Synthesis and Antiangiogenic Activity Study of New Hop Chalcone Xanthohumol Analogues

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Abstract

Angiogenesis induction is a hallmark of cancer. Antiangiogenic properties of Xanthohumol (XN), a naturally occurring prenylated chalcone from hops, have been widely reported. Here we describe the synthesis and study the antiangiogenic activity *in vitro* of a series of XN derivatives, where different substituents on the B-ring of the chalcone scaffold were inserted. The new XN derivatives inhibited human umbilical-vein endothelial cell (HUVEC) proliferation, adhesion, migration, invasion and their ability to form capillary-like structures *in vitro* at 10 μM concentration. The preliminary results indicate that the phenolic OH group in R, present in natural XN, is not necessary for having antiangiogenic activity. In fact, the most effective compound from this series, **13**, was characterized by a para-methoxy group in R and a fluorine atom in R_2 on B-ring. This study paves the way for future development of synthetic analogues of XN to be used as cancer angiopreventive and chemopreventive agents.

Keywords:

xanthohumol analogues

prenylated chalcones

antiangiogenic activity

chemopreventive agents

Abbreviations: XN, xanthohumol; HUVEC, human umbilical vein endothelial cells; EGCG, epigallocatechin-3-gallate; MOMCl, chloromethylmethyl ether; DEAD, diethyl azodicarboxylate; SAR, structure-activity relationship; FBS, foetal bovine serum; FITC, fluorescein isothiocyanate; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride.

1. Introduction

Angiogenesis refers to the formation of new blood vessels from pre-existing vasculature [1]. Physiological angiogenesis is necessary for key processes such as wound healing and tissue regeneration and repair. In pathological conditions, including cardiovascular diseases, diabetes, cancer, and other pathologies associated with chronic inflammation, angiogenesis represent a relevant hallmark contributing to disease insurgence and progression. Therefore, the induction of new blood vessels is a necessary condition for tumors to recruit oxygen, nutrients and disseminate to distant sites. Strategies aimed at blocking or delaying tumor angiogenesis, have been employed both in therapeutic and (chemo) prevention settings and promising results have been obtained in pre-clinical models and in the clinics [2, 3]. Cancer chemoprevention offers the possibility of long-term low toxic treatments that could keep initial tumors at bay, delaying their progression to a clinically relevant tumor [4-6]. Many chemopreventive agents are directly derived from natural sources (phytochemicals) that have been reported to exert anti-proliferative, pro-apoptotic, antioxidant and anti-inflammatory activities [7]. We have demonstrated that many of these phytochemical derived agents act also as anti-angiogenic and anti-inflammatory drugs, a concept we named "angioprevention" [8-10]. Substantial attention has been addressed to flavonoids and their synthetic precursors, chalcones, a class of polyphenolic compounds that, within their wide range of activity, also exhibit anti-angiogenic properties [11]. During the last decades, the prenylated chalcone Xanthohumol (XN, **1**, Fig. 1) has emerged as a cancer chemopreventive agent [12]. It is the major prenylated chalcone present in the female inflorescences of the hop plant *Humulus lupulus* L. (Cannabaceae) employed in the brewing process to preserve and to add bitterness and flavor to beer. The beneficial properties of hops are

