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Manuka honey's anti-metastatic impact on colon Cancer Stem-Like Cells: unveiling its effects on epithelial-mesenchymal transition, angiogenesis and telomere length

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Colorectal cancer often leads to metastasis, with cancer stem cells (CSCs) playing a pivotal role in this process. Two closely linked mechanisms, epithelial-mesenchymal transition and angiogenesis, contribute to metastasis and recent research has also highlighted the impact of telomere replication on this harmful tumor progression. Standard chemotherapy alone can inadvertently promote drug-resistant CSCs, posing a challenge. Combining chemotherapy with other compounds, including natural ones, shows promise in enhancing effectiveness while minimizing side effects. This study investigated the anti-metastatic potential of Manuka honey, both alone and in combination with 5-Fluorouracil, using a 3D model of colon spheres enriched with CSCs-like cells. In summary, it was observed that the treatment reduced migration ability by downregulating the transcription factors Slug, Snail, and Twist, which are key players in epithelial-mesenchymal transition. Additionally, Manuka honey downregulated pro-angiogenic factors and shortened CSC telomeres by downregulating c-Myc, demonstrating an effective anti-metastatic potential. This study suggests new research opportunities for studying the impact of natural compounds when combined with pharmaceuticals, with the potential to enhance effectiveness and reduce side effects.

Introduction

One of the distinctive features of colorectal cancer (CRC), which ranks in 2022 as the second leading cause of cancer-related mortality (with 904,019 deaths) and the third in terms of global incidence (1,926,425 new cases), is its significant propensity to develop metastases, particularly in the liver, lungs, and peritoneum. [1] . Up to 80% of CRC cases have the potential to become metastatic. However, in practice, about 50% of all cases reach this stage, with approximately 20% being metastatic at the initial diagnosis. The presence of metastases accounts for more than 90% of cancer-related deaths.[2] Several factors responsible for the manifestation of metastases have been identified, among which the presence of cancer stem cells (CSCs) in the tumor mass plays a fundamental role. These cells are highly tumorigenic and possess a marked ability for self-renewal, making them the only cells capable of directing tumorigenesis at distant sites. Additionally, their high resistance to chemotherapy and radiotherapy contributes to their critical role in metastasis.[3]

Closely associated with this phenomenon there are two mechanisms that contribute significantly to the presence of metastases: epithelial-mesenchymal transition (EMT) and angiogenesis.[4] Despite this, most of the mechanisms behind the metastasis process remain unknown. Recent research has shown that telomere replication and telomerase activity can also influence this detrimental tumor process..[5] Frequently, standard chemotherapy can be a double-edged sword due to its potential to inadvertently select for drug-resistant cells, known as CSCs. These, with their high survival rate, proliferation and dissemination capacity in distant organs, are able to induce the metastatic process above all through the activation of the EMT.[6] Furthermore, another important aspect to consider is that very often it has been observed that the use of these standard chemotherapeutics supports the angiogenesis process through the overexpression of vascular endothelial growth factors (VEGF) family members, creating a favorable environment for tumor dissemination through the bloodstream. [7]

There are a lot of scientific evidences confirming that the epithelial-mesenchymal transition (EMT) has a fundamental and complex role in CRC, as it is the mainly responsible for the presence of metastases and consequently for therapeutic failure and the low survival rate. EMT is defined as the process by which primary tumor cells lose their epithelial phenotype and adhesion capacity with a series of different biochemical changes by acquiring the mesenchymal phenotype, which allows them to have a higher migration and invasion capacity and greater resistance to apoptotic phenomena. These

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transformations allow the tumor cells to reach other areas in the body through the blood circulation, where they reacquire the epithelial phenotype, resuming tumor expansion. At the base of EMT there is a downregulation of Epithelial Cadherin (E-Cadherin) and an up-regulation of vimentin, whose levels are regulated by different factors including the transcription factors (TFs) Snail and Slug, belonging to the family of zinc-proteins, the basic helix-loop-helix family of TFs e.g. Twist and the distantly related zinc finger E-box-binding homeobox family proteins e.g. zinc-finger E homeobox-binding (Zeb). [8] In addition to these factors in the EMT process, different pathways are also involved, including three pathways (Wnt/β-catenin, Hedgehog (Hh), Notch) that are closely correlated with the self-renewal capacity of CSCs, confirming a responsibility of these cell subpopulation in the phenomenon of EMT in CRC.[9]

Angiogenesis is a crucial process both in the growth of the primary tumor and in the mechanism of metastasis. In fact, in this last process, in addition to providing nutrients and oxygen as for the primary tumor, it would also seem to contribute to the infiltration process of cells in the blood vessels, helping tumor dissemination in other districts. In CRC different growth factors have been identified which amental role in angiogenesis: among these the most studied are the VEGF family members, which are also widely used clinically as markers of metastatic risk in CRC and its progression.[10]

Telomeres are structures of both DNA and proteins situated at the tips of chromosomes. Their primary role is to safeguard the chromosomal DNA from fusion and deterioration caused by exonucleases and ligases. They also play a crucial role in regulating the identification and separation of chromosomes during mitosis, as well as anchoring the chromosomes to the cellular machinery within the nucleus. This anchoring aids in the efficient replication of DNA during the different phases of both the mitotic and meiotic cell cycles.[11]

Telomerase activity analysis could have diagnostic and prognostic utility in the context of malignant tumors. In fact, numerous studies have examined the telomerase activity of tumor samples of different types, comparing it in most cases with that of normal or non-neoplastic tissue. The results suggested that telomerase could be useful as a diagnostic marker of malignant neoplastic diseases. Unlike most normal somatic cells, telomerase activity was detected in about 90% of the tumors examined.[12] Analysing some of the most common cancers, one study initially identified telomerase activity in 89% of CRC, 88% of breast cancers, 78% of non-small cell lung cancers, and 100% of small cell lung carcinomas and this greater and elevated activity was then confirmed over the years. Numerous hopes have been placed on telomerase as a new target for anti-cancer drugs. The idea is that the selective inhibition of telomerase in telomerase-positive neoplasms could lead to the eventual degradation of chromosomal ends with consequent shortening of these up to a critical length which could lead to replicative senescence and cell death due to chromosomal damage.[13] However, much remains to be investigated in this area, as the relationship between telomere length/telomerase activity and cancer is not yet fully understood.

In conclusion, better understanding the main processes behind metastasis and looking for new strategies, including natural ones, that can support the effect of standard chemotherapy could chemosensitize those cells that are intrinsically resistant to drugs: the CSCs. These are in fact believed to be partly responsible for those phenomena certainly linked to metastasis such as EMT and angiogenesis as well as having a particular behaviour with regard to the length of telomeres.

