) HCT-116 and (B) LoVo cells was determined by Tali™ Image-based Cytometer. Results are expressed as % compared to control cells. Blue cells correspond to live cells, while red cells correspond to ROS-induced cells. TBARS and protein carbonyl content were measured in (C) HCT-116 and (D) LoVo cells. Results were measured as mmol/mg of protein and expressed as % compared to control cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs.* control group. Scale bar 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 1D).

3.3. The combination effect of STH and 5-FU on antioxidant enzyme activity and the expression

We further observed the expression and activity of antioxidant enzymes after the treatment with STH, 5-FU and STH+5-FU in HCT-116 and LoVo cells. As shown in Fig. 2, STH and 5-FU significantly decreased SOD-1 (72% and 57%), CAT (42% and 48%), GPx (49% and 43%), GR (57% and 60%) and GST (62% and 69%) activity in HCT-116 cells, whereas the STH+5-FU decreased the above activity of 61%, 51%, 35%, 54% and 45%, respectively.

Additionally, in LoVo cells, STH and 5-FU decreased 67% and 41% activity for SOD-1, 52% and 39% activity for CAT, 33% and 61% activity for GPx, 55% and 40% activity for GR and 54% and 49% activity for GST, respectively; these activities were reduced 46%, 57%, 39%, 44% and 47% after the combined treatment (Fig. 2B).

The treatment with STH and 5-FU suppressed the expression of Nrf2 and its downstream targets SOD-1, CAT and HO-1 by 0.57 and 0.77 fold, 0.51 and 0.80 fold, 0.41 and 0.78 fold, 0.72 and 0.75 fold in HCT-116 cells (Fig. 2C) and by 0.50 and 0.87 fold, 0.69 and 0.66 fold, 0.67 and 0.85 fold, and 0.70 and 0.84 fold, respectively in LoVo cells (Fig. 2D). In both cells, STH suppressed more compared to 5-FU, while in STH+5-FU group, the inhibitory effects were more prominent: for Nrf2 0.56 and 0.36 fold, for SOD-1 0.50 and 0.35 fold, for CAT 0.35 and 0.57 fold, for HO-1 0.55 and 0.49 fold, in HCT-116 and Lovo cell lines, respectively (Fig. 2C and D).

3.4. The combination effect of STH and 5-FU on ER stress and inflammation

Due to excess ROS and oxidative stresses, the expression of ER stress markers ATF-6 and XBP-1 significantly increased after the treatment with STH and/or 5-FU (Fig. 3). In HCT-116 cells, STH and 5-FU increased ATF-6 (1.63-fold and 1.82-fold) and XBP-1 (2.51 fold and 1.53 fold) expression but the combined treatment induced more effects in ATF-6 (1.86 fold), while in XBP1 (2.05 fold), it was less effective (Fig. 3A). Moreover, in LoVo cells, the expression of ATF-6 and XBP-1 increased 1.72-fold and 1.97-fold after STH and 1.68 fold and 1.71 fold after 5-FU, but the STH+5-FU significantly increased both proteins of 2.05 fold and 1.95 fold, respectively compared to single compound (Fig. 3B). Similarly, the expression of NF-κB and p-IκBα was suppressed after each treatment in both cell lines, while the combined treatment suppressed more the expression of NF-κB and a similar trend was observed in p-IκBα expression after the treatment with STH, 5-FU and STH+5-FU, respectively (Fig. 3A and £b).

3.5. The combination effect of STH and 5-FU on cell cycle in colon cancer cell lines

Cell cycle alteration in HCT-116 and LoVo cells treated with STH and/or 5-FU were evaluated. In HCT-116 cells, the treatment with STH, 5-FU and STH+5-FU induced a significant arrest at the S phase by accumulating 54%, 50% and 61% of cells, respectively (Fig. 4A).

Furthermore, in LoVo cells, the cells were accumulated in G2/M phase by 24%, 30% and 31% after the exposure to STH, 5-FU and STH+5-FU, respectively (Fig. 4B).

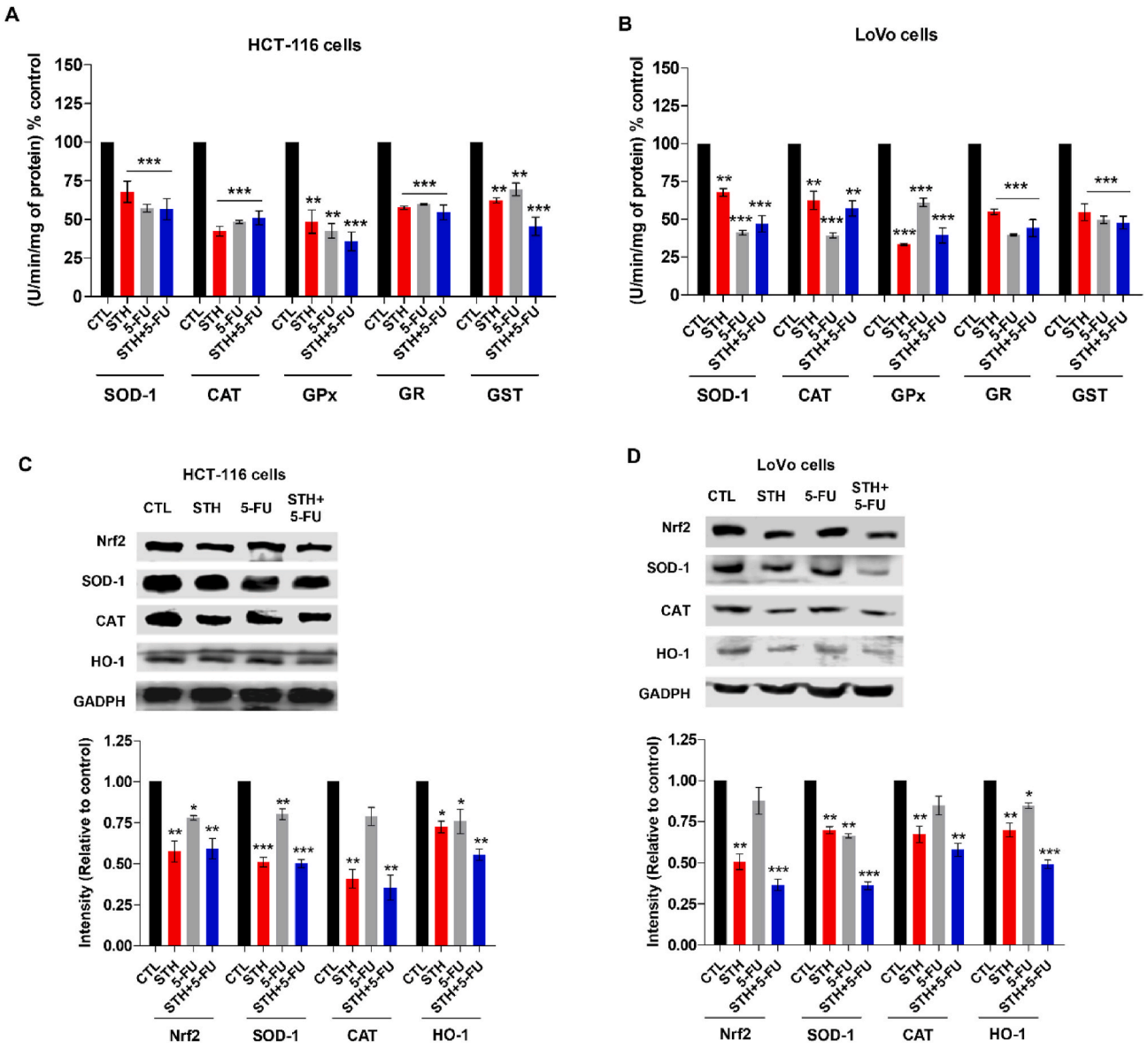


Fig. 2. Antioxidant enzymes activity and expression after the treatment with STH alone or in combination with 5-FU. SOD-1, CAT, GPx, GR and GST activities were measured as unit/mg protein and expressed as % compared to control cells in (A) HCT-116 and (B) LoVo cells. The protein expression of Nrf2, SOD-1, CAT and HO-1 was detected by western blotting (normalized to GADPH loading control) in (C) HCT-116 and (D) LoVo cells. Results are expressed as fold change compared to control cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group.