well known from ancient times and have been used in traditional medicines since the IX century. Xanthohumol was first isolated by Power in 1913 and its structure was elucidated in 1957 by Verzele [13]. However, only in the last decades, we assisted to an increasing interest to this molecule, since it is endowed with multiple biological activities including anti-diabetic, anti-inflammatory, anti-oxidant, anti-cancer, anti-invasive, and anti-angiogenic activities [14]. Given the promising results from *in vitro* and *in vivo* preclinical studies that have pointed out the numerous benefits exerted by XN, clinical trials are currently being developed evaluating the feasibility of XN treatment in the context of metabolic syndrome and prevention of DNA damage (https://clinicaltrials.gov/) [15]. We showed that XN has stronger anti-angiogenic activity as compared to the green tea flavonoid epigallocatechin-3-gallate (EGCG) [16]. Due to the multiple beneficial activities of XN, we were interested in determining whether chemical modification of the XN-scaffold resulted in more effective inhibition of angiogenesis, to identify novel potential chemopreventive agents. Therefore, we developed of a series of novel synthetic analogues of XN, compounds **2-14** [17], reported in Table 1. A series of XN analogues has been recently synthesized and shown to have toxicity toward HeLa cells by inhibiting the selenoprotein thioredoxin reductases (TrxRs) [18]. We chose to replace the phenolic group on B-ring of the XN (Fig. 1) with several substituents endowed with different electronic and steric properties such as halogens, methoxy or nitro groups, to evaluate the impact of these modifications on antiangiogenic activity. The prenyl group on A-ring was left unchanged, since previous studies [19] confirmed its importance for antiangiogenic activity. Derivatives **3**, **5**, **7**, **10** and **13** were synthesized and tested together with some analogues MOM-protected on A-ring, defined compounds **2**, **4**, **6**, **8**, **9**, **11**, **12** and **14**. Here, we compared the antiangiogenic effects of XN and our novel synthetic derivatives *in vitro*, where human umbilical vein endothelial cells

(HUVEC) were used as a model for cell proliferation, apoptosis, cell adhesion, migration, invasion and formation of capillary-like structures.

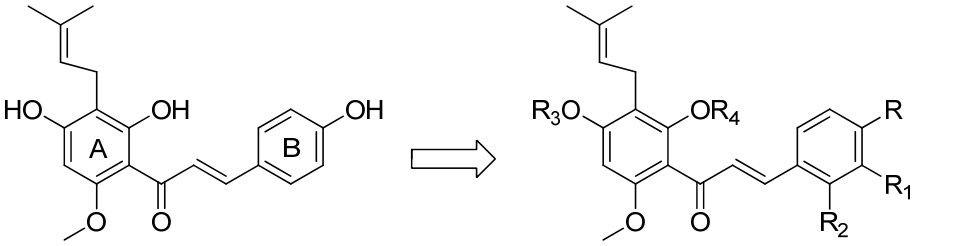
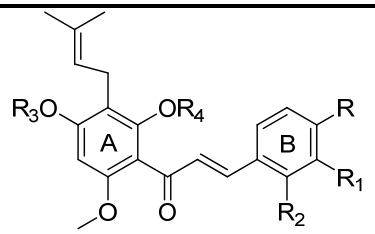


Fig. 1. Natural product Xanthohumol (XN) and its new synthetic analogues.

Table 1. Structure of the new synthetic XN analogues **2-14**.