In this context, the potential chemosensitizing and anti-metastatic effect of Manuka honey (MH) against 5-Fluorouracil (5-Fu), (a fluorinated pyrimidine analogue, widely used as chemotherapeutic agent for CRC) was investigated in colonspheres enriched with CSCs-like, derived from HCT-116 cell line. MH is rich in bioactive compounds (mainly quercetin, naringenin, pinocembrin, salicylic acid, and 3,4-dihydroxybenzoic acid) and has been shown in our preliminary work to have suppressive effects on colon CSC-like cells.[4] In this study, a direct effect of MH alone or in combination with 5-Fu on colon CSCs-like on (i) EMT was evaluated investigating the migration capacity and the gene expression of Slug, Snail and Twist; (ii) angiogenesis, testing the effect on 20 different pro- and anti-angiogenic proteins; (iii) telomere length and on the gene expression of the protooncogene c-Myc, strictly related with the enzymatic activity of telomerase. A comparison was also conducted using the original cells that were cultured as a monolayer, which were not enriched with CSCs-like characteristics.

Material and methods

Honey sample and treatment preparation

Manuka honey Nèctar Plus® UMF 15+ (Efit S.r.l) from New Zealand was imported to Italy and maintained at a temperature of 4°C until it underwent analysis. Prior to its application for colonsphere treatment, it underwent a series of steps: it was weighed, dissolved within the culture medium, and subsequently passed through a filtration process utilizing the MF-Millipore ™ Membrane Filter (0.45 µm).

Cells/spheroids culture and treatment

HCT-116 human colon adenocarcinoma cells (ATCC, Manassas, VA, USA) and the colonspheres enriched with CSCs-like resulting from these, were cultured and treated in the conditions extensively described in our previous article [15] and are resumed in Figure 1.

The ability to enrich the population of CSCs-like through the spheroid formation method was previously verified using the methodology described in our earlier work (data not shown). In the colonospheres, the protein expression of the surface marker of CSCs-like CD44 and of the stemness gene Nanog was evaluated through Western Blot. The results were compared with those obtained in parental cells grown in monolayer and the enrichment of the stem population was confirmed.[15]

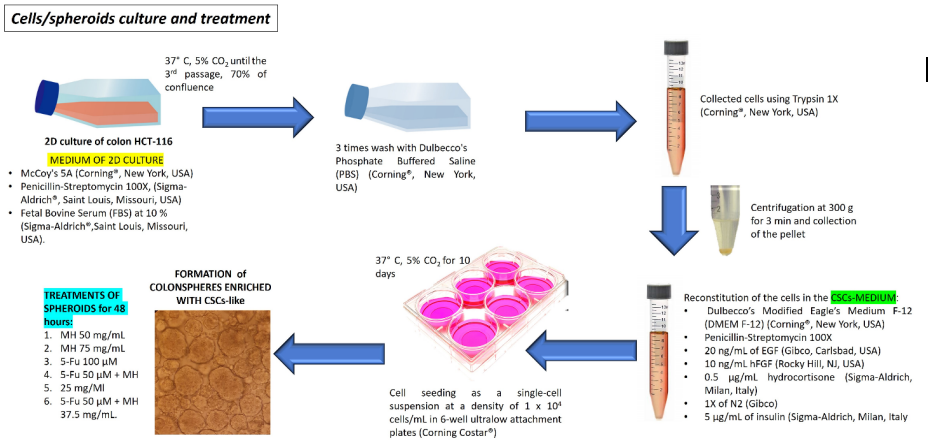


Figure 1. Cells/spheroids treatment. In this figure, the cell culture process, the formation and cultivation of cancer spheroids enriched with CSCs-like, and their subsequent treatment for further experiments are briefly summarized. FBS: fetal bovine serum; PBS: phosphate buffered saline; CSCs: cancer stem cells; MH: Manuka honey; 5-Fu: 5-Fluorouracil; EGF: epidermal growth factor; hFGF: human fibroblast growth factor.

The choice of concentrations used in this study (shown in Figure 1) was made in our previous works. [14,15] Multiparametric analyzes were carried out which allowed the evaluation of the physical and morphological effects deriving from the use of MH and/or 5-FU on colonospheres. **Preliminary microscopic analyses were conducted, followed by further analyses using specific software (ReViSP and AnaSP) [14] for spheroid analysis and measurements with a flow-based method balance** W8 (CellDynamics SRL).[15] **The minimum concentrations at which morphological and physical changes in the spheroids began to appear were selected.**

Protein array analysis

Protein array analysis was performed using Human Angiogenesis Antibody Array kits (ab134000) from Abcam®, Cambridge, UK, in accordance with the manufacturer's guidelines and was carried out across four of the six treatments employed, specifically focusing on the most illustrative ones (CTL, MH 75 mg/mL, 5-Fu 100 µM and 5-Fu 50 µM + MH 37.5 mg/mL). The membranes were visualized utilizing a chemiluminescent detection technique (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany) and the quantification of the array spots were performed by Image Studio 3 (C-DiGit Blot Scanner, LICOR, Bad Homburg, Germany), standardizing the signal was achieved by comparing it to the spots of the positive control found on each membrane as follows:

$$X(Ny)=X(y)\times P1/P(y)$$

Where:

P1= mean signal density of positive control spots on reference array

P(y)= mean signal density of positive control spots on array "y"

X(y)= mean signal density for spot "X" on array for sample "y"

X(Ny)= normalized signal intensity for spot "X" on array "y"

The results were expressed as fold increase of protein expression respect to the CTL group. All data were reported as a mean value of the two spots present in the membrane ± SD.

RNA isolation and quantitative Real-Time PCR analysis

Through PureLink® RNA mini kit (Invitrogen, Carlsbed, Ca, USA) following to the protocol, the RNA was extracted both from 2D cells (HCT-116 cell line) and from treated or not spheroids. After the extraction, the concentration and the purity of RNA were tested utilizing a microplate spectrophotometer (BioTek Sinergy HT, Winooski, USA). 5X All-in-one RT Mastermix kit (Applied Biological Materials Inc., Canada) was used for obtaining 100 ng of cDNA. For Real-time EvaGreen 2X qPCR MasterMix (Applied Biological Materials Inc., Canada) and the following froward and reverse primers (Sigma-Aldrich, Milan, Italy) were employed (Table 1):

GAPDH F :5'-GACCCCTTCATTGACCTCAACTACATG-3'

GAPDH R: 5'-GTGCACCACCCTGTTGCTGTAGCC-3'

Slug F: 5'-TGGTTGCTTCAAGGACACAT-3'

Slug R: 5'-GTTGCAGTGAGGGCAAGAA-3'

Snail F: 5'-GACCACTATGCCGCGCTCTT-3'

Snail R: 5'-TCGCTGTAGTTAGGCTTCCGATT-3'

Twist F: 5'-TTTCGGATGGGGTTGTTATC-3'

Twist R: 5'-AAACGACCTAACCCGAACG-3'

To standardize the quantitative data (comparing relative mRNA levels to the control), GAPDH was used in identical experimental conditions. For calculate the fold-change value was applied the $2^{-\Delta\Delta Ct}$ formula.