To further discover the molecular mechanisms involved in cell cycle arrest by STH and 5-FU alone or in combination, we examined the mRNA levels of cyclin-dependent kinases (CDK2 and CDK4), cycle regulatory proteins (cyclin D1 and cyclin E), cyclin-dependent kinase inhibitor (p21waf1/Cip1 and p27kip1) and p-retinoblastoma (p-Rb) at 48 h. In HCT-116 cells, all the treatments significantly decreased the levels of cyclin D1, cyclin E, CDK2, CDK4 and p-RB and the levels of p21Cip and p27Kip were increased, whereas the combined treatment induced more prominent effects (Fig. 4C). In LoVo cells, we did not observe any alteration of cyclin E, CDK2 and CDK4 mRNA levels, while cyclin D1 and p-Rb levels were suppressed similarly after the treatment of 5-FU and STH+5-FU and the levels of p21Cip and p27Kip increased slightly more after 5-FU compared to STH+5-FU, respectively (Fig. 4D).

3.6. *The combination effect of STH and 5-FU on apoptosis in colon cancer cells*

The Annexin V/PI staining was used for determining the apoptotic effects of STH and/or 5-FU in HCT-116 and LoVo cells with Tali™ Image-based Cytometer. The number of apoptotic cells was significantly increased after the treatment of STH (2.25 fold for HCT-115 and 3.31 fold for LoVo), 5-FU (5.49 fold for HCT-115 and 4.55 fold for LoVo) and STH+5-FU (3.89 fold for HCT-115 and 5.50 fold for LoVo), respectively, being the combined treatment remarkably effective in LoVo cells (Fig. 5A and B).

At the same time, each treatment significantly decreased the number of live cells and increased dead cells number.

To deeply investigate the molecular mechanism involved in apoptotic induction, we examined the mRNA expression of both intrinsic and extrinsic apoptotic markers (Fig. 5). In both cell lines, the combination induced a significant increase in p53 and c-PARP, while caspase-3 levels were similar in 5-FU and STH+5-FU (Fig. 5C and D). The expression of intrinsic apoptotic markers, Bax/Bcl2, Cyto C and caspase-9, were increased more after the combined treatment compared single compound in LoVo cells (Fig. 5F), while in HCT-116 cells the 5-FU increased more expression of Bax/Bcl2 and caspase-9 (Fig. 5E).

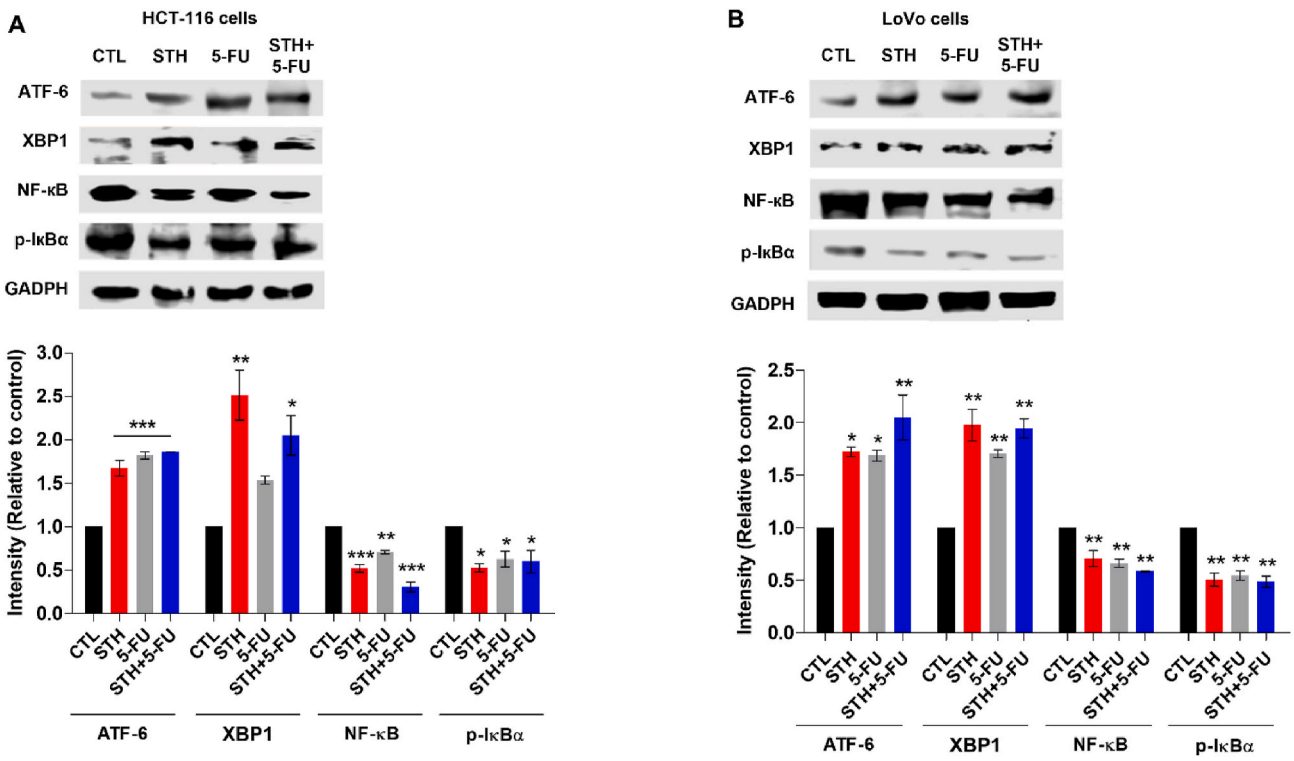


Fig. 3. STH sensitizes colon cancer cells to 5-FU induced ER stress and inflammation. (A) HCT-116 and (B) LoVo cells were treated with STH and 5-FU alone or in combination. The protein expression of ATF-6, XBP1, NF-κB and p-IκBα was detected by western blotting (normalized to GADPH loading control). Results are expressed as a fold change in comparison control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group.

Furthermore, the extrinsic apoptotic markers, FasL and caspase-8 increased similarly after the treatment of 5-FU and STH+5-FU in both cell lines, while in HCT-116 cells, 5-FU induced more expression of caspase-8 (Fig. 5G and H). Apoptotic effects were also confirmed by examining the protein expression of p53, c-PARP and caspase-3 (Fig. 5E and F). Each treatment significantly increased the expression of these proteins, whereas the combined treatment was more effective in the expression of c-PARP and caspase-3 in both cell lines (Fig. 5E and F).

3.7. STH enhances the anti-proliferative activity of 5-FU by regulating EGFR and MAPK signaling pathways

STH synergistically enhanced the effect of 5-FU on EGFR signaling pathways (Fig. 6): the expression levels of EGFR, HER2, p-Akt and p-mTOR were performed to evaluate the anti-proliferative effects of STH, 5-FU and STH+5-FU in HCT-116 and LoVo cells.

Compared to control, all treatments significantly suppressed the expression of the above markers, with the combination inducing more effects in both cell lines (Fig. 6A and B).

Similarly, the expressions of p-p38 MAPK and p-ERK1/2 were evaluated. As Fig. 6C and D illustrated, compared to control cells, all the treatments significantly increased the protein expression of p-p38 MAPK and p-ERK1/2 in HCT-116, while in LoVo cells, 5-FU induced slightly more effects compared to STH+5-FU.

3.8. The combination effect of STH and 5-FU on metabolic phenotype in colon cancer cells

In our previous part, we already evaluated the metabolic alteration exerted by STH treatment in HCT-116 and LoVo cells (Afrin et al., 2019a). In the present work, we observed the metabolic phenotype alteration as well as mitochondrial respiration and glycolysis after 48 h of treatment with IC_{50} of STH, 5-FU and STH+5-FU. As shown in Fig. 7A, STH treatment changed the baseline phenotype in the aerobic phase compared to the control (energetic state), but the treatment of 5-FU and STH+5-FU remarkably changed in the quiescent stage which is metabolically inactive. Moreover, in LoVo cells, all the treatments changed the phenotype profile in the quiescent stage compared to the control (energetic state) (Fig. 7B).

We also observed altered mitochondrial respiration after the treatment with STH and 5-FU in HCT-116 and LoVo cells (Fig. 7C and D). Similar effects were observed in both colon cancer cells: a decrease of ATP production, maximal and space respiratory capacity, and proton leak (Fig. 7C and D). However, when we used the combined treatment, the effects were more prominent compared to a single compound and in LoVo cells STH induced more effects compared to 5-FU (Fig. 7C and D).