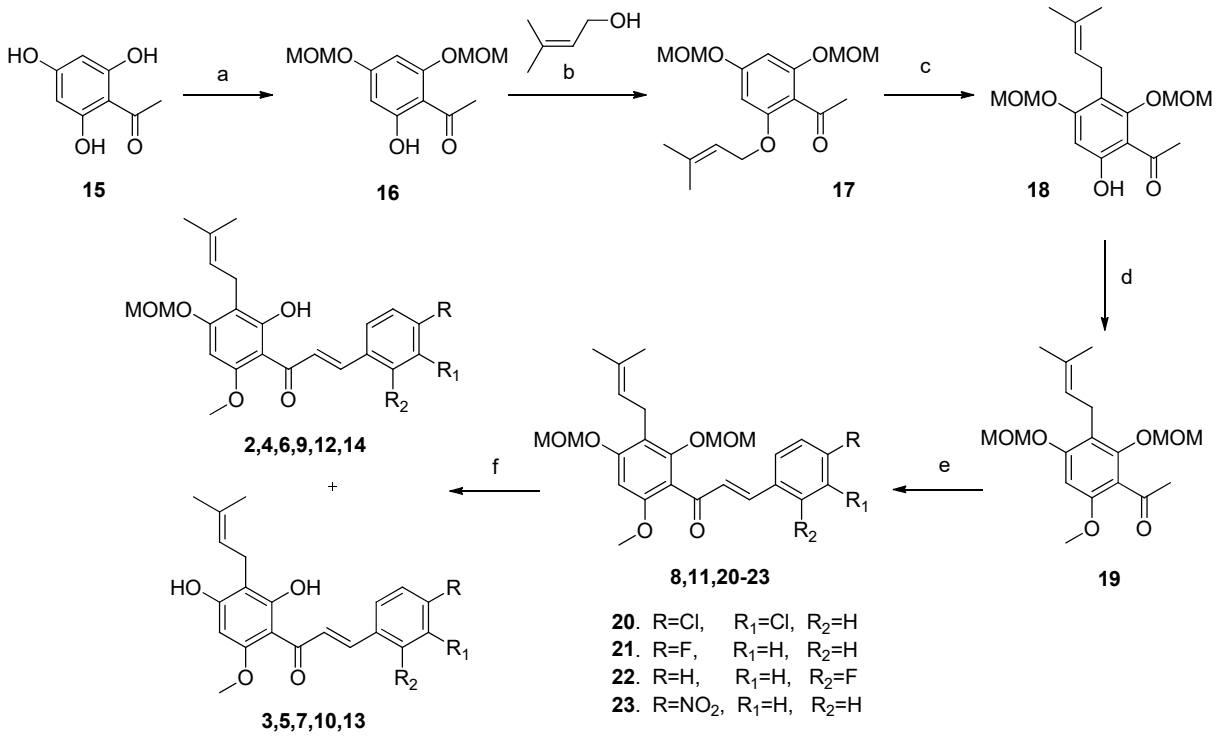
2-14

Compd	R	R_1	R_2	R_3	R_4
2	Cl	Cl	H	MOM	H
3	Cl	Cl	H	H	H
4	F	H	H	MOM	H
5	F	H	H	H	H
6	H	H	F	MOM	H
7	H	H	F	H	H
8	OCH_3	F	H	MOM	MOM
9	OCH_3	F	H	MOM	H
10	OCH_3	F	H	H	H
11	OCH_3	H	F	MOM	MOM
12	OCH_3	H	F	MOM	H
13	OCH_3	H	F	H	H
14	NO_2	H	H	MOM	H

2. Results and discussion.

2.1. Chemistry.

The novel synthetic XN analogues have been synthesized following the synthetic approach previously reported for the synthesis of XN and its natural derivatives [20, 21] and depicted in Scheme 1.



Scheme 1. Synthesis of XN analogues **2-14**. Reagents and conditions: (a) MOMCl, DIPEA, CH_2Cl_2, rt, 6 h, 57%; (b) 3-methyl-2-butene-1-ol, DEAD, PPh_3, THF, rt, 18 h, 60%; (c) *N*,*N*-dimethylaniline, 200 °C, 4 h, 42%; (d) $(CH_3O)SO_2$, K_2CO_3, acetone, reflux, 6 h, 65%; (e) the appropriate substituted benzaldehyde, aqueous NaOH, MeOH, reflux, 4 h, 24-62%; (f) HCl, MeOH/H_2O, 45 °C, 45 min, 7-77%.

Partial methoxymethyl (MOM) protection of commercially available 2,4,6-trihydroxyacetophenone with chloromethylmethyl ether (MOMCl) yielded derivative **16**. The prenyl group has been then introduced using two subsequent steps: a Mitsunobu reaction with prenyl alcohol to form the prenyl ether **17** followed by a Claisen rearrangement that provided