Migration assay

After 48 hours of the treatment of spheroids (with the following concentration: CTL, MH 50 mg/mL, MH 75 mg/mL, 5-Fu 100 µM, 5-

Fu 50 µM + MH 25 mg/mL and 5-Fu 50 µM + MH 37.5 mg/mL), they were mechanically disrupted using a 1000 µL pipette tip, employing gentle pipetting to separate the cells that formed the spheroids. Cell counting was performed with Tali® Image-based cytometer (Thermo Fisher Scientific, Milan, Italy) and then the cells were reseeded at a density of 1 x 10^6 cells/well in 1.5 mL of serum-free DMEM F-12 supplemented with Penicillin-Streptomycin 100X and 0.1 sterile BSA. The cellular suspension was added in the upper compartment of the transwell insert of the 6-wells plate Transwell® with 8.0 µm pore polycarbonate membrane (n.3428, Corning®, New York, USA). In the lower compartment 2.6 mL of DMEM F-12 supplemented with 10% FBS, which acted as a chemoattractant, were added. After 48 hours in incubator at 37°C with 5% CO_2 the migration capacity of the CSCs-like was evaluated; specifically migrated cells could be found in two different parts of the transwell: dropped in the media of the lower compartment or underside of the basket membrane. After removing the transwell insert from the plate and collecting the media from the lower compartment, cell counting was performed with Tali® Image-based cytometer (Thermo Fisher Scientific, Mi-lan, Italy), while for evaluating the migrated cells present in the underside of the basket's membranes, these were stained (a cell staining was performed as followed explained). Using a cotton-tipped applicator carefully the media and the cells present in the upper side of the membranes (non-migrated ones) were removed and discarded. The transwell inserts were placed in 70% EtOH for 10 minutes to allow fixation. After this time, the membranes were let dry for 15 minutes and then they were incubated for staining in 0.2% crystal violet (CV) solution (MeOH: H_2O 20:80) for 15 minutes at room temperature. The CV was removed from the top with cotton-tipped applicator very gently and after the membrane were washed with Milli-Q water for removing the excess of CV and let dry for 20 minutes (Figure 2).

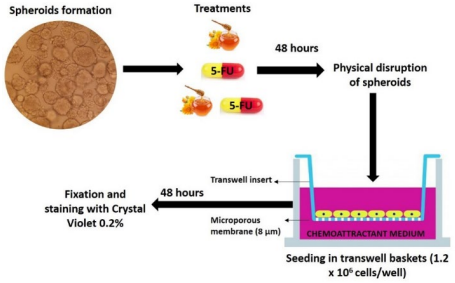


Figure 2. Schematic representation of the migration assay test. After treatment of spheroids, they were mechanically disrupted and then the cells were reseeded at a density of 1 x 10^6 cells/well. The cellular suspension was added in the upper compartment of the transwell insert. After 48 hours, the migration capacity of the CSCs-like was evaluated, using 0.2% crystal violet solution.

These membranes were observed under the inverted microscope Leitz Fluovert FU (Leica Microsystems) and the images were subjected to processing with an image editing free software (http://www.coolphptools.com/) for the evaluation of the percentage of colours present in the images, which is an estimation of migrated cells. To obtain quantitative information from the colours present in the images from microscope, the obtained images (three for each condition) were uploaded into the Internet-based Cool Hypertext Preprocessor (PHP) software tool (www.coolphptools.com/color_extract). The application software, which includes Hypertext Markup Language (HTML) embedded web scripting language, was executed setting the different options: "number of colours" set at 10 and "delta" (i.e. the gap or spacing between color shades) at 24. Based on the results of the color analysis, the hexadecimal color codes and the percentage values for each of all shades of violet present (corresponding to the migrated cells) generated by the computer were processed in Microsoft Excel.

DNA extraction and relative telomere length measurement

Genomic DNA was extracted from CSCs-like forming spheroids (treated or not) and HCT-116 grown in monolayer condition using Cells and Tissue DNA Isolation Kit (53100, Norgen Biotek Corp., Thorold, Canada) following the manufacturer's instructions. DNA purity and concentration were assessed using a microplate spectrophotometer system (BioTek Sinergy HT, Winooski, USA). The reference DNA was sequentially diluted for the construction of a standard curve (0.01-50 ng/µL). The samples were used at the concentration of 2 ng of DNA for each condition analysed and for both reactions. EvaGreen 2X qPCR Mastermix (Applied Biological Material Inc., Canada) was used for performing Real-Time PCR using the following forward (F) and reverse (R) primers (Sigma-Aldrich, Milan, Italy):

Telomere F 5'-GGTTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGT-3'

Telomere R 5'-TCCCGACTATCCCTATCCCTACCCTATCCCTATCCCTA-3'

Rplp0 F 5'-CAGCAAGTGGGAAGGTGTAATCC-3'

Rplp0 R 5'-CCCATTATATATCATCAACGGGTACAA-3'

Telomere (T) primers F was used at the final concentration of 270 nM and of 900 nM for TR. For the single (S) gene Rplp0 F was used the final concentration of 300 nM, for Rplp0 R that one of 500 nM. For telomere amplification there were used the following conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 58°C for 1 minutes. The Real-Time PCR reaction for Rplp0 consisted of 95°C for 5 minutes, 30 cycles of 95°C for 15 seconds and 54°C for 2 minutes. At the end of reactions, the mean Ct value for telomerase sequence and for Rplp0 single copy gene was calculated for each sample. The next calculation was ΔCt= CtT-CtS that is also called T/S ratio. The results were then expressed like relative telomere length (fold change relative to control) calculated with the following formula:

$$relative\frac{T}{S} = 2^{-[\frac{T}{S}(X)-\frac{T}{S}(CTL)]}$$

Statistical analysis

The data were displayed as the average value obtained from a minimum of three independent analyses ± standard deviation (SD). Statistical analysis was conducted using the STATISTICA software package (Statsoft Inc., Tulsa, OK, USA). To denote significant differences, letters obtained through one-way analysis of variance

were employed (ANOVA), subsequently, a Tukey's honest significant difference post hoc test was applied (p<0.05).