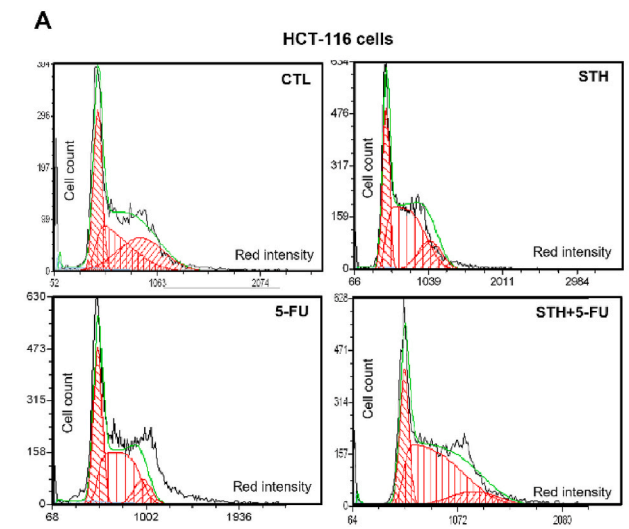
We checked whether STH and/or 5-FU might have any impact also on glycolysis. In HCT-116 cells, the treatment with STH and 5-FU decreased the glycolysis, glycolysis capacity and reserve and in the presence of STH+5-FU, similar effects were observed (Fig. 7E). Furthermore, in LoVo cells, the STH+5-FU treatment induced more effects compared to STH and 5-FU alone (Fig. 7F).

3.9. STH potentiates the anti-metastatic activity of 5-FU

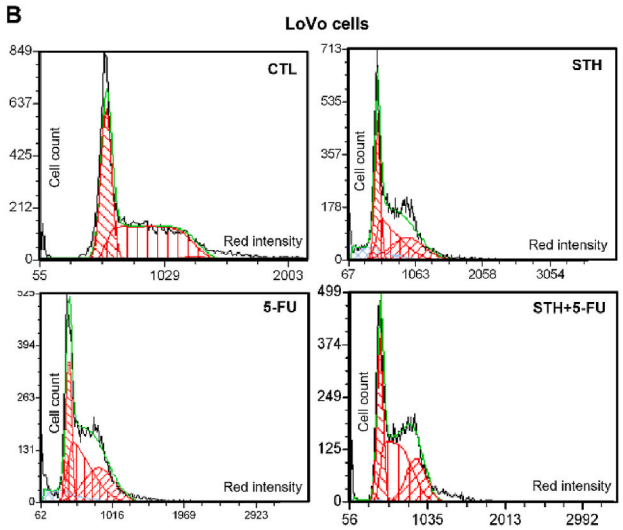
The anti-migratory effects of STH and/or 5-FU were performed by wound scratch assay. After 48 h of incubation, in HCT-116 cells, STH and 5-FU decreased wound closer by 44% and 60%, respectively, while combined treatment decreased 62% (Fig. 8A).

Furthermore, in LoVo cells, STH and 5-FU reduced wound closer around 60% and 57%, whereas exposure to STH+5-FU remarkably decreased 67% (Fig. 8B).

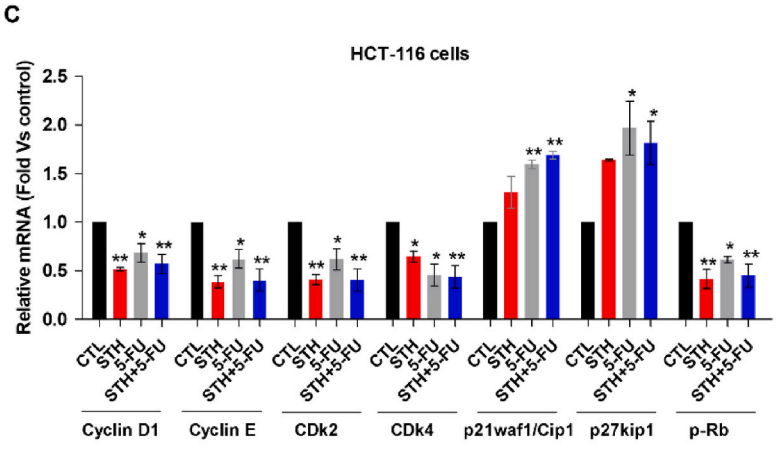
In order to further investigate the molecular mechanisms by which STH and/or 5-FU induced anti-migratory effects, we observed the protein expression of invasive (MMP-2 and MMP-9) and epithelial–mesenchymal transition EMT (E-cadherin, N-cadherin and β-catenin) markers in HCT-116 and LoVo cells (Fig. 8C and D). The combined treatment significantly suppressed the expression of MMP-2



Treatment	G1 phase	S phase	G2/M phase
CTL	31.10±2.81	33.92±1.88	34.98±2.65
STH	28.23±2.20	54.61±2.22	17.17±4.47
5-FU	30.74±2.01	50.01±1.87	12.26±3.13
STH+5-FU	24.24±2.61	61.31±2.19	14.45±2.83



Treatment	G1 phase	S phase	G2/M phase
CTL	39.89±7.48	51.54±2.24	6.11±2.57
STH	32.76±2.50	37.03±7.48	24.47±4.26
5-FU	28.60±2.92	42.93±2.01	30.21±7.48
STH+5-FU	24.98±3.92	41.13±2.01	31.17±3.21



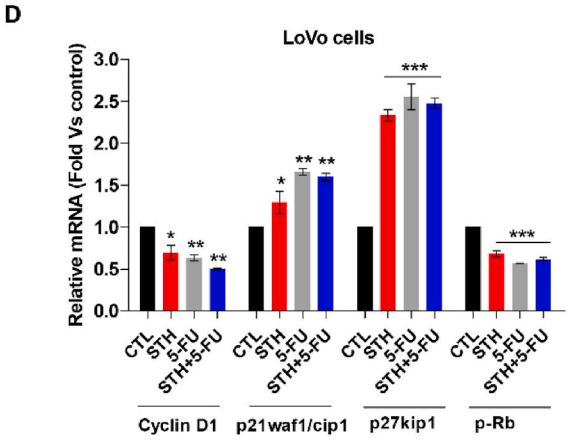


Fig. 4. Cell cycle analysis after the treatment with STH and 5-FU alone or in combination. Images represent an example of the response of (A) HCT-116 and (B) LoVo cells and percentage in each cell cycle phase G1, S and G2/M is presented in the table. The mRNA levels of cyclin D1, cyclin E, CDK2, CDK4, p21Cip, p27Kip and p-Rb were analyzed by RT-PCR and the data were normalized by GADPH mRNA levels in (C) HCT-116 and (D) LoVo cells. Results are expressed as fold change compared to control cells. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs.* control group.

and MMP-9 compared to STH and 5-FU alone, and in both cancer cells similar trend was observed (Fig. 8C and D). The expression of E-cadherin was significantly increased after each treatment, whereas in HCT-116 cells the combined treatment induced more effects (Fig. 8C), and in LoVo cells STH, 5-FU and STH+5-FU induced similar effects, respectively compared to control (Fig. 8D). Furthermore, N-cadherin expression was suppressed in both colon cancer cells and all the treatments induced similar effects, with more efficacy in LoVo cells. Similarly, the expression of β-catenin was suppressed in both cells: in HCT-116 cells the 5-FU and STH+5-FU induced similar effects, while in LoVo cells, 5-FU suppressed more compared to STH+5-FU (Fig. 8C and D).

