Nobiletin and Xanthohumol counteract the TNFα-mediated activation of endothelial cells through the inhibition of the NF-κB signaling pathway

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Abbreviations: XCF (Xanthohumol Containing Fraction); NCF (Nobiletin Containing Fraction); TNFα (Tumor Necrosis Factor α); HUVEC (human umbilical vein endothelial cells); VEGF (Vascular Endothelial Growth Factor); NF-κB (Nuclear Factor κ light chain enhancer of activated B cells); MDR (multidrug resistance); ECM (extracellular matrix).

Abstract

Angiogenesis, a process characterized by the formation of new blood vessels from pre-existing ones, is a crucial step in tumor growth and dissemination. Given the ability of tumors to interfere with multiple or different molecular pathways to promote angiogenesis, there is an increasing need to therapeutically block tumor progression by targeting multiple anti-angiogenic pathways.

Natural polyphenols present health-protective properties, which are likely attributed to their ability to activate multiple pathways involved in inflammation, carcinogenesis, and angiogenesis. Recently, increased attention has been addressed to the ability of flavonoids, the most abundant polyphenols in the diet, to prevent cancer by suppressing angiogenesis. Here we investigate the mechanisms by which xanthohumol (the major prenylated flavonoid of the hop plant Humulus lupulus L.) and nobiletin (flavonoid from red-orange Citrus sinensis) can modulate the effects of TNFα on human umbilical vein endothelial cells (HUVEC). The results reported in this paper show that xanthohumol and nobiletin pre-treatment of HUVEC inhibits the effects induced by TNFα on cell migration and invasion capability. Moreover, pre-treatment of HUVEC with these compounds affects colon cancer cell adhesion on the endothelial monolayer. Finally, our results highlight that xanthohumol and nobiletin can counteract the effects of TNFα on angiogenesis and invasiveness, mainly through VEGF and NF-κB pathways. Since angiogenesis plays an important pathological role in the progression of several diseases, our findings may provide clues for developing xanthohumol and nobiletin as therapeutic agents against angiogenesis-associated diseases.

1. Introduction

Cancer is the second reason of death after cardiovascular diseases all over the world and it is estimated that the percentage will grow exponentially to reach dramatic levels in the next decades.

Even if chemotherapy is the election therapy in cancer treatment, the scientific community is studying alternative strategies to solve the problem of resistance mainly due to the low accumulation of the drug in tumor cells (multidrug resistance, MDR) as well as the high toxicity of these compounds.

Natural compounds and natural plant-derived metabolites are promising alternative prevention and cancer therapy strategies due to their antiproliferative or proapoptotic effects (W. W. Li, Li, Hutnik, & Chiou, 2012; Rossi et al., 2014; Shuman Moss, Jensen-Taubman, Rubinstein, Viole, & Stetler-Stevenson, 2014). Several publications showed the chemopreventive activity of natural compounds such as epigallocatechin-3-gallate, quercetin, and resveratrol (Alam et al., 2022; H. Guo, Chen, Liao, & Wu, 2013; Jiang et al., 2020; Ward et al., 2018; H. Wu et al., 2019; J. Wu et al., 2014).

Among them, growing evidence suggests the beneficial effects of hop and citric fruits in cancer prevention and their possible use in clinics as combinational therapy with conventional drugs (Cirmi et al., 2017; Harish et al., 2021; J. Wang et al., 2021). Interestingly, the impact of these compounds mainly depends on their biologically active molecules, such as flavonoids, that can modulate several processes e.g. neurodegeneration, inflammation, anticancer or chemopreventive pathways (Calis, Mogulkoc, & Baltaci, 2020; Girisa et al., 2021; Kashyap et al., 2019; Kopustinskiene, Jakstas, Savickas, & Bernatoniene, 2020; Maleki, Crespo, & Cabanillas, 2019; Yi, 2018). Xanthohumol and Nobiletin, are the most abundant flavonoids extracted respectively from the hop plant and red-orange. Xanthohumol inhibits cell proliferation of leukemia by targeting the NF-κB and Akt pathways and inhibits non-small lung cancer cells by reducing cyclin D1 expression (Gao et al., 2020; Monteghirfo et al., 2008). Moreover, it sensitizes SW480 colon cancer cells to chemotherapy (Scagliarini, Mathey, Aires, & Delmas, 2020). Regarding endothelial cells, Albini and her collaborators demonstrated the anti-angiogenic potential of xanthohumol via AMPK activation (Gallo et al., 2016).

Nobiletin shares similar effects with xanthohumol: recent investigations show that nobiletin acts as a functional compound affecting colon cancer chemoprevention (Goh et al., 2019; X. Wu et al., 2015) and overcomes Multi-Drug Resistance (MDR) in cancer (Feng et al., 2020). Furthermore, nobiletin exerts its effect by targeting several oncogenes and oncosuppressor finally affecting several pathways such as NF-κB, Wnt, epithelial-to-mesenchymal transition (EMT), cell cycle, proliferation, and apoptosis pathways (Ashrafizadeh et al., 2020; X. Wu et al., 2017).

The recruitment in the tumor site of different cytotypes promotes cancer progression. Pathological and constitutive angiogenesis is one of the first events of tumor growth and dissemination so that it has been defined as one of the hallmarks of cancer (Hanahan & Weinberg, 2011).

Tumor Necrosis Factor-alpha (TNFα) is a member of the TNF/TNFR cytokine superfamily mainly involved in the inflammatory response acting as an important activator of endothelial cells (Bradley, 2008; Chen et al., 2018; Yoshimatsu et al., 2020). It enhances the expression of molecules produced and released by endothelial cells, thus interfering with cell adhesion, infiltration, and motility of tumor microenvironment cells (Cruceriu, Baldasici, Balacescu, & Berindan-Neagoe, 2020; Kobelt et al., 2020). Several authors focus, in the past decade, on the evaluation of anti-TNFα therapies in the treatment of patients with inflammatory diseases (Keystone & Ware, 2010).

In this study we investigate the capability of these natural compounds, Nobiletin-containing fraction (NCF) and Xanthohumol-containing fraction (XCF), alone or combined in a 50% mix of the two fractions (Mix), to revert the effects of TNFα on endothelial cells.

2. Material and Methods

2.1. Preparation of Nobiletin-Containing Fraction (NCF), Xanthohumol-Containing Fraction (XCF), and their combination (Mix)

NCF, XCF and Mix were prepared as previously described (Turdo et al., 2021). Briefly, the juice of Citrus sinensis var. Tarocco were centrifuged at 15000 rpm for 15 min, then lyophilized 24 hours at -52°C (LyoQuest-55, Telstar Technologies, Terrassa, Spain). Hop was treated with a mortar to obtain the powder, then treated with hexane for 10 minutes. Both compounds, reduced to powder, were extracted with MeOH three times for the complete recovery of the polyphenolic fraction. The methanolic evaporated extract was dissolved in MeOH: water 50:50 (v/v) to a final concentration of 1 mg/mL. Chromatographic profiles and chemical structure of Nobiletin and Xanthohumol fraction were described in (Turdo, et al., 2021). The Mix fraction is the combination (50% and 50%) of the two compounds, NCF and XCF. The concentration of the two compounds in the mix treatment is half of their concentration in the individual treatments.