Results and discussion

MH in combination with 5-Fu decrease the migration capacity of CSCs-like from spheroids downregulating the transcription factors Slug, Snail and Twist

The EMT process is considered to be a key point in tumor progression, activated especially during the phenomenon of metastasis and tumor invasion. CSCs play a key role in this important process. The molecular mechanisms behind the relationship between EMT and CSCs are still under study, but different associations have been found between this feature and the activation of the three pathways linked to self-renewal ability of CSCs (Wnt/β-catenin, Hh and Notch). In this small cellular subpopulation, the EMT process is largely regulated by some transcription factors (TFs), considered regulators of this important process which are Slug, Snail and Twist, which directly or indirectly inhibit the transcription of E-Cadherin.[16] As can be seen in Figure 3, which shows a representative image of what it was observed under the microscope and where the migrated cells are represented by the violet colour (derived from staining with CV, as described in the Materials and Methods section.), it was already possible to speculate that the used treatments strongly affected the migration capacity of CSCs-like. Furthermore, it is noteworthy how the use of the combined treatment (5-Fu 50 µM + MH 37.5 mg/mL) further decreases, in a statistically significant manner (p<0.05), the migratory capacity of CSC-like cells compared to chemotherapy alone.

Many studies in the literature proceeded to count migrated stained cells, in this study it was not possible to clearly distinguish the cells and make an assessment of their number, so to get a numerical idea on the effect of the treatments tested on the migratory capacity it was used a software (http://www.coolphptools.com/) that calculates the percentage of the colours present in a single image and in this case all the percentages of the violet colours shade obtained have been summed, which as previously mentioned represent the migrated cells. The analysis was carried out using three different fields of view for each condition used. However, this must be considered an indicative estimation as the real migrated cells have not been counted since it was not possible to count the number of single migrated cells as most likely the cells were aggregated, but this software and others similar for colorimetric analysis are used in other scientific studies.[17] From the histogram in Figure 3, it can be seen that the use of honey alone already decreased the migratory capacity of CSCs-like respectively by 1.23 (with MH 50 mg/mL) and 1.30 times (with MH 75 mg/mL) (p<0.05) compared to the CTL. The use of 5-Fu alone resulted in a decrease of 3.30 fold and the combination that used the lowest concentration of MH to 3.96 times (p<0.05). The observed reduction became even higher when the spheroids underwent the combined treatment 5-Fu 50 µM + MH 37.5 mg/mL: there was an estimated 7.51 fold reduction in migrated cells in comparison to CSCs-like originating from untreated spheroids.

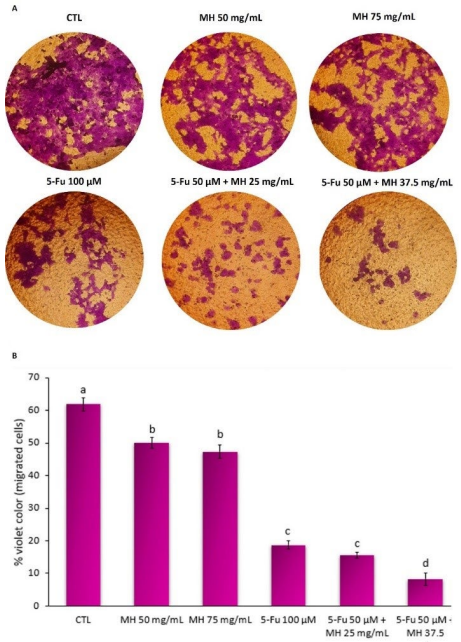


Figure 3. Transwell migration assay. (A) Transwell migration assay of CSCs-like originating from spheroids untreated (CTL) or treated with different conditions. After 48 hours the spheroids were collected and gently mechanically disrupted. The cells deriving from the disruption of treated or not spheroids were counted and seeded in same cell density in a basket transwells. After 48 hours they were stained with 0.2% of crystal violet the underside of the basket membrane that contained the migrated cells. The images were taken using a Leitz Fluovert FU (Leica Microsystems) microscope. (B) Histogram of the percentage of shade of violet color (representing an estimation of migrated cells) detected with http://www.coolphptools.com/ free software from three selected field of view for each analyzed condition. Values are presented as the mean ± SD of three different field (n=3). Distinct letters within the same column imply significant differences (p<0.05).

The EMT process in CSCs-like is regulated at a functional level both by self-renewal pathways and by TFs such as Slug, Snail and Twist. This study assessed how the tested conditions of MH, 5-Fu, and their combination affected the expression levels of these TFs by evaluating their gene expression. TFs regulate E-Cadherin protein expression, critical in this process. Its loss marks the shift from epithelial to mesenchymal phenotype, enabling invasion and initiating metastasis. Slug has been reported to be related to CSC traits in several types of cancer

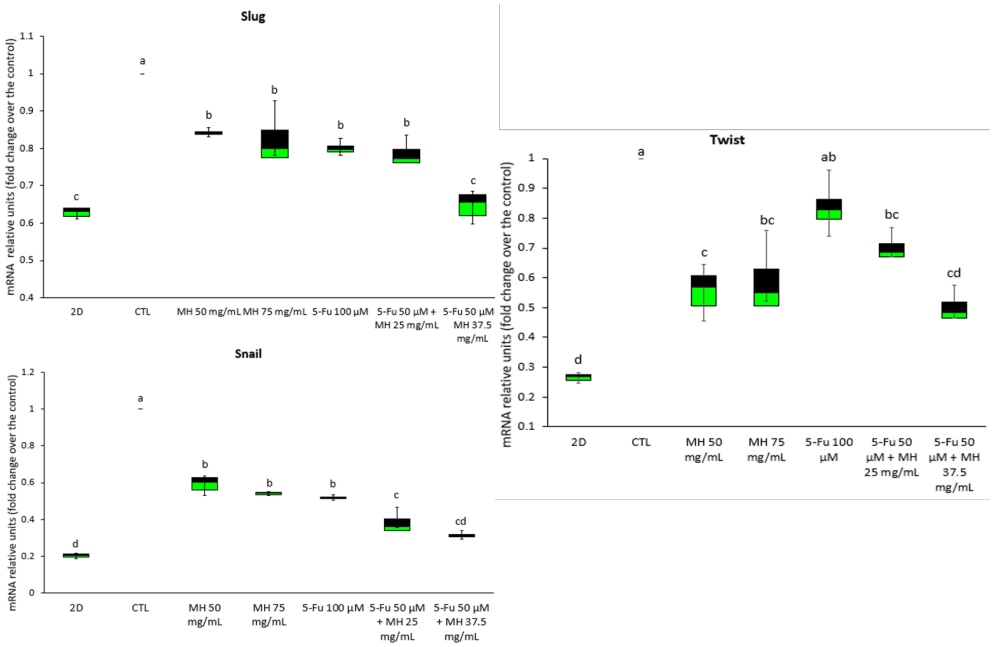


Figure 4. The effect of MH, 5-Fu and their combination on EMT TF related genes expression. Real-Time PCR was employed to analyze the mRNA expression of Slug, Snail and Twist in cells originating from monolayer culture (2D) and cells (CSCs-like) derived from untreated spheroids (CTL) or treated with different conditions. The data is presented as the mean ± standard deviation from three independent experiments (n=3). Distinct letters within the same column signify statistically significant differences (p<0.05).