prenylated acetophenone **18**. The Mitsunobu condensation of phenol **16** with prenyl alcohol was conducted in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphine (PPh_3) in THF (60% yield), then phenol **18** was obtained by treatment of **17** with *N,N*-dimethylaniline, at 200 °C for 4 h (42% yield). The phenolic hydroxyl group was then converted in the methoxy function by reaction with dimethyl sulfate in the presence of K_2CO_3 (65%yield) and the derivative **19** thus obtained was used in a Claisen-Schmidt condensation with diverse substituted benzaldehydes to build up the chalcone scaffold. When 2,4 and 3,4-difluoro benzaldehydes have been used, the chalcones obtained showed in 4 position a methoxy moiety, due to fluorine displacement reaction occurring in the reaction conditions (NaOH aq. in MeOH, at 65 °C). Finally, treatment of chalcones **20-23**, **8**, **11**, with HCl in MeOH/H_2O under controlled temperature and acidity conditions, allowed to obtain the deprotected XN analogues **3**, **5**, **7**, **10**, **13** together with MOM mono-protected derivatives **2**, **4**, **6**, **9**, **12-14**. Deprotected and mono-protected derivatives were separated by flash chromatography.

2.2. Biological activity.

In preliminary experiments the anti-angiogenic activity of XN was compared to the one of green tea epigallocatechin-3-gallate (EGCG), extensively described in literature as anti-angiogenic flavonoid, by analyzing human endothelial cells proliferation, viability, cell functions, the ability to interfere with migration, invasion and the capability to form capillary-like networks on matrix basement membrane (data not shown) [16]. All our analysis pointed out a more powerful anti-angiogenic-related activities of XN than EGCG. We therefore employed in all the assays unmodified original Xantohumol molecule as a positive control for the synthetic derivatives.

2.2.1. Effects of XN derivatives on HUVE cell proliferation and viability.

The newly synthesized compounds (**4**-**14**, Table 1) were first tested for cytotoxicity on human umbilical vein endothelial cells (HUVECs). Compounds **2** and **3** were considered unfit for testing due to some solubility issues. We treated HUVECs with increasing concentrations of XN (the positive control for antiangiogenesis) and its derivatives ranging from 1 μM to 20 μM for 24, 48, 72 and 96 h. Cell viability was then assessed by the MTT assay and cell death was demonstrated by Annexin-V/7-AAD double-staining and quantified on a flow cytometry. We observed that most of the XN derivatives inhibited HUVEC proliferation in a dose-dependent and time-dependent manner (Fig. 2). Since XN and many of its derivatives, such as **13** and **14** showed a relevant toxicity at 20 μM (Fig. 3), we used the 10 μM dose for the subsequent experiments and consistent with previous publications [16, 22]. At the 10 μM concentration and the 96h time point, several derivatives statistically inhibited HUVE cell growth as compared to XN alone: in particular **8** and **11** (Fig. 2). Noteworthy, derivative **14** was the only compound to show toxicity at 10 μM dose, as indicated by increased apoptosis on HUVECs (Fig. 3).