2.2 Cell culture

HUVEC (Human Umbilical Vein Endothelial cells) and SW620 (colorectal cancer cells) were obtained from ATCC and grown, respectively, in vascular cell basal medium supplemented with the Endothelial Cell Growth Kit (ATCC, LGC standards, MI, Italy) and in RPMI medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin) and 2mM glutamine (all from Euroclone, UK). Both cell lines were maintained in a humidified 5% of CO_2 atmosphere at 37°C. For all the experiments, cells were used at an early passage, following the good practice outlined in the UKCCCR Guidelines for the Use of Cell Lines

in Cancer Research. Moreover, all cell lines used are certified as being the designated type and have been checked to ensure they are free of contamination.

For all experiments HUVEC were treated as follows: cells were seeded to reach 60-70% of confluence the day after. 24 hours later, cells were treated overnight (o.n.) with 10 μg/mL of nobiletin containing fraction (NCF), xanthohumol containing fraction (XCF), or 10 μg/mL of their combination (Mix, 50% MCF plus 50% XCF), and finally exposed or not to TNFα (20 ng/mL) for 2 hours. DMSO, the solvent of each compound, was used as a control.

2.3 RNA Isolation and Real-Time PCR

Levels of ICAM-1 and VCAM-1 were measured by real-time PCR. HUVEC were seeded and treated as mentioned. RNA was isolated using the Illustra RNA spin Mini Isolation Kit (GE Healthcare; Chicago, IL, USA), according to the manufacturer's instructions. Total RNA from HUVEC was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). For quantitative SYBR Green Real-time PCR, the reaction was carried out in a total volume of 20 μl containing 2X SYBR Green I Master Mix (Applied Biosystems), 2 μL of cDNA, and 100 nM forward and reverse primers. The oligonucleotides used are reported in Table 1.

Real-time PCR was performed in 48-well plates using the Step-One Real-Time PCR System (Applied Biosystems). Relative changes in gene expression between control and treated samples were determined using the ΔΔCt method. Levels of the target transcript were normalized to a β-Actin endogenous control, constantly expressed in all samples (ΔCt). For ΔΔCt values, additional subtractions were performed between treated samples and control ΔCt values. Final values were expressed as fold change.

2.4 Enzyme-Linked ImmunoSorbent Assay (ELISA) Assays

HUVEC were seeded at $3{,}5\times10^5$ cells per well in 6-well plates and treated as described above. At the end of the experimental time, the conditioned medium was collected and centrifuged to remove cellular debris. The ELISA assays were then performed according to the manufacturer's instructions.

The amounts of VEGF or MMP-2 in culture supernatants were determined by using a human-specific ELISA kit (cat. number KHG0111, cat. number KHC3081, Thermo Fisher Scientific, Cambridge, MA, USA).

2.5 Western Blot Analysis

SDS-PAGE and Western blotting were performed according to standard protocols. Briefly, cells were washed in PBS and lysed for 1h in lysis buffer containing 15 mM Tris/HCl pH 7.5, 120 mM NaCl, 25 mM KCl, 1 mM EDTA, 0.5% Triton X100, and protease inhibitor cocktail (100X, Sigma-Aldrich, St. Louis, MO, USA) to obtain total protein lysates. Cell debris was removed by centrifugation at 17.000 rpm for 20 min at 4°C. For subcellular extracts cells were harvested and washed in PBS. Nuclear and cytoplasmatic protein lysates were obtained with Nuclear extracts kit (cat. number 40010, Active Motif) according to manufacturing instructions. The protein concentrations were determined by the Bradford microassay method (Sigma-Aldrich) using bovine serum albumin (BSA, Sigma-Aldrich) as a standard. A total of 20 μg protein from each sample was separated using Bolt Bis-Tris gel 4%–12% (Thermo Fisher Scientific) and transferred on nitrocellulose membranes (GE Healthcare). The membrane was then blocked in 5% BSA solution (5% BSA, 20 mM Tris, 140 mM NaCl, 0.1% Tween-20) and probed overnight with specific primary antibodies: anti-tubulin (1:1000, cat. number sc-398103), and anti-ICAM-1 (1:500, cat. number Sc107) from Santa Cruz Biotechnology (Dallas, Texas, USA); anti-VCAM-1 (1:500, cat. number PA5-86042) anti-MMP14 (1:500, cat. number MA537473) and anti-NF-κB (1:1000, cat. number 51-3500) and pNFκB (1:1000, cat. number MA515160) from Thermo Fisher Scientific. The membranes were incubated with mouse or rabbit HRP-conjugated secondary antibody (1:5000, Thermo Fisher Scientific) and the signal was detected by the Chemidoc Biorad acquisition instrument. The obtained images were analyzed with the Image Lab software (Bio-Rad; Hercules, CA, USA).

2.6 Immunofluorescence assay

For immunofluorescence, assay cells were fixed in 4% paraformaldehyde, and stained with NFκB primary antibody (1:100, cat number 51-3500 Thermo Fisher Scientific). The secondary antibody used was Alexa-Fluor 488 (Life Technologies). Samples were counterstained with Hoechst 3342 (1:1000, Life Technologies) for 10 min at room temperature to detect nuclei, and analyzed by confocal microscopy (Nikon A1) median nuclear planes. Five fields for each condition were captured and the percentage of cells with nuclear signal were calculated versus the total cell number.

2.7 Adhesion assay of SW620 on HUVEC monolayer

HUVEC were grown as a tight monolayer in 24 well plates and treated as described above. After treatment, cells were washed with PBS and $4x10^5$ SW620 cells/well were added for 1.5 hours at 37°C. Adherent cells were stained with hematoxylin/eosin. Each test group was assayed in triplicate; six fields for each condition were photographed by phase-contrast microscopy and counted using ImageJ software.

2.8 HUVEC tube formation on Matrigel

Matrigel was used to test the effects of the different compounds on *in vitro* vascular tube formation. 150 µl of Matrigel (BD Biosciences Europe, cat. number 356230) was added to each well of a 48-well plate and then incubated at 37°C for 30 min. $8x10^4$ HUVEC pretreated or not with different compounds were seeded in each well, incubated for 4 hours at 37°C, evaluated by phase-contrast microscopy and photographed. The numbers of branching points were calculated and quantified using ImageJ software.

2.9 HUVEC invasion 3D assay

Matrigel was added to angiogenesis µ-slides (cat. number 81506, Ibidi, Germany) at 10 µL/well, and then incubated at 37°C for 30 min. HUVEC ($1×10^4$ cells) pretreated or not with different compounds were resuspended in 50 µl of a 1:9 mixture of HUVEC medium: RPMI medium and added to the wells. After 4 hours of incubation at 37°C, for analyzing the outcome of tube formation and invasion into the Matrigel, immunofluorescence staining was performed. The cells were fixed in 1% PAF, permeabilized with 0.1% Triton X-100/PBS, and stained with ActinGreen™ 488 Ready Probes™ reagent (AlexaFluor 488 phalloidin, cat. number R37110, Thermo Fisher Scientific). Samples were counterstained with Hoechst to detect nuclei. Finally, specimens were analyzed by Nikon A1 confocal microscope.

2.10 Statistical analysis

Unpaired two-tailed Student's T-test for comparison of two independent groups was performed with GraphPad software (Graphpad Holdings, LLC, USA). The differences between the data sets were considered significant for $P \leq 0.05$. Quantitative data are represented as the mean ± standard deviation (SD) of at least three independent experiments.