4. Discussion

The effectiveness of a single chemotherapeutic agent in the treatment of CRC is partial or incomplete due to chemoresistance or adverse side effects. Therefore, the mixture of numerous chemo-sensitizers is currently employed in clinics to enhance their efficacy and concurrently overwhelm resistance and unwanted reaction (Longley et al., 2003). In our previous works (Afrin et al., 2017; Afrin et al., 2019b), we found that STH possesses several phenolic compounds, including gallic acid and kaempferol, and a good antioxidant activity; additionally, STH displayed important anti-cancer effects by modulating diverse molecular pathways (Afrin et al., 2019a, 2019b). The chemo preventive effects was accompanied by the induction of apoptosis via extrinsic (Fas L and caspase-8) and intrinsic (Bax/Bcl2, Cyto C and caspase-9) pathway, arresting cell cycles in S and G2/M phase, and modulating several cell cycle regulated genes (cyclin D1, cyclin E, CDK2, CDK4, p21Cip, p27Kip and p-RB) (Afrin et al., 2019b). Additionally, STH also induced oxidative stress by increasing intracellular ROS generation and suppressing antioxidant activities. Suppressed mitochondrial respiration and glycolysis were observed after STH treatment: all these effects could be ascribed to the high amount of bioactive compounds of SHT as reported in our previous study (Afrin et al., 2019a). In this study, we observed that STH

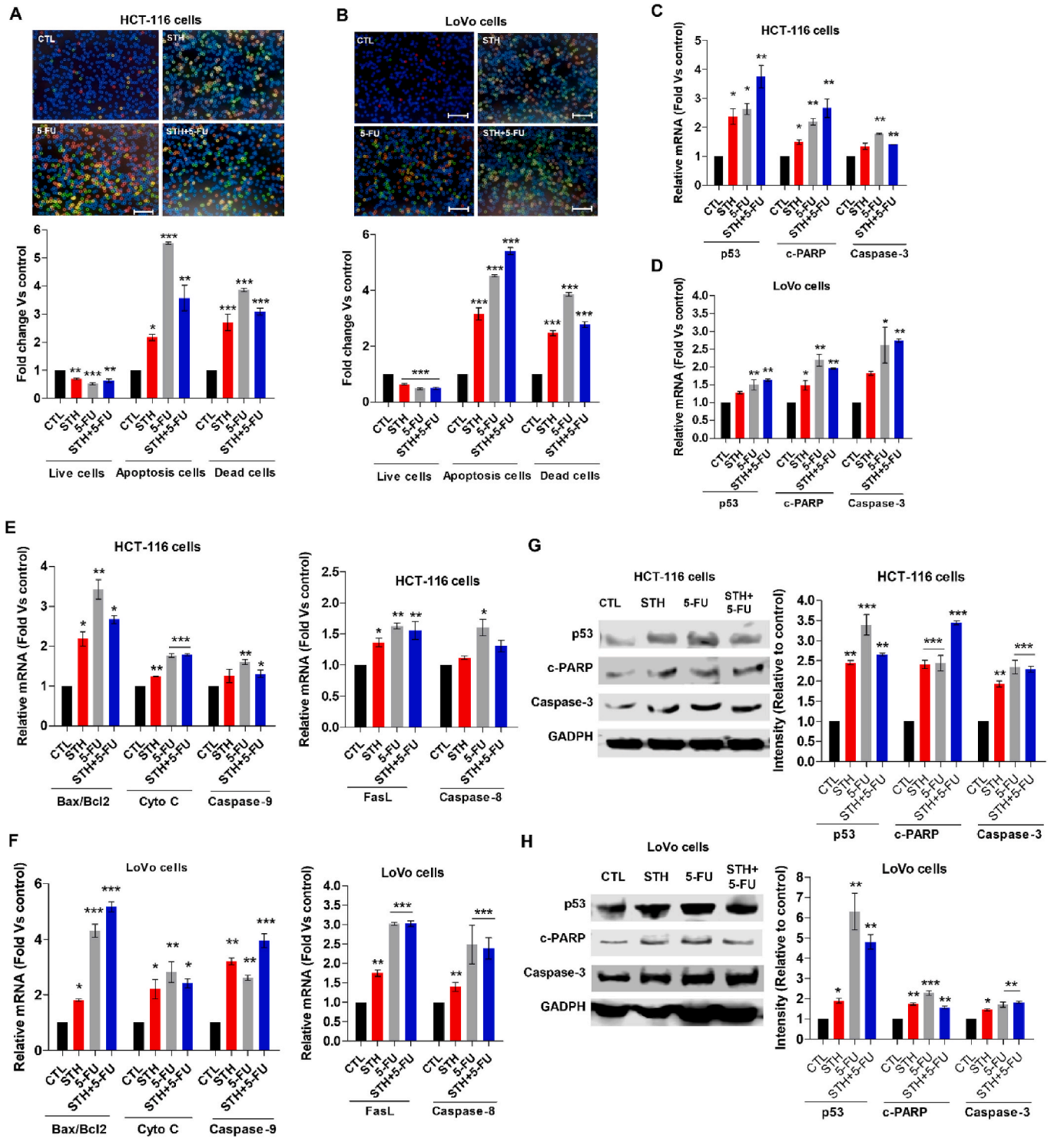


Fig. 5. Apoptosis responses after the treatment with STH and 5-FU alone or in combination. (A) HCT-116 and (B) LoVo cells apoptosis was detected by Tali™ Image-based Cytometer after Annexin V Alexa Fluor® 488 and PI staining. Fluorescence images show the apoptotic effect with or without treatment: blue colour represents live cells, green colour represents apoptotic cells and red and yellow colours represent dead cells. The mRNA levels of p53, c-PARP, caspase-3 Bax/Bcl2, Cyto C, Fas L, caspase-8 and caspase-9 were analyzed by RT-PCR and the data were normalized by GADPH mRNA levels in (C, E) HCT-116 and (D, F) LoVo cells. The protein expression of p53, c-PARP and caspase-3 were detected by western blotting (normalized to GADPH loading control) in (G) HCT-116 and (H) LoVo cells. Results are expressed as fold change compared to control cells. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs.* control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

exhibits an impressive synergistic effect, enhancing the 5-FU activities in colon cancer cells by growth reduction, suggesting that STH could reduce 5-FU doses limiting undesired reactions and drug resistance. A previous study has demonstrated that the potent inhibitory effects of 5-FU against colon cancer cells are enhanced by the presence of various polyphenols, even at very lower doses (Hakim et al., 2014). At the same time, chemo-sensitization effect of various honey, such as Manuka honey, Tulang honey and Gelam honey are already investigated in rat