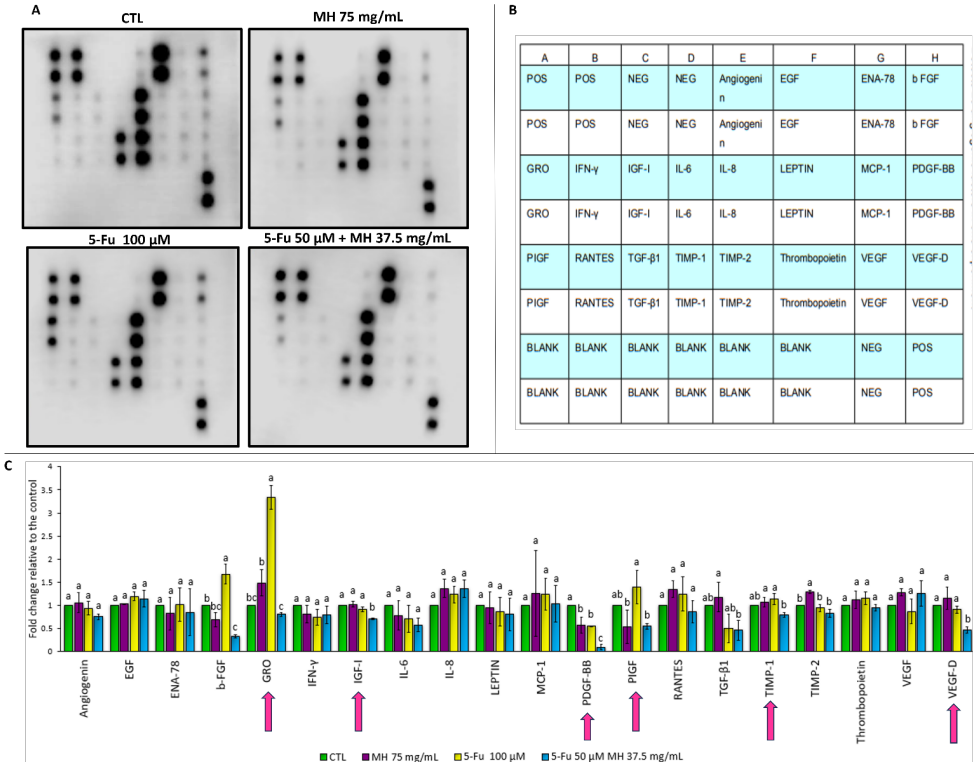
and its expression is linked with metastasis, invasiveness and adverse prognosis, also in colon cancer.[18] In this study, as can be seen in Figure 4, there is a clear statistical difference (p<0.05) in the expression levels of this zinc finger TF between conventionally cultured cells and CSCs-like originating from untreated spheroids, where it was expressed 1.59 times more than the parental cells, confirmed in the same cell line and in HT-29 colon cancer cell line also by Han et al. (2013).[19] When colonspheres were treated with both concentrations of honey alone, with 5-Fu and with the combination of these with the lowest concentration of MH, there was a downregulation (p<0.05) which brings to very similar values in the expression levels (with a decrease range of 1.18-1.27 fold). The use of the combined treatment 5-Fu 50 µM + MH 37.5 mg/mL made the Slug expression levels even more down-regulated, reaching 1.56 times lower than those observed in the CTL, equalizing the expression levels to be statistically comparable (p<0.05) to their adherent counterparts. A downregulation of this TF was also observed in CSCs-like from H1299 and Lu99 human non-small cell lung cancer cell lines treated with EGCG [20] and breast CSCs-like subjected to treatments with baicalin, baicalein,[21] chrysin and honeycomb.[22] Similarly, quercetin, alone or in synergy with EGCG, inhibited the invasive and metastatic characteristics of prostatic CSCs [23] and resveratrol and carnosol had the same effects in glioblastoma CSCs-like.[24] The other zinc finger TF capable of inducing EMT phenomena in CSCs, analysed in this study is Snail, whose over-expression is also associated with a higher EMT activity and CSCs-like phenotype in human CRC cells.[25] From the results obtained in this study, Snail was also found to be up-regulated in spheroids compared with their parental cells, where expression levels were found to be 4.95 times lower (p<0.05) than those obtained in untreated spheroids. These results were also found by Han et al., (2013) in HCT-116 and HT-29 colon cancer cell lines. [19] When the colonspheres were treated with both concentrations of MH alone and with chemotherapeutic drug alone, there was a significant decrease (p<0.05) in its expression level, with similar values between them

with a range that goes from 1.71 to 1.92 times lower compared to the CTL. The observed decrease became greater with the use of combined treatment and reached a reduction of 2.62 times when the tumorspheres were treated with 5-Fu 50 µM + MH 25 mg/mL, and of one of 3.20 times when they were treated with the combination which involved the highest concentration of MH in association with 5-Fu. As discussed for the Slug gene, in the same studies that found a downregulation, an associated downregulation of Snail was also observed by the different compounds discussed above. The third EMT TF discussed in this study is Twist, overexpressed in CRC and closely associated not only with a metastatic phenotype with higher invasiveness and migration capacity but also with a high chemoresistance, so much to be considered a marker of colon CSCs.[26,4] Again, a clear significant difference (p<0.05) was found between conventionally cultured cells and CSCs-like from untreated spheroids, where expression levels were 3.80 times higher. Similar results were also found by Ayinde et al. (2019) in HCT-116 and SW620 colon cell lines.[27,5] Treatment of the spheroids with MH at a concentration of 50 mg/mL resulted in a statistically significant reduction (p<0.05) compared to the CTL of 1.83 times, the highest concentration of MH let to a decrease of 1.71 fold (p<0.05). The reduction was less when the colonspheres were treated with chemotherapeutic agent alone (1.20 times less expressed), while the use of combined treatments led to a decrease of 1.43 times when the condition 50 µM + MH 25 mg/mL was used, and an even greater one, of 2.00 fold, when the concentration of MH equal to 37.5 mg/mL was used in association with 5-Fu. A downregulation of Twist was also observed in colon CSCs-like (SW480 cell line) treated with quercetin,[26,8] in prostatic CSCs-like from PC-3 and LNCaP cell lines by EGCG [23,1] and in breast CSCs-like from different cell lines by flavonoid wogonoside (a glucuronide of wogonin).[29,7] It was also observed that another natural non-polyphenolic compound, the triptolide isolated from a Chinese herbs, was able to inhibit the spheroid formation capacity, EMT processes and migration ability in colon CSCs-like from HT-29 cell line by lowering the expression levels of Twist.[30,28]

From the results obtained it can be asserted that MH is able to lower the migration capacity of colon CSCs-like alone but even more when it is used in combination with 5-Fu downregulating some inhibitors (the EMT TFs) of the key factor of the epithelial phenotype, E-Cadherin. The combined treatments of MH and 5-Fu, in fact, were able to significantly downregulate the gene expression of Slug, Snail and Twist, inhibitors of E-Cadherin.