2 Results

3.1 The pre-treatment of HUVEC with NCF and XCF reverts the TNFα-mediated production of VEGF

TNFα treatment with the concentration of 20ng/mL for 2h is able to induce the activation of endothelial cells (Q. Guo, Zhang, Zhang, Zhang, & Wu, 2019). Here, to study the potentiality of XCF, NCF, and their combination (Mix) to revert the effects of TNFα stimulation, HUVEC endothelial cells have been treated with 10µg/mL of NCF and XCF alone or in combination before receiving

TNFα. NCF and XCF concentrations were decided according to the cell viability results published by Turdo and colleagues (Turdo, et al., 2021).

Figure 1 shows that TNFα can activate endothelial cells thus increasing the secretion of VEGF in the conditioned medium, as evaluated by ELISA assay. Interestingly, the pre-treatment with all compounds is able to significantly revert the effects observed.

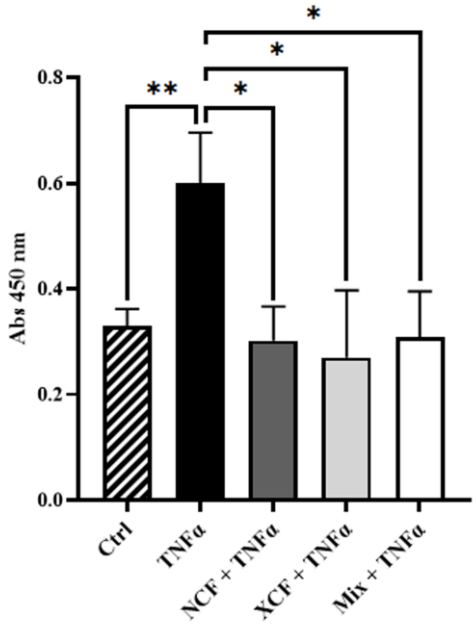

Figure 1. The pre-treatment of HUVEC with NCF and XCF reverts the TNFα-mediated production of VEGF. ELISA assay for secreted-VEGF was performed with conditioned media from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data, expressed as absorbance (Abs) at 450 nm, are the mean ± SD of three independent experiments. *p< 0.05; **p< 0.005.

3.2 The pre-treatment of HUVEC with NCF and XCF reduces cell invasion capability mediated by TNFα

To further characterize the effects of the compounds on TNFα treated cells we investigated their activity with respect to the TNFα-induced invasion capability.

Tube formation assay on unstimulated cells demonstrated that NCF, XCF, and their combination (Mix) decrease the number of branching points on matrigel. Branching points represent the first step of vasculogenesis since they are an intermediate structure between growing and forming sprouts during vascular network growth and remodeling (De Smet, Segura, De Bock, Hohensinner, & Carmeliet, 2009; Horowitz & Simons, 2008) (https://www.ncbi.nlm.nih.gov/books/NBK6295). Figure 2 shows that all compounds significantly reduce the number of branching points, leading us to suppose that they could affect neo-vasculogenesis.

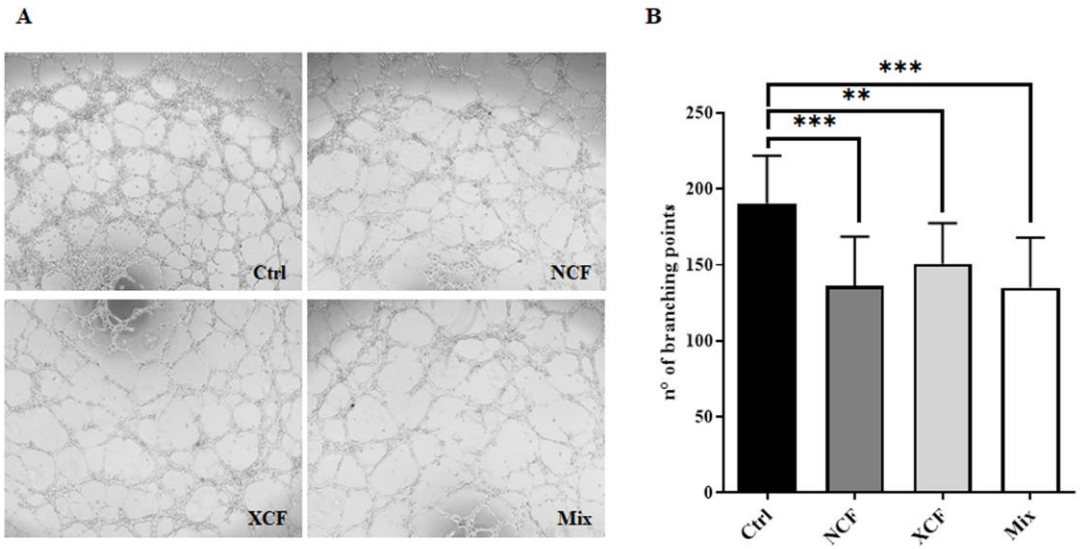

Figure 2. Nobiletin and xanthohumol decrease the number of branching points on matrigel of unstimulated endothelial cells. HUVEC treated with NCF, XCF alone or in combination (Mix), were plated on Matrigel and analyzed after 4h of incubation by phase-contrast microscopy. (A) representative images of matrigel assay observed by phase-contrast microscopy. (B) quantitative analysis of branching points calculated and quantified using ImageJ software. **p< 0.05; *** p< 0.005.

To better clarify if the compounds can interfere with the ability of endothelial cells to invade the matrix forming new tubes, we perform the 3D invasion assay on HUVEC treated with NCF, XCF and Mix before TNFα activation. Figure 3 shows that the invasivity prompted by TNFα is strongly inhibited by pretreatment of cells with all compounds alone or in combination.