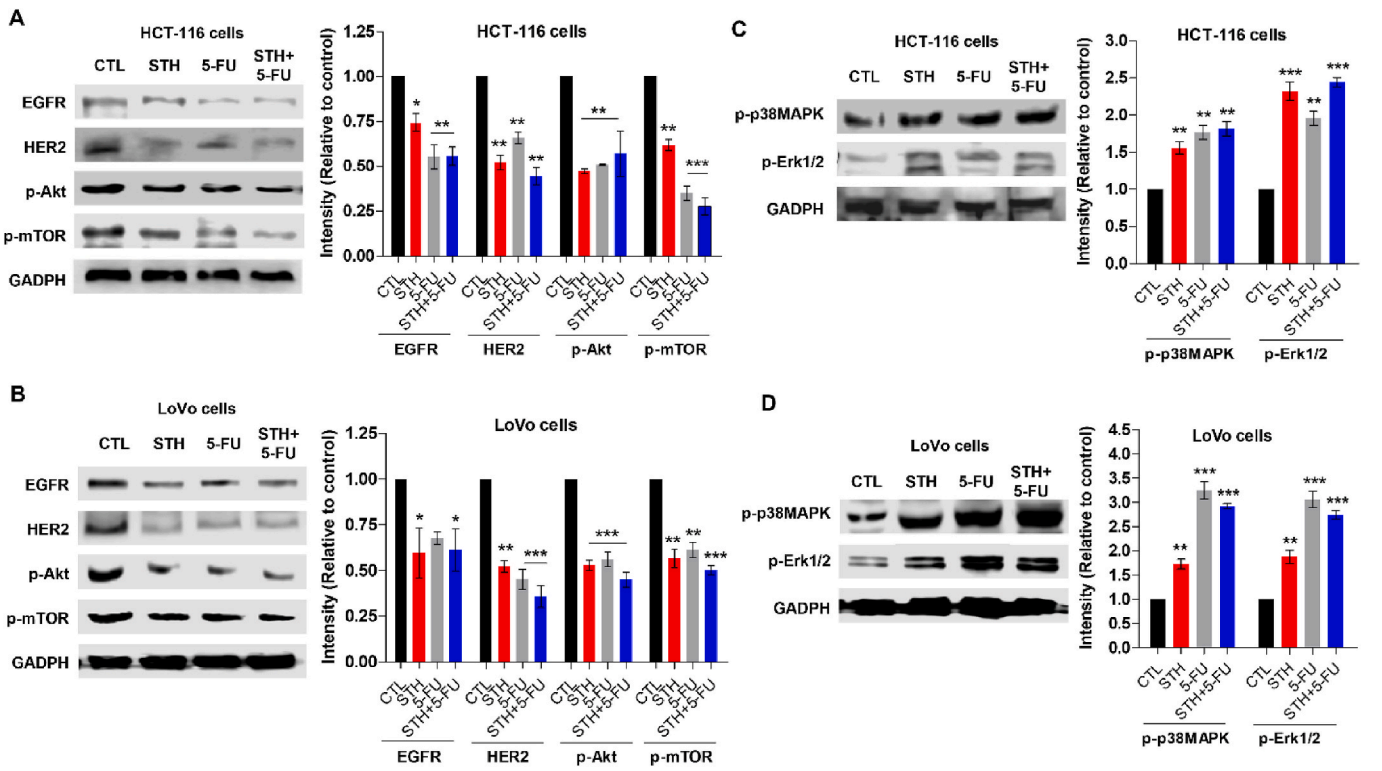


Fig. 6. STH enhances the antitumor activity of 5-FU through regulating EGFR and MAPK signaling. HCT-116 and LoVo cells were treated with STH and 5-FU alone or in combination. The protein expressions of EGFR, HER2, p-AKT, p-mTOR, p-p38 MAPK and p-ERK1/2 were detected by western blotting (normalized to GADPH loading control) in (A, C) HCT-116 and (B, D) LoVo cells. Results are expressed as a fold change in comparison control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group.

models of melanoma cancer (Fernandez-Cabezudo et al., 2013) and *in vitro* models of colon cancer (Hakim et al., 2014). Overall, our results are in agreement with those obtained in previous studies, in which the IC_{50} doses of 5-FU is remarkably reduced in the presence of STH, exerting synergistic effects on cell viability assay in colon cancer HCT-116 and LoVo cells (Table 1).

Induction of oxidative stress is one of the most common therapeutic strategies of the modern drug discovery system. Elevated ROS has been used as effectors for various drugs by damaging cancer cells preferentially through injuries of cell bio-molecules (lipid, protein and DNA) and suppressing antioxidant pathways (Gorrini et al., 2013). STH suppressed HCT-116 and LoVo cells defence system, by inducing oxidative stress through ROS production and increased TBARS and PCC levels, which further decreased antioxidant enzyme expression and activity; at the same time, it induced protective effects on oxidative stress in non-cancer cells, such as human dermal fibroblasts, where it exhibited no toxic effects until 48 h of treatment up to 40 mg/mL, did not induce intracellular ROS production or any significant changes in TBARS and PCC levels, did not affect the activities of the main antioxidant enzymes and the energetic metabolism, both in term of glycolysis and mitochondrial respiration and promoted the migration ability for healing the wound area (Afrin et al., 2019a). In the present work, we found that the IC_{50} doses of STH and 5-FU induced oxidative stress, and the combined treatment was more effective in HCT-116 cells compared to LoVo cells (Figs. 1 and 2). These results are consistent with other studies that evaluated the chemo-sensitizing effects of quercetin, gypenosides and selenium on ovarian (Li et al., 2014), colon (Kong et al., 2015) and melanoma (Fan et al., 2013) carcinoma cells through triggering oxidative stress.

Enhanced Nrf2 expression also induces resistance of 5-FU and creates a favorable condition for CRC growth (X.-Q. Zhao, Zhang, Xia, Zhou and Cao, 2015). In our work, the expression of Nrf2 and other antioxidant enzymes SOD-1, CAT and HO-1 was suppressed after the treatment with STH and 5-FU; 5-FU induced less effects compared to STH, but the suppressive activity of 5-FU was increased when it was combined with STH at 2-fold less concentration (Fig. 2). In agreement with our results, several natural compounds, such as luteolin, apigenin and chrysin, suppressed Nrf2 activity and expression for enhancing the sensitivity of cancer cells to chemotherapeutic drugs in lung cancer (Tang et al., 2011) and liver cancer (Gao et al., 2013a, 2013b) cells.

Due to excess ROS, the inner mitochondrial membrane is weakened and releases Cyto C, while ER releases Ca^{2+} which activates ER stress markers for stimulating apoptotic cell death (Malhotra and Kaufman, 2007). In the present work, STH and 5-FU induced ER stress through increasing ATF-6 and XBP-1 expression, and the combined treatment was more effective in LoVo cells (Fig. 3). In colon cancer cells, quercetin and p-coumaric acid were able to stimulate ER stress cell death through elevating the expression of ATF-6, ATF-4, XBP-1, PERK, eIF2α and CHOP, and the fold changes were compared to chemotherapeutic drugs (Khan et al., 2016; Sharma et al., 2018), but still now, to the best of our knowledge, there is not any study observing the chemo-sensitizing effects of STH on ER stress.

Increasing the therapeutic efficacy of 5-FU by targeting the NF-κB represents an interesting tool for decreasing the chemoresistance. A large number of studies highlighted that natural compounds have the ability to overcome the therapeutic resistance by suppressing the expression NF-κB (Shakibaei et al., 2015). In the present work, we observed that the combined treatment was more effective in suppressing the expression of NF-κB, while similar suppressed expressions were observed for p-IκBα in both cell lines (Fig. 3).

The cytotoxic effects exerted by 5-FU is associated with cell cycle arrest and apoptosis induction (Longley et al., 2003). The exposure to STH and 5-FU induced S phase arrest in HCT-116 cells and G2/M phase arrest in LoVo cells (Fig. 4). In HCT-116 cells, STH induced more effects compared to 5-FU, while in LoVo cells 5-FU was more effective. Furthermore, the arresting ability of 5-FU was increased when it was