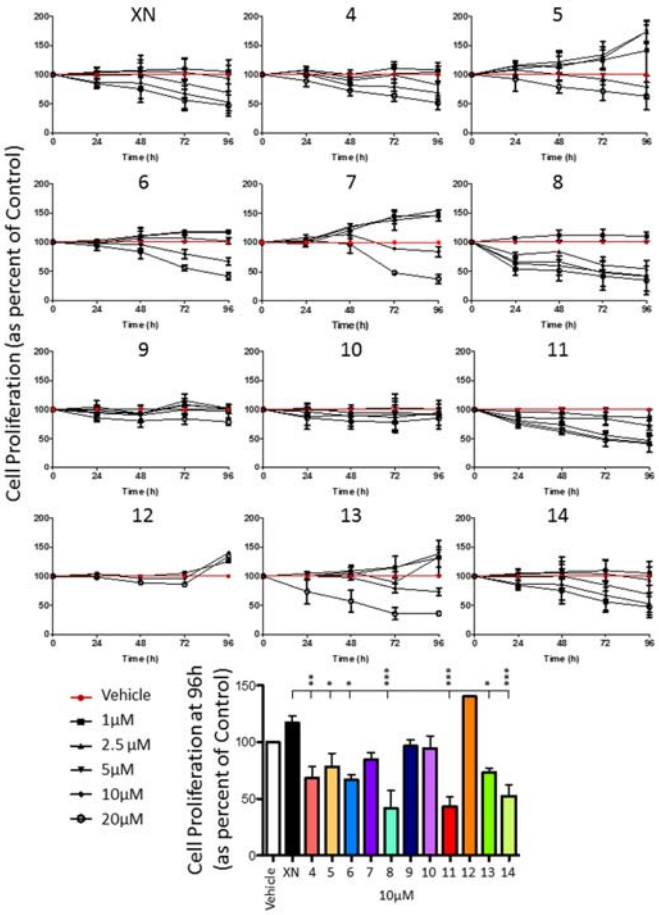


Figure 2. Effects of XN derivatives on endothelial cell proliferation *in vitro* –XN and XN derivatives interfere with endothelial cell (HUVEC) proliferation, as assessed by MTT assay. Results are showed as Mean ± SEM; *p <0.05, ***p<0.001 compared to XN alone. **“**Vehicle” indicates cells treated with vehicle (DMSO in the medium).

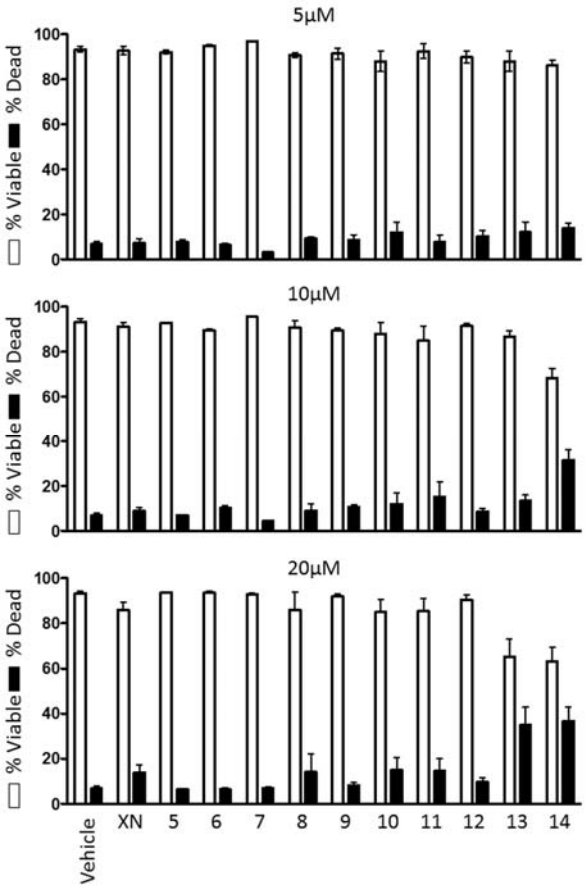


Figure 3. Effects of XN derivatives on endothelial cell viability *in vitro* - Flow cytometry analysis showed that 24 h treatment of endothelial cells with 20 μM XN derivatives resulted in increased rates of apoptotic (AnnexinV^{+}7-AAD$^{-/+}$) cells. **"**Vehicle" indicates cells treated with vehicle (DMSO in the medium). Results are showed as Mean ± SEM.

These results suggest that the presence of a MOM-protective group in R_4 positively impacts on the ability to block cell proliferation, considering that **8** and **11** (the only two derivatives of the series bearing two MOM-protecting groups on A-ring) are the most potent inhibitors of HUVE cell growth (Fig. 2). Moreover, the introduction of a strong electron-withdrawing group such as a nitro group in R on B-ring seems to cause toxicity, as shown by the increased apoptosis on

HUVECs given by compound **14** already at 10 μM concentration. Finally, only compound **12** characterized by a para-methoxy substituent on B-ring, a fluorine atom in R_2 and a MOM-group in R_3 position was not able to affect endothelial cell growth.