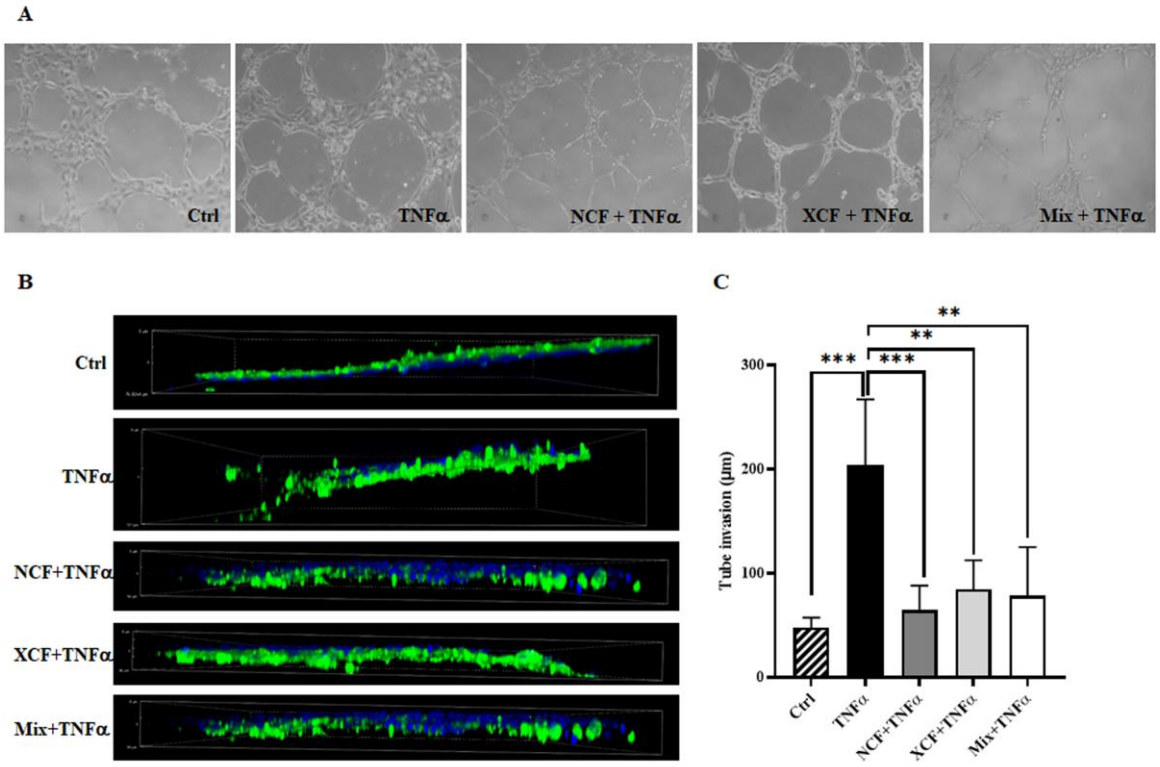

Figure 3. HUVEC invasion capability induced by TNFα is inhibited by pretreatment with all compounds. HUVEC pre-treated with NCF, XCF alone or in combination (Mix), were stimulated for 2h with TNFα and plated on Matrigel. (A) representative images of matrigel assay observed by phase contrast microscopy. (B) immunofluorescence staining of HUVEC 3D invasion capability, evaluated by Nikon A1 confocal microscope analysis. (C) quantitative analysis of HUVEC 3D invasion capability. **p< 0.005; *** p< 0.0005.

3.3 The pre-treatment of HUVEC with NCF and XCF decreases colon-cancer cell adhesion

Activated endothelial cells represent the site where adhere for tumor cells during metastasizing processes. So, we decided to verify if the different compounds play a role in regulating the adhesion capacity of tumor cells on the endothelial monolayer.

Adhesion assay has been performed by adding metastatic colorectal cancer SW620 on unstimulated or TNFα activated HUVEC monolayer, pre-treated or not with the compounds. Figure 4 shows that the natural compounds, alone or in combination, did not affect the adhesion of cancer cells on unstimulated endothelial monolayer. Interestingly, while TNFα treatment induced tumor cell adhesion on HUVEC monolayer, the pre-treatment with all compounds significantly inhibited this process.

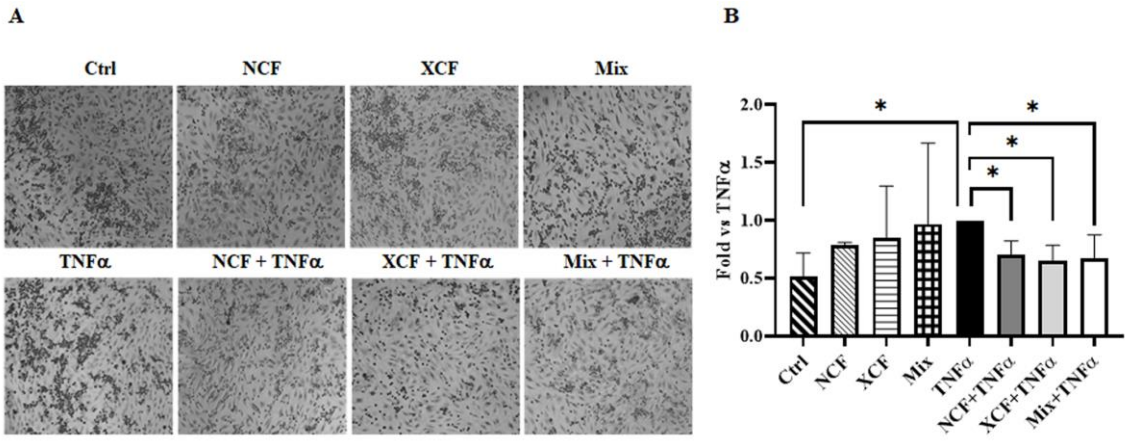

Figure 4. The pre-treatment of HUVEC with NCF and XCF decreases colon-cancer cell adhesion. Adhesion assay has been performed adding metastatic colorectal cancer SW620 on HUVEC monolayer, pre-treated or not with the compounds and activated by TNFα. (A) representative images of adhesion assay. (B) quantitative analysis of tumor cells adherent to endothelial monolayer. *p< 0.05.

3.4 NCF and XCF pre-treatment reduces metalloproteinases expression

MMPs play a pivotal role in controlling angiogenesis and adhesion. In particular, MMP-14 (MT1-MMP) is involved in capillary tube formation and activates several MMPs, including MMP-2. For this reason and to clarify the mechanism through which the natural compounds affect invasion and vasculogenesis we analyzed the expression of these two MMPs.

Figure 5 shows that while TNFα treatment induces MMPs expression on HUVEC, the pre-treatment with all compounds significantly counteracted MMP-14 increase and MMP-2 release, as demonstrated by western blot and ELISA assay.

Overall these data shows the ability of these natural compounds to affect metalloproteinases expression, endothelial tube formation and vasculogenesis.