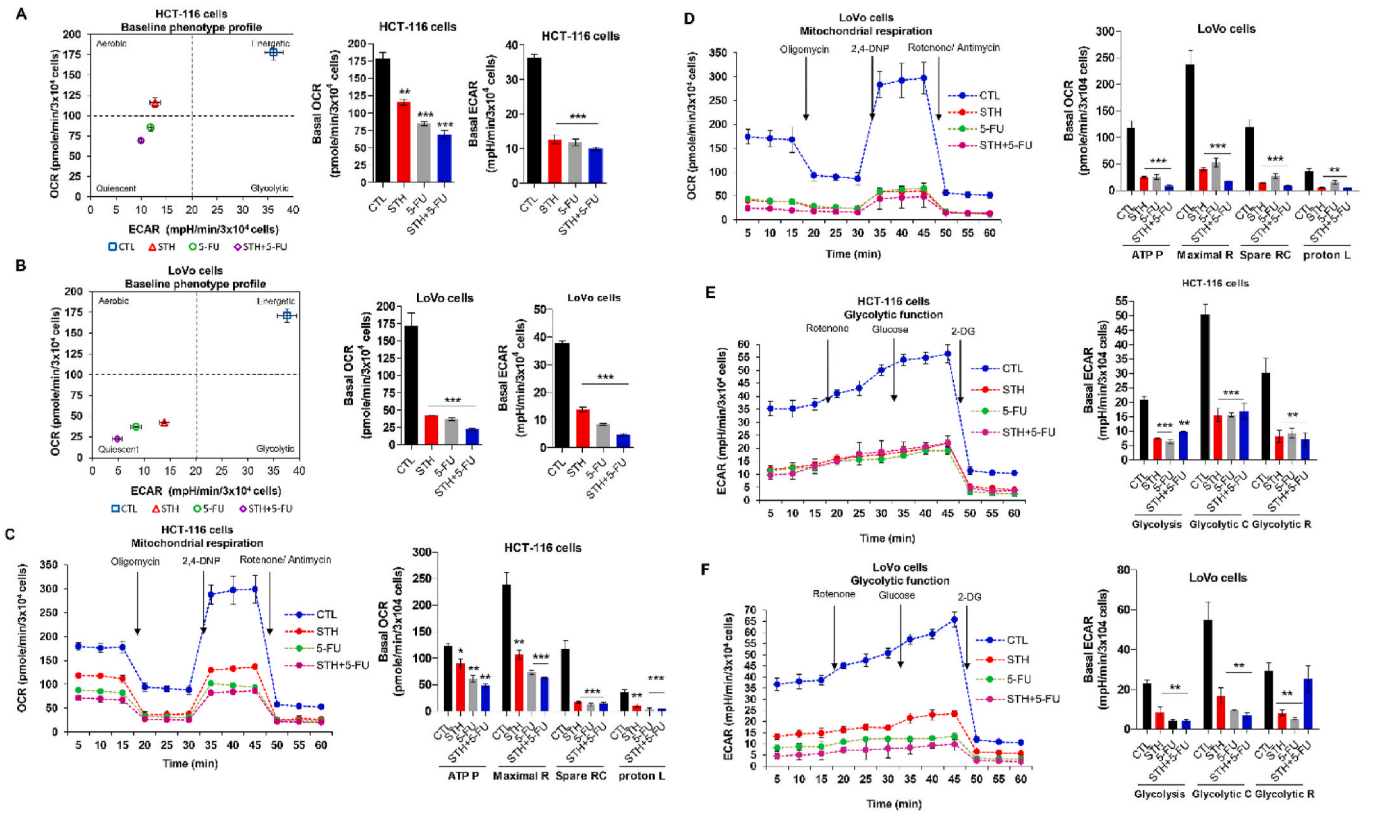


Fig. 7. STH potentiates the anti-metastatic activity of 5-FU in colon cancer cells. HCT-116 and LoVo cells were treated with STH and 5-FU alone or in combination. The ratio between basal OCR and basal ECAR showed the metabolic phenotype in (A) HCT-116 and (B) LoVo cells. OCR was determined by sequential injections of oligomycin, 2,4-DNP, and rotenone/antimycin in (C) HCT-116 and (D) LoVo cells. ATP production (ATP p), maximal respiration capacity (Maximal R), spare respiration capacity (Spare RC) and proton leak (proton L) were calculated from the XF cell Mito stress test profile. ECAR was determined by sequential injections of rotenone, glucose, and 2-DG in (E) HCT-116 and (F) LoVo cells. The parameters shown in the figure (glycolysis, glycolytic capacity and glycolytic reserves) were measured according to the kit instruction. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group.

combined with STH, at lower concentrations, in both cell lines (Fig. 4). The molecular mechanisms behind the arresting effects were also confirmed by mRNA analysis with the suppression of mRNA levels of CDK2, CDK4 and cyclin E after STH+5-FU treatment in HCT-116 cells, and with the increase of p21Cip and p27Kip and decrease of cyclin D1 and p-Rb in both cancer cells (Fig. 4). Only a few studies have demonstrated that the natural compounds have the ability to enhance the chemo-sanitizing effects of 5-FU by arresting the cell cycle trough modulating different molecular pathways in colon cancer cells (González-Sarrías et al., 2015; Kong et al., 2015). For example, in colon SW-480 cancer cells, gypenosides enhanced the 5-FU augmented G1 to S phase cell cycle arrest whereas suppressed cyclin E and CDK2 expression (Kong et al., 2015). Furthermore, the metabolites of ellagic acid and 5-FU arrested the cell cycle at S and G2/M phase in a panel of colon cancer cells, and more prominent effects were observed after the combined treatment of these compounds (González-Sarrías et al., 2015).

The intrinsic and the extrinsic apoptotic pathways are the two major pathways involved in the activation of caspases and protease cascade. Flow-cytometry analysis revealed the apoptotic induction after the treatment of STH and 5-FU alone or in combination in both cancer cells with the combined effect more prominent in LoVo cells (Fig. 5). All the treatments remarkably increased both the mRNA and protein expression of the main apoptotic markers p53, c-PARP and caspase-3, with the combined treatments inducing more expressions (Fig. 5). Additionally, the present study also confirmed that STH and/or 5-FU triggered apoptosis in HCT-116 and LoVo cells through intrinsic (Bax/Bcle, Cyto C and caspase-9) and extrinsic (Fas L and caspase-8) apoptotic pathways (Fig. 5). Our results are reliable with previous findings that confirmed that natural bioactive compounds increased the therapeutic efficacy of anti-cancer drugs through activating apoptosis by targeting different molecular pathways, such as p53, caspase-3,-8, -9, Bax, PARP in diverse CRC cells, as demonstrated for quercetin (Xavier et al., 2011), ellagic acid (González-Sarrías et al., 2015), gypenosides (Kong et al., 2015), curcumin (Shakibaei et al., 2015), among other compounds.

The EGFR/HER2 and PI3K-Akt-mTOR pathways are vital targets for colon cancer growth and survival and are correlated with 5-FU chemoresistance (Misale et al., 2012). The expression of EGFR, HER2, p-Akt and p-mTOR were suppressed after the treatment of STH and 5-FU in both cancer cells; the combined treatment induced more or similar effects (Fig. 6). Our results are in agreement with the previous finding that highlighted that polyphenols augmented the effects of 5-FU by suppressing phosphorylation of EGFR, HER2, IGR-1R, PI3K-Akt-mTOR in HCT-116, HT-29 and SW-480 colon cancer cells and $FC^{1}3K^{1}$ApcMin mouse model of CRC (Patel et al., 2010).

MAPKs pathway plays an important role in regulating the mitochondrial related proteins, such as p38 MAPK, ERK1/2 and JNK and recent evidence displays the potential relationship between oxidative stress and MAPKs in apoptosis (Guo et al., 2016). In the present study, we found a significant increase of p-p38 MAPK and p-ERK1/2 in HCT-116 and LoVo cells after 5-FU treatment (Fig. 6). Furthermore, in the presence of STH, 5-FU induced similar effects at lower concentrations in HCT-116 cells and slightly less effects in LoVo cells (Fig. 6). These results are consistent with previous studies that examined the chemo-sensitizing capacity of other polyphenols in elevating the expressions of p-p38 MAPK, p-ERK1/2 as well as p-JNK in different cancer models, such as epigallocatechin-3-gallate in hepatoma cancer cells (Suganuma et al., 2006) and resveratrol in colon cancer (Mohapatra et al., 2011) cells. It has also been evaluated that the activated MAPK