MH in combination with 5-Fu downregulate pro-angiogenic proteins

Angiogenesis is a complicated process based on the balancing of inhibitors and inducers of angiogenesis, in which new blood vessels are formed from endothelial precursors. In cancers it is of fundamental importance as it guarantees the supply of nutrients and oxygen necessary for the mass tumor to be able to develop, contributing to its invasiveness in secondary districts and therefore to the formation of metastases. Recent studies have reported that CSCs could be defined as “angiogenic cells” since some of their properties, including the high migration and self-renewal capacity of these rare tumor cells, are essential for endothelial vessel formation.[31] In this study The Human Angiogenesis Antibody Membrane Array (ab134000, Abcam) was used for investigating the expression of various proteins involved in angiogenesis processes. . This experiment was carried out using only the four most significant treatments among the six total used in the study to understand how these acted on the complicated process of angiogenesis (Figure 5).



A	B	C	D	E	F	G	H
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
PlGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
PlGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
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Figure 5. **The effect of MH, 5-Fu and their combination on the protein expression of angiogenesis**. (A) Human Angiogenesis Antibody Array. The spheroids were subjected to various conditions for a 48-hour period and then compared to untreated spheroids. (B) Array map used in this study. (C) The data is displayed as the average ± standard deviation derived from three distinct experiments (n=3). Distinct letters within the same column signify statistically significant differences ($p<0.05$). The pink arrows indicate the proteins that show variation with the treatments and are extensively discussed in the text. EGF: epidermal growth factor; ENA-78: Epithelial Neutrophil-Activating peptide 78; b-FGF: basal fibroblast growth factor; GRO: Growth-regulated oncogene ; IFN-γ: Interferon gamma; IGF-I: Insulin growth factor; IL-6: Interleukin-6; IL-8: Interleukin-8; MCP-1: Monocyte Chemoattractant Protein-1; PDGF-BB: Platelet Derived Growth Factor-BB; PlGF: Placental growth factor ; TGF-β1: Transforming growth factor beta 1; TIMP-1: Tissue inhibitor of metalloproteinase 1; TIMP-2: Tissue inhibitor of metalloproteinase 2; VEGF-A: Vascular endothelial growth factor-A; VEGF-D: Vascular endothelial growth factor-D.

Proteins that exhibited statistically significant differences ($p<0.05$) in expression levels between untreated spheroids and different treatment conditions have been found will be extensively discussed. On the other hand, no statistically significant differences ($p<0.05$) were found in the protein expression of the following factors related to the angiogenesis process: angiogenin, EGF, Epithelial Neutrophil-Activating peptide 78 (ENA78), Interferon gamma (IFN-γ), IL-6, IL-8, Leptin, Monocyte Chemoattractant Protein-1 (MCP-1), RANTES, Thrombopoietin and Vascular endothelial growth factor (VEGF) A (Figure 5 A,C).

Basic (b)-FGF (Figure 5 A, C), whose the main bio-physiological function is to promote proliferation, was the first pro-angiogenic factor identified and it was found that in different types of cancer, including CRC one, its over-expression is associated with the presence of lymph node metastasis. In cancer cells it acts both by promoting an over-proliferation of these cells and by stimulating angiogenesis 35. In this study it was observed that the MH at a concentration of 75 mg/mL was able to statistically ($p<0.05$) down-regulate the levels of b-FGF compared to the CTL, with a protein expression 1.44 fold lower. It is interesting to observe that there was instead an increase in the levels of this pro-angiogenic factor when the spheroids were treated with 5-Fu alone (resulting 1.67 times

higher), which could be indicative of a greater chemoresistance of CSCs-like; in fact it has been observed in some colon cancer cell lines (LoVo, DLD1 and HT-15) that 48 h of incubation of these cells with 5-Fu increased the expression levels of b-FGF from 1.96 times to 3.51 times and that a knockdown of b-FGF led to a decrease in the IC50 of 5-Fu.[32] Finally, it was possible to observe that the treatment of spheroids with the combined treatment 5-Fu 50 µM + MH 37.5 mg/mL led to a reduction in the levels of protein expression of b-FGF of 3.01 times compared to the CTL (p<0.05). In colon cancer cells LoVo it was found that EGCG was able to suppress the protein levels of b-FGF and to decrease angiogenesis in EGCG-treated mice.[33]

Another factor involved in angiogenesis where statistically significant differences (p<0.05) were noted in this study is Growth-regulated oncogene (GRO) (Figure 5 A,C), a chemokine also called chemokine (C-X-C motif) ligand 1 (CXCL1) that appears to be over-expressed in different types of cancer, including CRC, melanoma, hepatocellular and melanoma where it is associated with a higher proliferation rate, metastasis, leukocyte infiltration and angiogenesis. The angiogenesis process associated with this oncogene seems to be linked to the activation of thrombin.[34] In this study it was observed that both MH used alone and 5-Fu statistically significantly increased (p<0.05) GRO protein levels compared to CSCs-like deriving from untreated spheroids by 1.49 and 3.34 times, respectively, while their use in combination caused a decrease in the protein level of 1.23 fold compared to CTL. Similar results were observed in a study that demonstrated *in vivo* that the administration of 5-Fu increased the mRNA expression level of GRO in the colon of mice and this up-regulation was instead suppressed by the use of curcumin in combination with 5-Fu by targeting NF-κB signaling pathway, also reducing some side effects of chemotherapy, such as the presence of diarrhea.[35]

IGF-I (Figure 5 A, C) has an anti-apoptotic and cell proliferation-promoting effect, but its local expression would also seem to be associated with the angiogenesis process in CRC.[36] The intensity of the spots observed in this experiment is not particularly evident for any sample, but in any case the software used for the detection was able to identify the intensity of the bands, differentiating them from the negative spots: it was observed that single treatments, both with MH and with 5-Fu alone, did not lead to statistically significant changes (p<0.05) in the IGF-I protein levels, while the combined treatment significantly lowered (p<0.05)) its levels, which were found to be 1.39 times lower than those observed in CSCs-like deriving from untreated spheroids. It has been observed in other studies that EGCG alone or in combination with chemotherapy was able to inhibit angiogenesis in lung cancer cell lines and *in vivo* in mice by downregulating the expression of IGF-I.[37]