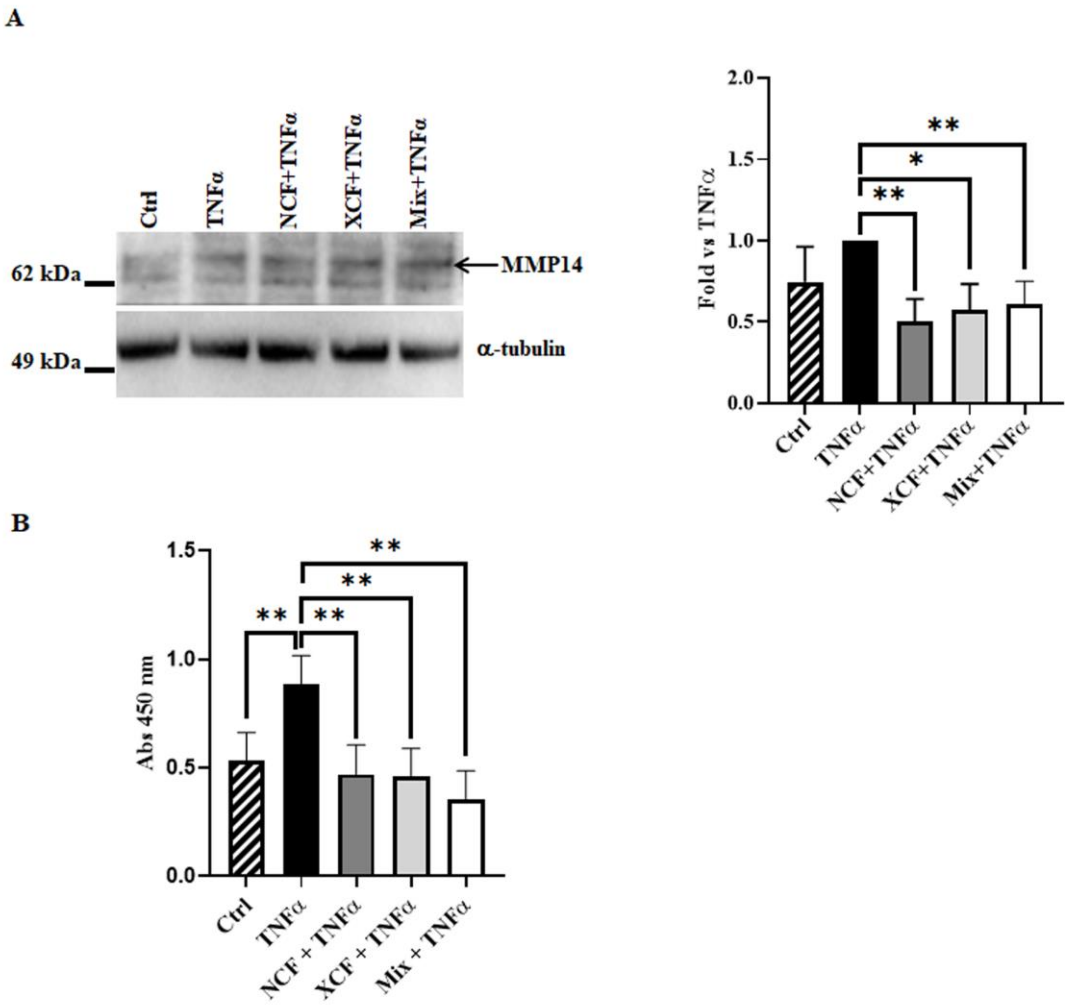

Figure 5. NCF and XCF pre-treatment reduces metalloproteinases expression. (A) Representative images (left) and densitometric analyses (right) of the Western blots for MMP14 on total extract proteins from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data are expressed as the mean ± SD of three independent experiments. (B) ELISA assay for secreted-MMP2 was performed with conditioned media from HUVEC pre-treated with NCF, XCF alone or in combination, and then stimulated for 2h with TNFα. Data, expressed as absorbance (Abs) at 450nm, are the mean ± SD of three independent experiments. *p< 0.05;**p< 0.005.

3.5 The pre-treatment of HUVEC with NCF and XCF reduces the expression of the adhesion molecules ICAM-1 and VCAM-1

To better clarify the molecular mechanism through which the two compounds exert their effects on angiogenesis and cell adhesion, we investigated the expression of the two cell adhesion molecules: VCAM-1 and ICAM-1, both upregulated in endothelial cells in response to cytokines stimulation, i.e. TNFα (Ozawa et al., 2003). Transcriptional assays demonstrated that the pre-treatment of cells with natural compounds reduces ICAM-1 and VCAM-1 mRNA level (figure 6A). The inhibitory effects

are less marked in protein levels, for which we found a reduction only in ICAM-1 expression after cell pretreatment (figure 6B).

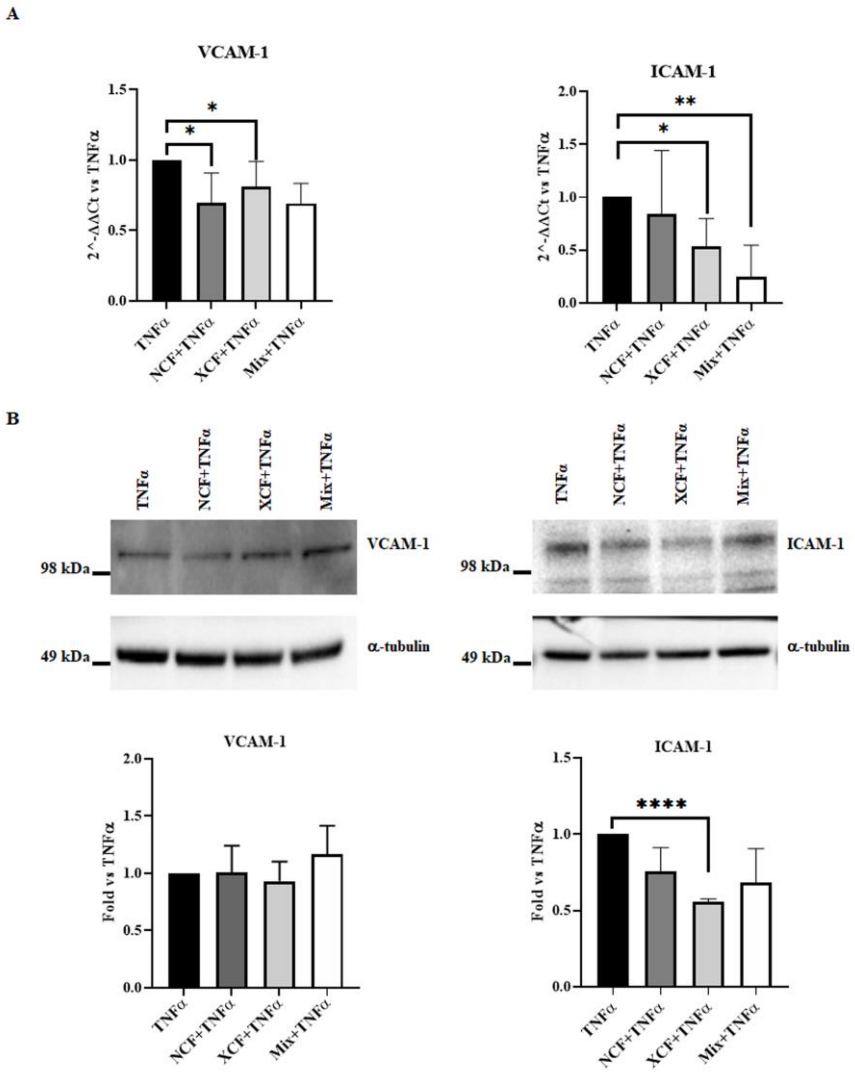

Figure 6. Nobiletin and xanthohumol reduce the expression of adhesion molecules in HUVEC stimulated by TNFα. (A) Real-time PCR for VCAM-1 (left) and ICAM-1 (right) in HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. VCAM-1 or ICAM-1 data were normalized for β-actin, ΔΔCt is expressed as fold of increase (FOI) with respect to TNFα treated sample. Data are expressed as the mean ± SD of three independent experiments. (B) Representative images (top) and densitometric analyses (bottom) of the Western blots for VCAM-1 (left) and ICAM-1 (right) on total extract proteins from HUVEC pre-treated with NCF, XCF alone or in combination and then stimulated for 2h with TNFα. Data are expressed as the mean ± SD of three independent experiments. * $p<0.05$; ** $p<0.005$; **** $p<0.0005$.

3.6 NCF and XCF inhibit NF-κB expression and revert its TNFα mediated nuclear traslocation

It is well known that the cytokine's transcriptional modulation, induced by the TNF family, is mediated by the activation of the NF-κB pathway (Hayden & Ghosh, 2014). Here, we investigated whether natural compounds could interfere with TNF-mediated NF-κB activation.

Figure 7 shows that pre-treatment of HUVEC with the different compounds alone or in combination, significantly reduces NF-κB protein expression. Figure 8 further indicates that TNFα mediates the nuclear traslocation of NFkB and interestingly the compounds are able to reduces the amount of nuclear NFkB, thus unveiling the molecular mediator behind the inhibitory effects outlined above.