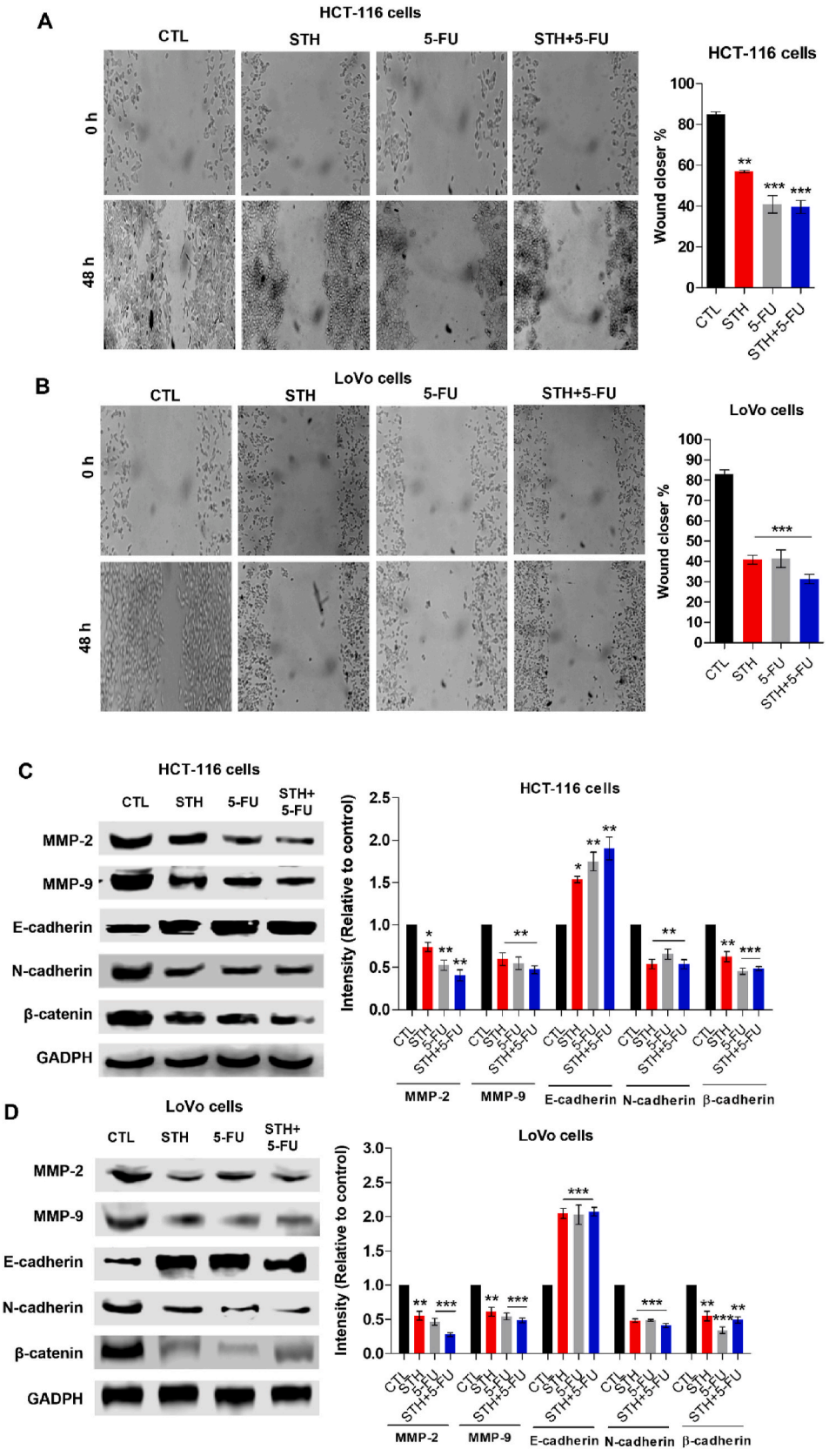


Fig. 8. STH potentiates the anti-metastatic activity of 5-FU in colon cancer cells. HCT-116 and LoVo cells were treated with STH and 5-FU alone or in combination. The wound closure percentages were analyzed by Image J software in (A) HCT-116 and (B) LoVo cells. The results were expressed as % compared to control. The protein expression of MMP-2, MMP-9, E-cadherin, N-cadherin and β-catenin were detected by western blotting (normalized to GADPH loading control) in (C) HCT-116 and (D) LoVo cells. Results are expressed as a fold change in comparison control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group.

pathway alters the pro and/or anti-apoptotic markers (Bcl2 and Bax) which translocate to mitochondria and are responsible for the generation of ROS, leading to the activation of mitochondria-dependent apoptosis (Guo et al., 2016).

Metabolic phenotype is one of the hallmarks of the modern drug discovery system, since uncontrolled metabolism is connected with therapeutic resistance (Zhao et al., 2013). Chemoresistant proliferative cancer cells maintain their required energy levels through oxidative phosphorylation (Denise et al., 2015) or higher aerobic glycolysis (Zhou et al., 2012). Previously, we found that STH had a significant impact on HCT-116 and LoVo cells metabolism by decreasing mitochondrial respiration and glycolysis (Afrin et al., 2019a). In the present work, STH enhanced the therapeutic efficacy of 5-FU at 2-fold lower concentration by switching quiescent phenotype from energetic phenotype (Fig. 7). Additionally, the combined treatment was more efficient in reducing mitochondrial respiration and glycolysis, especially in LoVo cells (Fig. 7). Moreover, no metabolic changes were observed in non-cancer cells after exposure to STH (Afrin et al., 2019b). Overall, these results suggested that STH has a significant chemo-sensitizing impact on colon cancer cells metabolism.

The anti-metastatic effects by reducing cell migration, invasion and EMT markers expression has been well studied for several natural food stuffs or isolated polyphenols but the chemo-sensitizing effects on metastatic mechanisms are still elusive. In the present report, 5-FU decreased wound closer percentage, and the suppressing activity increased when combined with STH in LoVo cells (Fig. 8). Moreover, the combination of STH and 5-FU treatment suppressed the MMP-2, MMP-9, N-cadherin and β-catenin and elevated N-cadherin expression in both cell lines (Fig. 8). Similar effects were observed when standard chemotherapy was combined with phytochemicals by inducing anti-metastatic effects through suppressing the MMP-9, MMP-13 and EMT markers in colon cancer cells (Shakibaei et al., 2015).

5. Conclusions

The improvement of chemo-sensitizing effects of 5-FU by natural compounds in colorectal cancer has been investigated. The present study demonstrated that STH enhances the 5-FU effect in colon cancer cells and could lead to fewer effective doses and consequently lower side effects. The molecular mechanisms were associated with the suppression of proliferative markers EGFR, HER2, p-Akt and p-mTOR expression; elevated p-p38 MAPK and p-ERK1/2 were observed for apoptosis induction. STH enhanced 5-FU induced cell death by oxidative stress by generating ROS and decreasing antioxidant enzyme defence system. The arrest in cell cycle and the induction of apoptosis were also confirmed after the combined treatment through the regulation of cell cycle genes and mitochondrial-dependent and non-dependent apoptotic markers. The metabolic phenotypes were significantly altered after the combination treatment and suppressed migration, invasion and EMT were also shown. Overall, this study highlights a new opportunity of using a combined treatment of 5-FU and STH for the treatment of colon cancer, which needs to be confirmed in animal models to discover the possible chemo-sensitizing effects of STH. However, our work has some limitations, including the evaluation of the chemosensitivity with only one type of honey or only on *in vitro* model, the complete lack of the assessment of the honey polyphenols stability in culture media or of the metabolic transformation, highlighting the necessity to validate our results with different approaches by using both *in vitro* and *in vivo* models.

CRediT authorship contribution statement

Sadia Afrin: Conceptualization, Formal analysis, Writing – original draft. **Francesca Giampieri:** Conceptualization, Formal analysis, Writing – original draft. **Danila Cianciosi:** Formal analysis. **José M. Alvarez-Suarez:** Data curation, Visualization. **Beatriz Bullon:** Investigation. **Adolfo Amici:** Formal analysis, Investigation. **Josè L. Quiles:** Visualization. **Tamara Y. Forbes-Hernández:** Writing – review & editing, Supervision. **Maurizio Battino:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.112484.

Conflict of interest

The authors declare no conflicts of interest.

References

Afrin, S., Forbes-Hernández, T.Y., Cianciosi, D., Pistollato, F., Zhang, J., Pacetti, M., et al., 2019a. Strawberry Tree honey as a new potential functional food. Part 2: strawberry tree honey increases ROS generation by suppressing Nrf2-ARE and NF-κB signaling pathways and decreases metabolic phenotypes and metastatic activity in colon cancer cells. J. Funct. Foods 57, 477–487.

Afrin, S., Forbes-Hernandez, T.Y., Gasparrini, M., Bompadre, S., Quiles, J.L., Sanna, G., et al., 2017. Strawberry-tree honey induces growth inhibition of human colon cancer cells and increases ROS generation: a comparison with Manuka honey. Int. J. Mol. Sci. 18 (3), 613.

Afrin, S., Giampieri, F., Cianciosi, D., Pistollato, F., Ansary, J., Pacetti, M., et al., 2019b. Strawberry Tree honey as a new potential functional food. Part 1: strawberry tree honey reduces colon cancer cell proliferation and colony formation ability, inhibits cell cycle and promotes apoptosis by regulating EGFR and MAPKs signaling pathways. J. Funct. Foods 57, 439–452.

Afrin, S., Giampieri, F., Forbes-Hernandez, T.Y., Gasparrini, M., Amici, A., Cianciosi, D., et al., 2018. Manuka honey synergistically enhances the chemopreventive effect of 5-fluorouracil on human colon cancer cells by inducing oxidative stress and apoptosis, altering metabolic phenotypes and suppressing metastasis ability. Free Radic. Biol. Med. 126, 41–54.

Battino, M., Giampieri, F., Cianciosi, D., Ansary, J., Chen, X., Zhang, D., et al., 2021. The roles of strawberry and honey phytochemicals on human health: a possible clue on the molecular mechanisms involved in the prevention of oxidative stress and inflammation. Phytomedicine 86, 153170.

Cianciosi, D., Forbes-Hernández, T.Y., Ansary, J., Gil, E., Amici, A., Bompadre, S., et al., 2020. Phenolic compounds from Mediterranean foods as nutraceutical tools for the prevention of cancer: the effect of honey polyphenols on colorectal cancer stem-like cells from spheroids. Food Chem. 325, 126881.