2.2.2. Effect of XN derivatives on HUVE cell adhesion, migration and invasion

Since adhesion, migration and invasion are crucial steps for angiogenesis, we also investigated the abilities of XN and its derivatives to inhibit these processes. We observed that XN and its synthetic derivatives were able to decrease HUVEC adhesion compared with the nontreated control (FBS 10% with dissolved vehicle, DMSO) (Fig. 4A). However, only one of the synthetic derivatives, **13**, was statistically more active than the parent compound XN. Comparable results were obtained in terms of migration with XN and its derivatives (except for **12**) which were able to decrease HUVEC migration (Fig. 4B) compared with the control. Again, **13** showed significantly greater inhibition than XN, along with compound **5**. In the invasion assay, XN and its derivatives (except for **9** and **12**) showed statistical differences compared to that of the control (Fig. 4C). However, in this case, none of the derivatives showed significantly more activity compared to the parent compound XN.

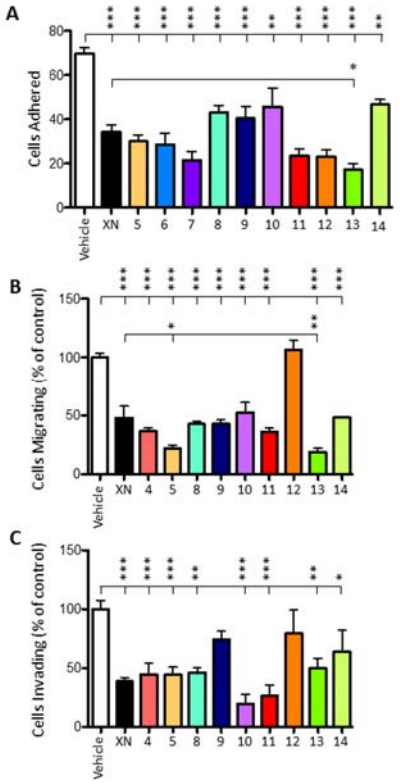


Figure 4. Effects of xanthohumol derivatives on HUVE cell adhesion, invasion and migration. XN and XN derivatives at 10 μM concentration can interfere with crucial steps of angiogenesis by decreasing HUVEC A**)** adhesion B) migration and C) invasion, as compared to vehicle-treated cells. **"**Vehicle" indicates cells treated with vehicle (DMSO in the medium). Results are showed as Mean ± SEM. *p <0.05; **p<0.001; ***p<0.001 as indicated by the bars.

These results point out the importance of a phenolic OH group in R_3 together with a fluorine atom on ring-B for having inhibitory activity on cell migration (see activity of **5** vs **4** and of **13** vs **12**, Fig. 4B). In particular, compound **13**, characterized by a para-methoxy substituent on B-

ring and a fluorine atom in R_2 position, showed the best activity on migration and adhesion inhibition on HUVECs also compared to XN.

2.2.3. Effect of XN derivatives on morphogenesis *in vitro*

When cultured on a membrane-basement matrix layer, HUVE cells form capillary-like networks within 6 h *in vitro*, mimicking the events occurring during vessel network formation *in vivo*. We evaluated the ability of XN derivatives to effect HUVEC morphogenesis *in vitro* at 10 μM concentration. We observed that all the XN derivatives were able to decrease the formation of capillary-like structures in a statistically significant manner as compared to the control (FBS 10% without vehicle) (Fig. 5). Noteworthy, almost all XN derivatives were more potent than the parental compound XN for all parameters considered, and among them **5**, **8** and even more so **13** showed statistically significant differences in number of master segments.