Platelet Derived Growth Factor-BB (PDGF-BB) (Figure 5 A, C) isomer mitogen has been found to be a critical factor in angiogenesis and appears to be over-expressed in tumor cells and also closely associated in colon carcinogenesis, where it also plays a role in stimulating autocrine growth of cancer cells.[34] In this current study, a downregulation (p<0.05) of this pro-angiogenic factor with respect to the CTL was noted, both with the use of MH alone (1.73 times less expressed) and with 5-Fu alone (1.79 times less expressed). When MH and 5-Fu were used as a combined treatment, the observed decrease was very higher (p<0.05), in fact it was 10.08 times less expressed than in the CTL. Also in this case an inhibitory action was observed by EGCG against PDGF-BB in hepatic cancer cell line and moreover a downregulatory effect against this growth factor was also observed with baicalin in the same cancer cell line.[38]

Placental growth factor (PlGF) (Figure 5 A,C), a glycoprotein with high homology to VEGF, is another proangiogenic factor that is very often responsible for antiangiogenic drug resistance in CRC and it would also seem to be correlated with less survival rate in patients with this cancer.[39] In the present study, the intensity of the observed spots is not particularly evident for any sample, but the software used for the detection was able to identify the intensity of the bands, differentiating them from the negative spots: homologue of VEGF was found to be significantly (p<0.05) downregulated and in a similar manner, both with treatment with MH alone and with the combination of this with 5-Fu (respectively 1.85 and 1.82 fold less than the CTL), while treatment colonspheres with 5-Fu alone led to a statistically significant (p<0.05) increase in protein levels of PlGF compared to CTL (1.39 fold greater). There are no studies in the literature that evaluated how polyphenols could affect this factor in cancer, but there is a study that evaluated the levels of this protein in human retinal endothelial cells treated with kaempferol and it was observed that it was able to downregulate its protein level.[40]

TGF-β1 (Figure 5 A, C) is an inducer of angiogenesis which, however, seems to have a dualistic role as it has been observed that *in vivo* it is capable of inducing angiogenesis, while *in vitro* it inhibits cell proliferation,[41] for this reason it will not be discussed in this work also because the intensity of the detected spots is very weak.

Tissue inhibitor of metalloproteinase (TIMP) 1 (Figure 5 A, C) is an inhibitor of MMPs and therefore is able to inhibit by forming noncovalent 1:1 stoichiometric complex their proteolytic activity towards the extracellular matrix, which is a necessary process in angiogenesis. To assess the effect of this MMP inhibitor on angiogenesis, the levels of the latter should also be evaluated. On the other hand, however, as regards TIMP-1, it has been observed that this inhibitor of MMPs plays a fundamental role in the regulation of tumor cell proliferation as well as decreases the drug sensitivity to many chemotherapy drugs.[42] Recently, a series of clinical studies have also shown that increased TIMP-1 expression is closely associated with a poor prognosis in several types of cancer, including CRC.[43] In this study, TIMP-1 protein levels were observed to rise slightly, albeit statistically significantly (p<0.05) when the colonspheres were treated with both MH (1.06 fold higher compared to CTL) and only chemotherapeutic agent (1.13 fold). On the other hand, when the spheroids were treated with the combination of MH and 5-Fu, the expression levels were statistically significantly (p<0.05) lower than in the CTL (1.24 fold). A downregulatory effect of this protein was observed in HT1080 fibrosarcoma cells, where both myricetin and quercetin were able to lower its protein level, while having an inhibitory effect on MMPs and therefore on angiogenesis.[44]

TIMP-2 (Figure 5 A, C) is also an inhibitor of MMPs, in particular of MMP2 and MMP9 and therefore the expression of MMPs should be

evaluated simultaneously for the evaluation of its effect on angiogenesis. In this case, an association with tumor staging was found in CRC, also related in this case to the relationship with MMP2: a high MMP2/TIMP-2 ratio is correlated with a poor prognosis.[45] In the present work it has been observed that TIMP-2 levels were statistically significantly up-regulated (p<0.05) only with the treatment of colonspheres with MH alone (1.29 times more expressed than CTL). Treatment of the spheroids with both 5-Fu and with this in combination with MH did not cause statistically significant (p<0.05) changes in the expression levels of this MMPs inhibitor. The effects of polyphenols on the levels of this protein in cancer cells are contrasting, in fact it was found that in human CRC cell lines DLD-1 the Hibiscus sabdariffa polyphenol-enriched extract was able to increase the protein level of TIMP-2 [46] and the same happened in HCT-116 treated with a polyphenol extract deriving from green tea.[47] In contrast in SCC4 oral cancer cells, kaempferol was able to downregulate TIMP-2 mRNA and protein expression [48] and in B16/F10 mouse melanoma cells carnosol was shown to do the same, decreasing invasion and angiogenic capacity of these cells.[49]

The last protein analysed in this Antibody array in which changes in expression levels were noted is VEGF-D (Figure 5 A, C), which is considered to be a potent pro-angiogenic factor as well as lymphoangiogenic one in colon cancer.[50] In the present study no significant differences (p<0.05) were found in the protein levels of this pro-angiogenic factor when the spheroids were treated with the single treatments, either MH or 5-Fu. While the combined use of these led to a strong downregulation (p<0.05) in the protein levels of VEGF-2, which are 2.10 times lower than those found in CSCs-like deriving from untreated spheroids. Similarly, in breast CSCs-like (from SUM-149 and SUM-190) EGCG in association with the chemotherapeutic agent was found to downregulate this lymphoangiogenesis promoting protein.[51]

In conclusion it can be affirmed that through this experiment which analysed the levels of different proteins involved in angiogenesis it is possible to note that some of the pro-angiogenic factors (b-FGF, GRO, PIGF) were strongly up-regulated by the use of chemotherapeutic agent alone; this could be due to the fact that it has been found in several studies that different stressful conditions are able to up-regulate the levels of these factors [52] and it could be speculated that the 5-Fu could act as a stressful agent favouring an up-regulation of these pro-angiogenic proteins. It is also interesting to observe how MH acted synergistically in the downregulation of some pro-angiogenic factors (b-FGF, IGF-I, PDGF-BB, PIGF, TGF-β1 and VEGF-D) respect to single treatments. It could therefore be said that the use of MH in combination with 5-Fu was able to decrease the protein levels of some pro-angiogenic factors and could also be able to mitigate a physiological increase in the protein levels of these pro-angiogenic factors due to the chemotherapeutic drug, which as seen for GRO could be also related to some side effects encountered after taking chemotherapeutic agents.

MH in combination with 5-Fu reduces telomere length of colon CSCs-like from spheroids

There are some studies that link the ability to self-renewal of stem-cells, EMT in cancer cells, resistance to apoptotic phenomena also to telomere (repetitive hexamer of TTAGGGs) length, which, however, has a controversial role in the onset and in the progression of cancer and is still being studied especially regarding CSCs. In fact, there are some scientific studies that associate a shortening of telomeres with carcinogenesis phenomena, on the other hand there are many others that instead attribute a greater length of telomeres to increased survival capacity with associated immortality in tumor cells; (cancer-telomere length paradox is associated with the type of cancer and the relationships between chromosomal ends and cancer have yet to be fully studied and understood.[53]

In this last part of this work, it was evaluated the effect of the different treatments on telomere length by quantitative Real-Time PCR using telomere specific primers and DNA extracted from cells deriving from monolayer culture or from colonspheres treated or not. The results were adjusted to the single copy gene Rplp0 evaluated also for each condition.