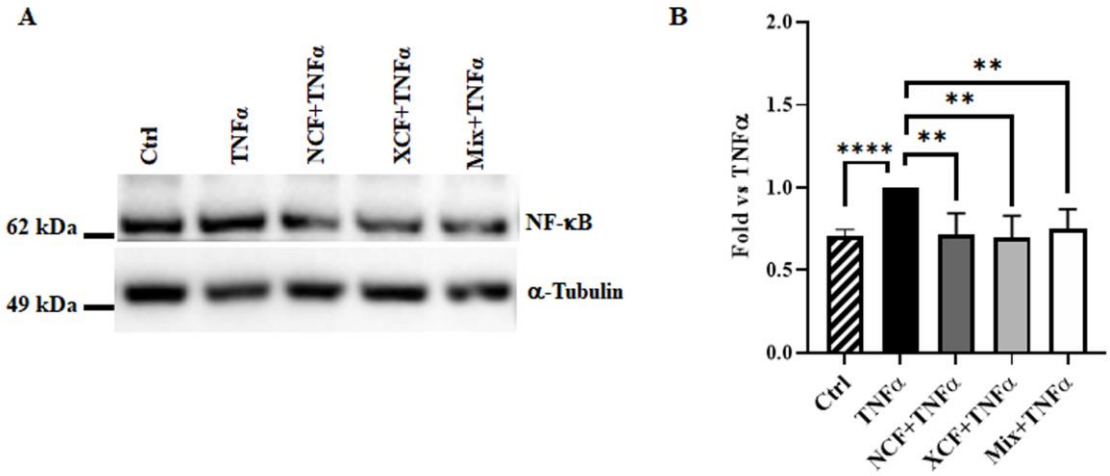

Figure 7. Nobiletin and xanthohumol reduce NF-κB protein expression on HUVEC activated by TNFα. Representative image (A) and densitometric analysis (B) of the western blot for NF-κB on total extract proteins from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data are represented as the mean ± SD of three independent experiments. ** $p<0.05$.

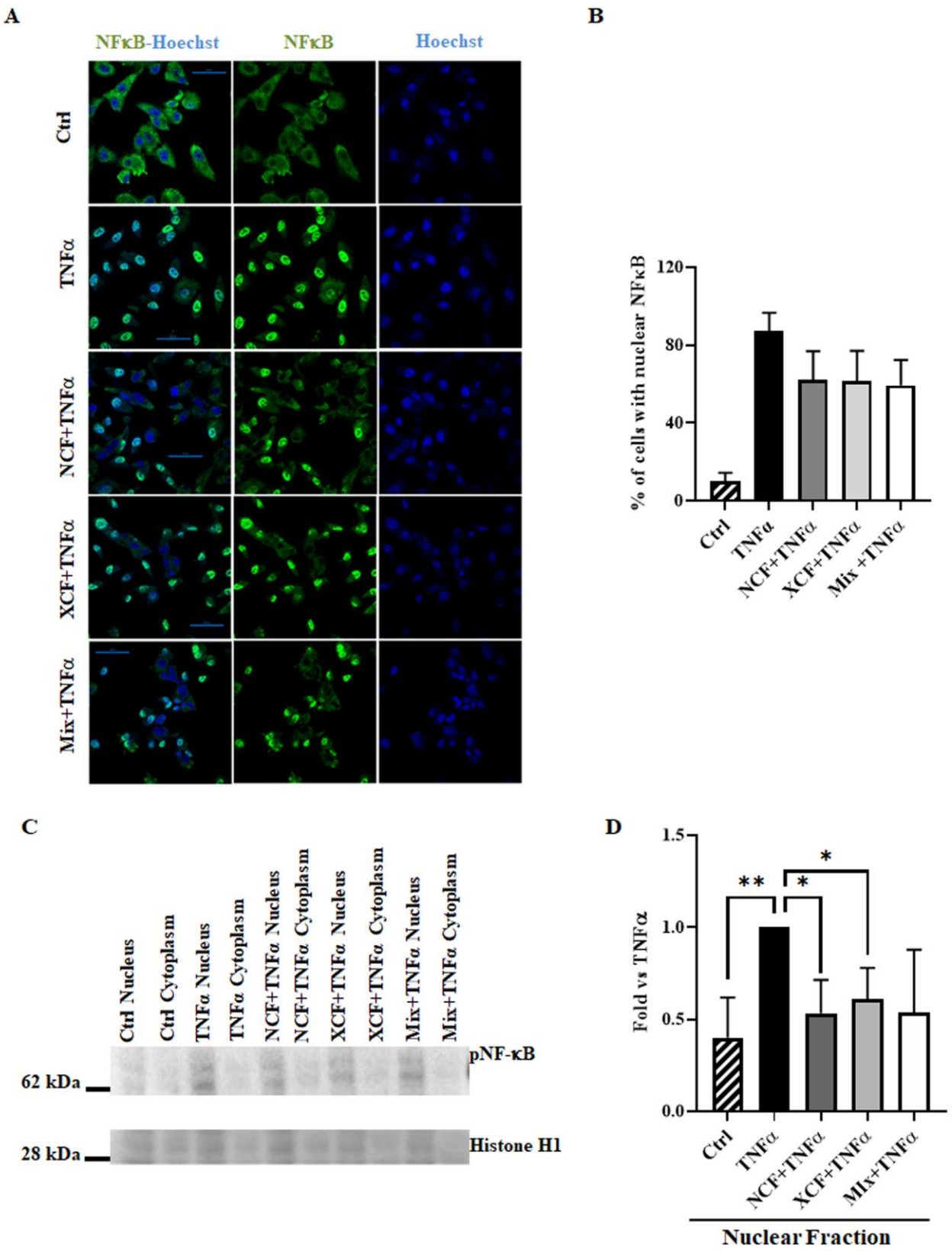

Figure 8. Nobiletin and xanthohumol revert TNFα mediated NF-κB nuclear traslocation.

(A) Immunofluorescence analysis for NFkB nuclear localization of HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. NFκB in green and Hoechst stained nuclei in blue. The scale bar is 50 μm (B) Analysis of the percentage of cells with nuclear signal respect to the total cell

number/field. Data are represented as the mean ± SD of three independent experiments. (C) Representative image and (D) densitometric analysis of the western blot for NF-κB on nuclear protein extract from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data are represented as the mean ± SD of three independent experiments. * $p<0.05$; ** $p<0.005$;

4. Discussion

Scientific advancement allows us to discover the beneficial effects of compounds extracted by plants and offered by nature. In the last years, researchers have turned their interest in evaluating the possible use of natural compounds in the clinical field as they are easily available, often included in every day-diets, and display low toxicity. Several studies have observed the anti-angiogenic property of natural compounds found in vegetables, as carotenoids, polyphenols, terpenoids, and their synthetic counterparts. Many of these compounds received increasing attention as agents for cancer prevention but also for cancer therapy. These compounds exerts antioxidant, anti-proliferative, and pro-apoptotic effects on a variety of cancer cells, including leukemia, prostate, breast, colon, brain, melanoma, and pancreas (W. W. Li, et al., 2012; Quader, Bello-DeOcampo, Williams, Kleinman, & Webber, 2001; Rossi, et al., 2014; Shuman Moss, et al., 2014; Tosetti, Noonan, & Albini, 2009).

It was previously reported that selected fractions from Citrus sinensis and Humulus lupulus L. extracts, containing nobiletin and xanthohumol as the main components, have a broad range of biological effects (Lust et al., 2005; Miranda et al., 1999). In recent years, these compounds have gained attention for their anti-angiogenic and anti-inflammatory activity both *in vitro* and *in vivo* (Dell'Eva et al., 2007; Luo, Guan, & Zhou, 2008; Negrao et al., 2012).