Denise, C., Paoli, P., Calvani, M., Taddei, M.L., Giannoni, E., Kopetz, S., 2015. 5-Fluorouracil resistant colon cancer cells are addicted to OXPHOS to survive and enhance stem-like traits. Oncotarget 6 (39), 41706.

Fan, C., Chen, J., Wang, Y., Wong, Y.-S., Zhang, Y., Zheng, W., 2013. Selenocystine potentiates cancer cell apoptosis induced by 5-fluorouracil by triggering reactive oxygen species-mediated DNA damage and inactivation of the ERK pathway. Free Radic. Biol. Med. 65, 305–316.

Fernandez-Cabezudo, M.J., El-Kharrag, R., Torab, F., Bashir, G., George, J.A., El-Taji, H., 2013. Intravenous administration of manuka honey inhibits tumor growth and improves host survival when used in combination with chemotherapy in a melanoma mouse model. PloS One 8 (2), e55993.

Gao, A.-M., Ke, Z.-P., Shi, F., Sun, G.-C., Chen, H., 2013a. Chrysin enhances sensitivity of BEL-7402/ADM cells to doxorubicin by suppressing PI3K/Akt/Nrf2 and ERK/Nrf2 pathway. Chem. Biol. Interact. 206 (1), 100–108.

Gao, A.-M., Ke, Z.-P., Wang, J.-N., Yang, J.-Y., Chen, S.-Y., Chen, H., 2013b. Apigenin sensitizes doxorubicin-resistant hepatocellular carcinoma BEL-7402/ADM cells to doxorubicin via inhibiting PI3K/Akt/Nrf2 pathway. Carcinogenesis 34 (8), 1806–1814.

Giampieri, F., Alvarez-Suarez, J.M., Cordero, M.D., Gasparrini, M., Forbes-Hernandez, T. Y., Afrin, S., et al., 2017. Strawberry consumption improves aging-associated

impairments, mitochondrial biogenesis and functionality through the AMP-activated protein kinase signaling cascade. Food Chem. 234, 464–471.
González-Sarrías, A., Tomé-Carneiro, J., Bellesia, A., Tomás-Barberán, F.A., Espín, J.C., 2015. The ellagic acid-derived gut microbiota metabolite, urolithin A, potentiates the anticancer effects of 5-fluorouracil chemotherapy on human colon cancer cells. Food Funct 6 (5), 1460–1469.
González-Vallinas, M., Molina, S., Vicente, G., de la Cueva, A., Vargas, T., Santoyo, S., 2013. Antitumor effect of 5-fluorouracil is enhanced by rosemary extract in both drug sensitive and resistant colon cancer cells. Pharmacol. Res. 72, 61–68.
Gorrini, C., Harris, I.S., Mak, T.W., 2013. Modulation of oxidative stress as an anticancer strategy. Nat. Rev. Drug Discov. 12 (12), 931.
Guo, C., Yang, M., Jing, L., Wang, J., Yu, Y., Li, Y., 2016. Amorphous silica nanoparticles trigger vascular endothelial cell injury through apoptosis and autophagy via reactive oxygen species-mediated MAPK/Bcl-2 and PI3K/Akt/mTOR signaling. Int. J. Nanomed. 11, 5257–5276.
Hakim, L., Alias, E., Makpol, S., Ngah, W.Z.W., Morad, N.A., Yusof, Y.A., 2014. Gelam honey and ginger potentiate the anti cancer effect of 5-FU against HCT 116 colorectal cancer cells. Asian Pac. J. Cancer Prev. APJCP 15 (11), 4651–4657.
Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Thun, M.J., 2009. Cancer statistics, 2009. CA Cancer J. Clin. 59 (4), 225–249.
Khan, I., Paul, S., Jakhar, R., Bhardwaj, M., Han, J., Kang, S.C., 2016. Novel quercetin derivative TEF induces ER stress and mitochondria-mediated apoptosis in human colon cancer HCT-116 cells. Biomed. Pharmacother. 84, 789–799.
Kong, L., Wang, X., Zhang, K., Yuan, W., Yang, Q., Fan, J., 2015. Gypenosides synergistically enhances the anti-tumor effect of 5-fluorouracil on colorectal cancer in vitro and in vivo: a role for oxidative stress-mediated DNA damage and p53 activation. PloS One 10 (9), e0137888.
Lee, K.W., Bode, A.M., Dong, Z., 2011. Molecular targets of phytochemicals for cancer prevention. Nat. Rev. Canc. 11 (3), 211–218.
Li, N., Sun, C., Zhou, B., Xing, H., Ma, D., Chen, G., 2014. Low concentration of quercetin antagonizes the cytotoxic effects of anti-neoplastic drugs in ovarian cancer. PloS One 9 (7), e100314.
Liu, R.H., 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J. Nutr. 134 (12), 3479S–3485S.
Longley, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Canc. 3 (5), 330.
Malhotra, J.D., Kaufman, R.J., 2007. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxidants Redox Signal. 9 (12), 2277–2294.
Misale, S., Yaeger, R., Hobor, S., Scala, E., Janakiraman, M., Liska, D., 2012. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. Nature 486 (7404), 532.
Mohapatra, P., Preet, R., Choudhuri, M., Choudhuri, T., Kundu, C.N., 2011. 5-fluorouracil increases the chemopreventive potentials of resveratrol through DNA damage and MAPK signaling pathway in human colorectal cancer cells. Oncol. Res. 19 (7), 311–321.
Núñez-Sánchez, M.A., González-Sarrías, A., Romo-Vaquero, M., García-Villalba, R., Selma, M.V., Tomás-Barberán, F.A., 2015. Dietary phenolics against colorectal cancer—from promising preclinical results to poor translation into clinical trials: pitfalls and future needs. Mol. Nutr. Food Res. 59 (7), 1274–1291.
Patel, B.B., Gupta, D., Elliott, A.A., Sengupta, V., Yu, Y., Majumdar, A.P.N., 2010. Curcumin targets FOLFOX-surviving colon cancer cells via inhibition of EGFRs and IGF-1R. Anticancer Res. 30 (2), 319–325.
Schmoll, H.J., Van Cutsem, E., Stein, A., Valentini, V., Glimelius, B., Haustermans, K., 2012. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. Ann. Oncol. 23 (10), 2479–2516.
Shakibaei, M., Kraehe, P., Popper, B., Shayan, P., Goel, A., Buhrmann, C., 2015. Curcumin potentiates antitumor activity of 5-fluorouracil in a 3D alginate tumor microenvironment of colorectal cancer. BMC Canc. 15 (1), 250.
Sharma, S.H., Rajamanickam, V., Nagarajan, S., 2018. Antiproliferative effect of p-Coumaric acid targets UPR activation by downregulating Grp78 in colon cancer. Chem. Biol. Interact. 291, 16–28.
Suganuma, M., Kurusu, M., Suzuki, K., Tasaki, E., Fujiki, H., 2006. Green tea polyphenol stimulates cancer preventive effects of celecoxib in human lung cancer cells by upregulation of GADD153 gene. Int. J. Canc. 119 (1), 33–40.
Tang, X., Wang, H., Fan, L., Wu, X., Xin, A., Ren, H., 2011. Luteolin inhibits Nrf2 leading to negative regulation of the Nrf2/ARE pathway and sensitization of human lung carcinoma A549 cells to therapeutic drugs. Free Radic. Biol. Med. 50 (11), 1599–1609.
Xavier, C.P.R., Lima, C.F., Rohde, M., Pereira-Wilson, C., 2011. Quercetin enhances 5-fluorouracil-induced apoptosis in MSI colorectal cancer cells through p53 modulation. Canc. Chemother. Pharmacol. 68 (6), 1449–1457.
Zhao, X.-Q., Zhang, Y.-F., Xia, Y.-F., Zhou, Z.-M., Cao, Y.-Q., 2015. Promoter demethylation of nuclear factor-erythroid 2-related factor 2 gene in drug-resistant colon cancer cells. Oncol. Lett. 10 (3), 1287–1292.
Zhao, Y., Butler, E.B., Tan, M., 2013. Targeting cellular metabolism to improve cancer therapeutics. Cell Death Dis. 4 (3), e532.
Zhou, Y., Tozzi, F., Chen, J., Fan, F., Xia, L., Wang, J., 2012. Intracellular ATP levels are a pivotal determinant of chemoresistance in colon cancer cells. Canc. Res. 72 (1), 304–314.