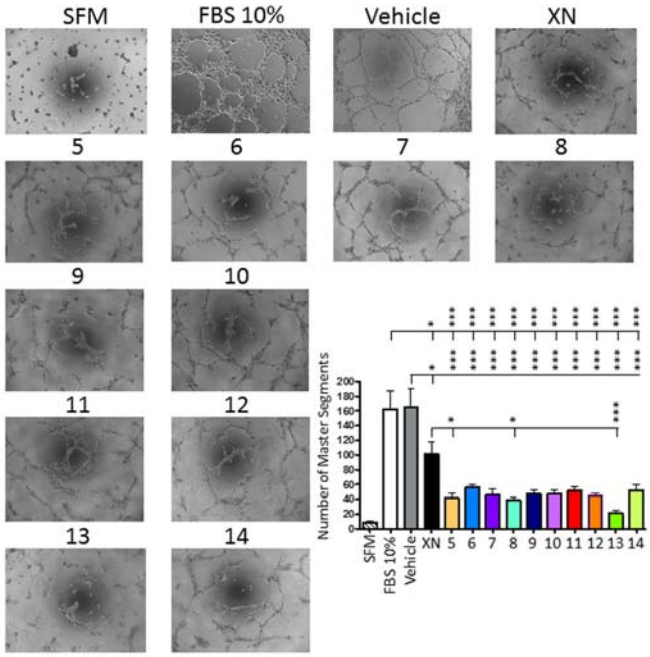


Figure 5. Effects of XN derivatives on HUVEC morphogenesis – Following 6 h treatment, XN and all XN derivatives at 10 μM concentration can inhibit HUVEC ability to form capillary-like structures on matrigel compared with vehicle-treated cells. SFM: cells treated with serum-free EGM-2 medium as a negative control. FBS 10%: cells treated with medium containing 10% FBS without vehicle. **"**Vehicle" indicates cells treated with vehicle (DMSO in the medium). Results are showed as Mean ± SEM. *p <0.05; **p<0.001; ***p<0.001 as indicated by the bars.

Also in this assay, the best activity results were given by compound **13**, characterized by a para-methoxy substituent on B-ring and a fluorine atom in R_2 position. Taken together, all these data suggest that the new XN derivatives exert antiangiogenic activities that can be even higher than the XN ones.

3. Conclusions.

A new series of synthetic derivatives of XN has been prepared and their effects on angiogenesis have been evaluated in comparison with XN ones. In particular, compounds **4-14** have been tested *in vitro* on cell proliferation, apoptosis, cell adhesion, migration, invasion and morphogenesis with HUVE cells at 10 μM concentration. Despite the limited number of derivatives synthesized in the present study, some comments about structure-activity relationships (SAR) can be made. The results obtained so far suggest the importance of a phenolic OH group in R_3 on A-ring together with a fluorine atom on B-ring for having inhibitory activity on cell migration (see activity of **5** vs **4** and of **13** vs **12**, Fig. 4B). The presence of a MOM-protective group in R_4 seems to positively influence the ability to block cell proliferation, considering that **8** and **11** are the most potent inhibitors of HUVE cell growth (Fig. 2). Moreover, the introduction of a nitro group in R on B-ring seems to cause toxicity, as shown by the increased apoptosis on HUVECs given by compound **14** already at 10 μM. Noteworthy, the

phenolic OH group in R, present in natural XN, does not seem necessary for having antiangiogenic activity. In fact, among all synthesized compounds, the best activity results were exhibited by compound **13**, which was the strongest inhibitor of cell adhesion, migration and morphogenesis even compared to XN. This derivative is characterized by a para-methoxy group in R and a fluorine atom in R_2 on B-ring. Given the different activity between **13** and its analogue bearing a fluorine in R_1, compound **10**, the position of F substitution on B-ring seems to be determinant for activity.

Overall, these preliminary data highlight that the newly synthesized XN derivatives may represent relevant compounds able to affect crucial steps of angiogenesis. Further studies must be conducted to assess the pharmacokinetic properties of the most promising compounds in comparison with naturally produced XN.