Albini and coworkers showed that xanthohumol is among the chemo-preventive natural compounds with the ability to target the tumor vasculature. They demonstrated that, *in vitro*, Xanthohumol negatively interferes with endothelial cell growth, migration, invasion, and the ability to form a network of tubular-like structures (Albini et al., 2006). In the same way, nobiletin acts as an anti-carcinogenic compound through anti-proliferative activity, induction of apoptosis and cell cycle deregulation in human lung cancer and colon cancer (Luo, et al., 2008; Miyamoto, Yasui, Tanaka, Ohigashi, & Murakami, 2008). Furthermore, nobiletin was shown to cause cell cycle arrest at G1 phase, and to inhibit gastric carcinoma cell migration *in vivo* (Minagawa et al., 2001; Morley, Ferguson, & Koropatnick, 2007).

Recently, Turdo et al. showed the effects of Nobiletin containing fraction (NCF), Xanthohumol containing fraction (XCF), and their combination (Mix) on colon cancer cells, demonstrating that these compounds sensitize colorectal cancer stem cells to 5-fluorouracil and oxaliplatin (FOX)–based chemotherapy (Turdo, et al., 2021).

In this study, we firstly investigated the ability of XCF, NCF, or their combination to affect endothelial cell functions *in vitro*.

Angiogenesis plays an important role in the development of cancer. It has been associated with the growth, dissemination, and metastasis of solid tumors and increased levels of angiogenic molecules have been correlated with poor prognosis in several solid tumors (Nishida, Yano, Nishida, Kamura, & Kojiro, 2006). In recent studies, increased microvessel density in areas of intense neovascularization is a significant and independent prognostic indicator in early-stage breast, prostate, gastrointestinal, ovarian, and melanoma cancers (Aguayo et al., 2000; Gomez-Raposo et al., 2009; S. Li et al., 2019; Melegh & Oltean, 2019).

Here we focused our attention on the possible role of these compounds to revert the effects of TNFα on endothelial cells. For this reason, endothelial cells have been treated with NCF and XCF singularly or in combination before receiving TNFα-induced activation. TNFα is considered one of the most important factors that control endothelial cells activation and its role in cell adhesion has been investigated in several studies. For example, in HUVEC cell culture, the TNFα can stimulate cell adhesion that is been found to correlate with the NF-κB induction of several cell adhesion molecules including ICAM-1, and VCAM-1 (Hayden & Ghosh, 2014; Liu, Zhang, Joo, & Sun, 2017; Sakurada, Kato, & Okamoto, 1996; Sasaki et al., 2003). Regarding tumor cell adhesion on endothelial monolayer, here we revealed that while TNFα treatment induces colon cancer cell line adhesion on HUVEC monolayer, the pre-treatment with all compounds significantly inhibits this process affecting the expression of the adhesion molecules ICAM-1, and VCAM-1. It is very interesting to note that the treatment with the natural compound alone or in combination did not affect the adhesion of cancer cells. Therefore, it seems that the compounds are able to affect the adhesion of tumor cells to endothelial cells only when the latter are activated by TNFα.

Moreover, the results obtained from the 3D invasion assay and the matrigel assay demonstrated that pre-treatment with all compounds inhibits the invasive ability and reduces the number of branching points induced by TNFα. This result lets us to hypothesize that these compounds can affect neo-angiogenesis. Several pieces of evidence support the important function of MMPs in angiogenic processes (Bajbouj, Ramakrishnan, & Hamid, 2021; X. Wang & Khalil, 2018). Comprehensive analysis of the effects of MMPs mediated ECM component degradation in various types of cancer systems is described elsewhere (Giannelli, Falk-Marzillier, Schiraldi, Stetler-Stevenson, & Quaranta, 1997; Koshikawa, Giannelli, Cirulli, Miyazaki, & Quaranta, 2000; Seftor et al., 2001). It is well known that MMP-14 (MT1-MMP), a transmembrane MMP, plays an important role in angiogenesis and tumor cell invasion (Kessenbrock, Plaks, & Werb, 2010; Page-McCaw, Ewald, & Werb, 2007) as well as in the capillary tubes formation (Galvez, Matias-Roman, Albar, Sanchez-

Madrid, & Arroyo, 2001). In addition to exerting direct effects on angiogenesis and ECM remodeling, MMP-14 also activates several downstream MMPs, such as MMP-2 (Al-Raawi, Abu-El-Zahab, El-Shinawi, & Mohamed, 2011; Du et al., 2008; Ohno-Matsui et al., 2003). More recently it was shown that MT1-MMP-mediated collagen degradation is required for matrix tunnel formation in 3D collagen gels, and this mechanism is essential for tumor and endothelial cell motility in 3D matrices in an MMP-dependent mode (Fisher et al., 2009; Stratman et al., 2009). Here we demonstrated that TNFα treatment induces the MMP14 and MMP2 expression, interestingly the pre-treatment with all compounds significantly inhibits the expression of MMP14 and MMP2, mainly actors of angiogenesis. The ability of XCF and NCF to reduce the expression and activity of metalloproteinases, together with the reduction of the migratory and invasive capacity of the cells treated with the compounds, could us suggest a possible correlation between MMPs and these processes.

Dell'Eva et al. demonstrated that XCF has antiangiogenic properties *in vitro* and *in vivo* associated with inhibition of the Akt/NF-κB pathway (Dell'Eva, et al., 2007). Our data (summarized in Figure 9) reveal that the effects of xanthohumol and nobiletin seem to involve the NF-κB pathway which is responsible for the regulation of the adhesion capability of tumor cells to the vascular endothelium through the two adhesion molecules VCAM-1 and ICAM-1.

We decided to test the effects of the single fraction (NCF or XCF) and their combination because we were also interested in a possible synergistic or additive effect of the combination and we hoped to have a similar effect with lesser concentrations, as our results effectively demonstrated.

Interestingly, the use of 50% mix of NCF and XCF exert results at least comparable to those of the treatment with the single fraction but using the half dose of each compound.