As can be seen in Figure 5, there was a statistically significant difference (p<0.05), albeit slight, in telomere length between HCT-116 conventionally cultured and CSCs-like deriving from untreated spheroids (1.08 fold decrease). Similarly, when the colonspheres were treated with the two MH concentrations, the telomere length was less than the control, by 1.53 and 1.57 times, respectively. Treatment with 5-Fu alone and the combination with the lowest concentration of MH also lowered the values compared to the CTL respectively 1.27 and 1.21 times. When, on the other hand, the tumorsphere were treated with the 5-Fu 50 µM + MH 37.5 mg/mL treatment, the reduction observed was more evident, in fact a statistically significant reduction (p<0.05) was detected, equal to a value 1.74 times smaller than that observed in CSCs-like deriving from untreated spheroids. Similar results were observed in colon CSCs-like (from HCT-116 cell line) treated with walnut phenolic extracts in association with a decreased enzymatic activity of telomerase (enzyme that adds repetitive sequences of non-coding DNA), associated with a reduction of c-Myc, which seems to be involved in the activation of telomerase.[54] Similar results were also observed by Chung et al., (2018) [55] in colon cancer cells from HCT-116 and DLD1 cell lines, where resveratrol and even more the combination of this phenolic compound with 5-Fu significantly decreased telomerase activity in association with a decrease in typical stem cell markers.

For c-Myc (Figure 6) no statistically significant difference (p<0.05) was detected between parental cells and CSCs-like originating from untreated colonspheres. Also for the spheroids treated with MH 50 mg/mL alone and with the chemotherapy drug alone, no statistically significant change (p<0.05) was note. While when the speroids were treated with MH 75 mg/mL led to a reduction of 1.08 fold in c-Myc expression in comparison to the CTL. The decrease in the expression levels of this proto-oncogene was bigger when the CSCs were exposed to the combined treatments, resulting 1.25 and 1.28 times less than in the control.

In conclusion it can be said that in this study it was found that MH, in particular in combination with 5-Fu, was able to influence the length of telomeres, which is reduced in treated CSCs-like, result related to the downregulation of c-Myc. .

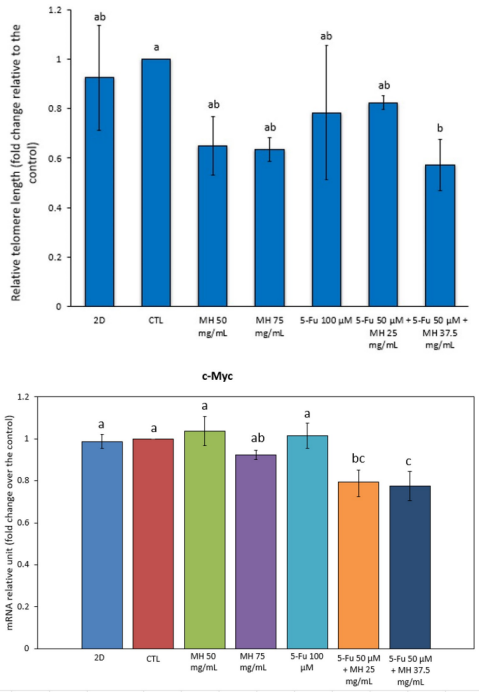

Figure 6. The effect of MH, 5-Fu and their combination on telomere length and on c-Myc expression. Telomere length and c-Myc mRNA expression was examined via Real-Time PCR in cells originating from monolayer culture (2D) and cells (CSCs-like) derived from spheroids, both untreated (CTL) and treated with various conditions. The data is presented as the mean ± standard deviation from three independent experiments (n=3). Distinct letters within the same column signify statistically significant differences ($p<0.05$). c-Myc: cellular Myelocytomatosis.

Conclusion

From the results obtained it can be stated that the use of MH, especially in combination with 5-Fu, is able to mitigate the migration capacity of CSCs-like. This ability in tumor cells and CSCs-like is closely related to the EMT process and therefore to the ability to initiate metastasis. In this study, a downregulation of TFs such as Slug, Snail and Twist was noted, key factors of EMT as they directly or indirectly regulate the expression of E-cadherin.

Furthermore, it has been found that some of the pro-angiogenic factors (b-FGF, GRO, PIGF) resulted to be strongly up-regulated by the use of chemotherapeutic agent alone, this could be due to the fact that different stressful conditions are able to up-regulate the levels of these factors and it could be speculated that 5-Fu could act as a stressful agent favouring an up-regulation of these pro-angiogenic proteins. It was observed that instead MH acted synergistically with 5-Fu in the downregulation of some pro-angiogenic factors (b-FGF, IGF-I, PDGF-BB, PlGF, TGF-β1 and VEGF-D). Therefore the use of MH in combination with 5-Fu was able to decrease the protein levels of some pro-angiogenic factors, and to mitigate a physiological increase in the protein levels of these pro-angiogenic factors due to the chemotherapeutic drug, which, as seen for GRO, could be also related to some side effects encountered after taking chemotherapeutic agents. Based on the results obtained, it also appears that MH, in particular in combination with 5-Fu, was able to influence the length of telomeres, which appears to be shorter in treated CSCs-like; it may be speculated that it may act on telomerase activity through the downregulation of c-Myc.

In conclusion, the results obtained in this research suggest that MH possesses an anti-metastatic effect in an *in vitro* cellular model by acting through various mechanisms that contribute to the metastatic potential of tumor cells, particularly of CSCs. Further studies, both *in vitro* and *in vivo*, as well as clinical trials, are unquestionably necessary to assess whether a specific bioactive compound or a combination of these within MH are responsible for this anti-metastatic and chemosensitizing effect against 5-Fu. Of particular interest and worth noting is the finding regarding the regulation of the pro-angiogenic factor GRO, which is responsible, as previously mentioned, for various adverse effects of chemotherapy in colon cancer patients and that in this study was found to be significantly downregulated with the combined treatment. For this reason, it deserves further exploration in the future.

Author Contributions

Danila Cianciosi and Tamara Y. Forbes-Hernandez conceived and designed the experiments. Danila Cianciosi and Maria Elexpuru-Zabaleta performed the *in vitro* assays. José L. Quiles analyzed the data. Danila Cianciosi and Tamara Y. Forbes-Hernandez wrote the manuscript with input from all authors. Maurizio Battino and Francesca Giampieri supervised the project. All authors discussed the results and provided the final approval of the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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