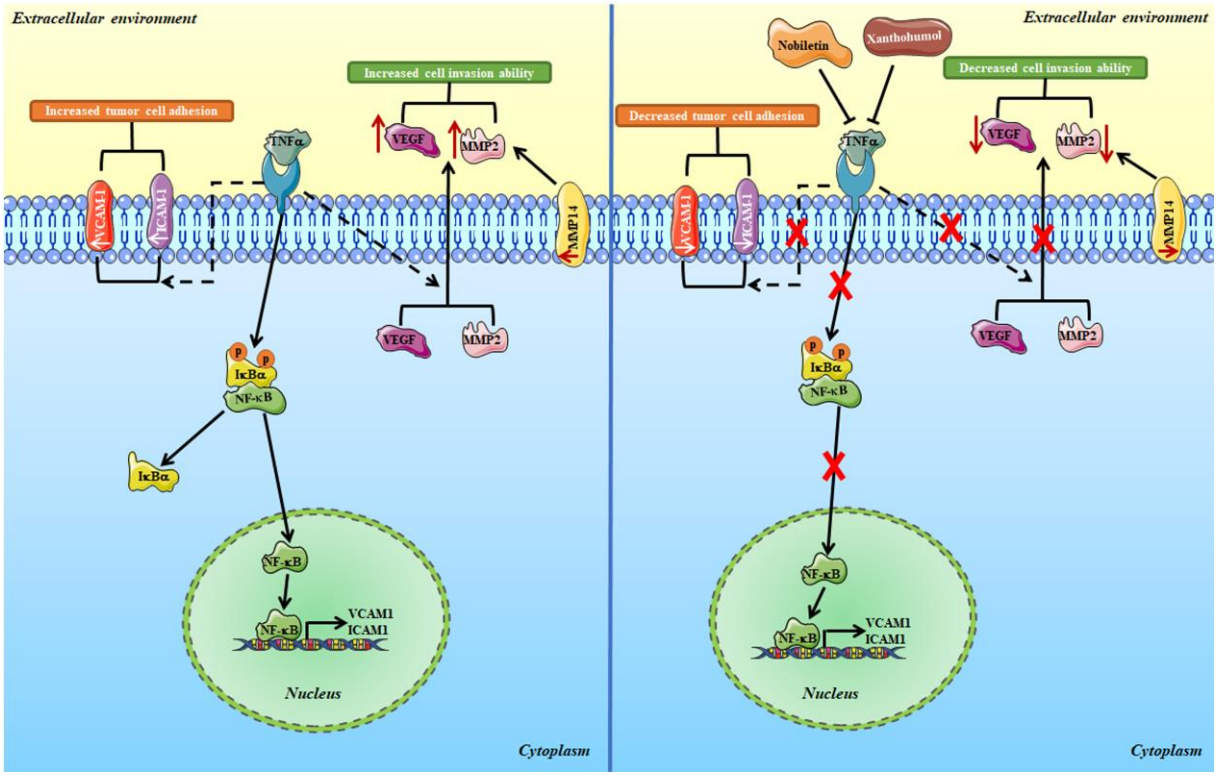

Figure 9. A schematic drawing of the proposed mechanisms by wich Nobiletin and Xanthohumol modulate TNFα-mediated activation of endothelial cells. On the left the binding of TNFα to its receptor results in the activation of the NFκB's target transcription. Moreover, TNFα mediates release of VEGF and MMP-2 and it induces MMP14 protein expression. This processes are involved in angiogenesis and invasiveness. On the right of the picture are reported the inhibitory effect of Nobiletin and Xanthohumol that counteract TNFα induced pathways.

5. Conclusions

Here we showed the potential effects of NCF and XCF, specific fractions enriched in flavonoids from natural extracts Citrus sinensis and Humulus lupulus, on activated endothelium.

The results reported here provide evidence that NCF and XCF can counteract and subvert the effects of TNFα on angiogenesis and invasiveness, and in parallel reduce the expression of VEGF and NF-κB**.** Further studies are required to demonstrate how the natural compounds affect the NF-κB pathway. Since angiogenesis plays an important pathological role in the progress of a wide range of diseases, our findings suggest a rationale for the future development of these compounds as potential chemopreventive supplements to target diseases that involve angiogenesis.

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SR, AC, PD, GP, MGB and RA. All authors have read and agreed to the published version of the manuscript.

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Figure legends

Figure 1. The pre-treatment of HUVEC with NCF and XCF reverts the TNFα-mediated production of VEGF.

ELISA assay for secreted-VEGF was performed with conditioned media from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data, expressed as absorbance (Abs) at 450 nm, are the mean ± SD of three independent experiments. *p< 0.05; **p< 0.005.

Figure 2. Nobiletin and xanthohumol decrease the number of branching points on matrigel of unstimulated endothelial cells. HUVEC treated with NCF, XCF alone or in combination (Mix), were plated on Matrigel and analyzed after 4h of incubation by phase-contrast microscopy. (A) representative images of matrigel assay observed by phase-contrast microscopy. (B) quantitative analysis of branching points calculated and quantified using ImageJ software. **p< 0.05; *** p< 0.005.

Figure 3. HUVEC invasion capability induced by TNFα is inhibited by pretreatment with all compounds. HUVEC pre-treated with NCF, XCF alone or in combination (Mix), were stimulated for 2h with TNFα and plated on Matrigel. (A) representative images of matrigel assay observed by phase contrast microscopy. (B) immunofluorescence staining of HUVEC 3D invasion capability, evaluated by Nikon A1 confocal microscope analysis. (C) quantitative analysis of HUVEC 3D invasion capability. **p< 0.005; *** p< 0.0005.

Figure 4. The pre-treatment of HUVEC with NCF and XCF decreases colon-cancer cell adhesion. Adhesion assay has been performed adding metastatic colorectal cancer SW620 on HUVEC monolayer, pre-treated or not with the compounds and activated by TNFα. (A) representative images of adhesion assay. (B) quantitative analysis of tumor cells adherent to endothelial monolayer. *p< 0.05.

Figure 5. NCF and XCF pre-treatment reduces metalloproteinases expression. (A) Representative images (left) and densitometric analyses (right) of the Western blots for MMP14 on total extract proteins from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data are expressed as the mean ± SD of three independent experiments. (B) ELISA assay for secreted-MMP2 was performed with conditioned media from HUVEC pre-treated with NCF, XCF alone or in combination, and then stimulated for 2h with TNFα.

Data, expressed as absorbance (Abs) at 450nm, are the mean ± SD of three independent experiments. *p< 0.05;**p< 0.005.

Figure 6. Nobiletin and xanthohumol reduce the expression of adhesion molecules in HUVEC stimulated by TNFα. (A) Real-time PCR for VCAM-1 (left) and ICAM-1 (right) in HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. VCAM-1 or ICAM-1 data were normalized for β-actin, ΔΔCt is expressed as fold of increase (FOI) with respect to TNFα treated sample. Data are expressed as the mean ± SD of three independent experiments. (B) Representative images (top) and densitometric analyses (bottom) of the Western blots for VCAM-1 (left) and ICAM-1 (right) on total extract proteins from HUVEC pre-treated with NCF, XCF alone or in combination and then stimulated for 2h with TNFα. Data are expressed as the mean ± SD of three independent experiments. * p<0.05; ** p<0.005; **** p<0.0005.

Figure 7. Nobiletin and xanthohumol reduce NF-κB protein expression on HUVEC activated by TNFα. Representative image (A) and densitometric analysis (B) of the western blot for NF-κB on total extract proteins from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data are represented as the mean ± SD of three independent experiments. ** p<0.05.

Figure 8. Nobiletin and xanthohumol revert TNFα mediated NF-κB nuclear traslocation. (A) Immunofluorescence analysis for NFκB nuclear localization on HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. NFκB in green and Hoechst stained nuclei in blue. The scale bar is….(B) Analysis of the percentage of cells with nuclear signal respect to the total cell number/field. Data are represented as the mean ± SD of three independent experiments. (C) Representative image and (D) densitometric analysis of the western blot for NF-κB on nuclear protein extract from HUVEC pre-treated with NCF, XCF alone or in combination (Mix), and then stimulated for 2h with TNFα. Data are represented as the mean ± SD of three independent experiments. * p<0.05; ** p<0.005;

Figure 9. A schematic drawing of the proposed mechanisms by wich Nobiletin and Xanthohumol modulate TNFα-mediated activation of endothelial cells. On the left the binding of TNFα to its receptor results in the activation of the NFκB's target transcription. Moreover, TNFα mediates release of VEGF and MMP-2 and it induces MMP14 protein expression. This processes are

involved in angiogenesis and invasiveness. On the right of the picture are reported the inhibitory effect of Nobiletin and Xanthohumol that counteract TNFα induced pathways.