4. Experimental section

4.1. Chemistry. Melting points were determined on a Kofler hotstage apparatus and are uncorrected. ^{1}H, ^{13}C and ^{19}F NMR spectra were determined with a Varian Gemini 200 MHz spectrometer or a Bruker Avance III HD 400 MHz spectrometer. Chemical shifts (δ) are reported in parts per million and coupling constants (*J*) are given in hertz (Hz). The following abbreviations are used: singlet (s), doublet (d), triplet (t), double-doublet (dd), double-triplet (dt), broad (br) and multiplet (m). Chromatographic separations were performed on silica gel columns by flash column chromatography (Kieselgel 40, 0.040–0.063 mm; Merck) or using ISOLUTE Flash Si II cartridges (Biotage). Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F254) sheets that were visualized under a UV lamp. Evaporation was performed *in vacuo* (rotating evaporator). Sodium sulfate was always used as

the drying agent. Commercially available chemicals were purchased from Sigma-Aldrich (Milan, Italy). Elemental analysis has been used to determine the purity of target compounds. Analytical results are within ± 0.40% of the theoretical values.

4.2. 2′-Hydroxy-4′,6′-dimethoxymethylacetophenone (16).

To an ice-cooled suspension of 2′,4′,6′-trihydroxyacetophenone monohydrate (2.00 g, 10.74 mmol) in 21.50 mL of CH_2Cl_2, *N*,*N*-diisopropylethylamine (5.60 mL, 32.22 mmol) and then dropwise MOMCl (2.40 mL, 32.22 mmol) were added. The reaction was gradually allowed to reach room temperature and stirred for 6 h. The reaction was quenched with aqueous saturated NH_4Cl solution (20 mL). The resulting mixture was extracted with CH_2Cl_2/H_2O (1:1). The organic layers were combined, dried over Na_2SO_4, filtered, and evaporated. The yellowish residue was purified by flash chromatography (*n*-hexane/EtOAc 6:1) using a Isolute Flash Si II cartridge to afford **16** [23] as a colorless oil (1.57 g, 57% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.65 (s, 3H), 3.47 (s, 3H), 3.52 (s, 3H), 5.17 (s, 2H), 5.25 (s, 2H), 6.24 (d, J = 2.4 Hz, 1H), 6.27 (d, J = 2.4 Hz, 1H).

4.3. 4′,6′-Dimethoxymethyl-2′-(3-methylbut-2-en-1-yloxy)acetophenone (17).

To an ice-cooled solution of **16** (917 mg, 3.57 mmol) in 18 mL of dry THF, PPh_3 (1.123 g, 4.30 mmol) and 3-methyl-2-buten-1-ol (0.54 mL, 5.35 mmol) were added. Diethyl azodicarboxylate (DEAD) (0.90 mL, 5.71 mmol), was added dropwise and the resulting solution was allowed to reach room temperature and stirred for 18 h. The solvent was evaporated, the residue suspended in ether and the solid filtered. The filtrate was evaporated and the residue was purified by silica gel flash chromatography (*n*-hexane/EtOAc 6:1) affording **17** [24] as a colorless oil (684 mg, 60% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.70 (s, 3H), 1.75 (s, 3H), 2.47

(s, 3H), 3.45 (s, 3H), 3.47 (s, 3H), 4.49 (d, *J* = 6.6 Hz, 2H), 5.12 (s, 2H), 5.15 (s, 2H), 5.40 (t, *J* = 6.6 Hz, 1H), 6.30 (d, *J* = 1.8 Hz, 1H), 6.43 (d, *J* = 1.8 Hz, 1H).

4.4. 2′,4′-Dimethoxymethyl-6′-hydroxy-3′-(3-methylbut-2-en-1-yloxy)-acetophenone (18).

Intermediate **17** (680 mg, 2 mmol) was dissolved in 21 mL of *N,N*-dimethylaniline and heated at 200 °C for 4 h. Ethyl acetate (100 mL) was added and the mixture washed with 1 N HCl (2 × 100 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash chromatography (*n*-hexane/EtOAc 20:1) using a Isolute Flash Si II cartridge to afford **18** [24] as yellow oil (275 mg, 42% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.68 (s, 3H), 1.76 (s, 3H), 2.70 (s, 3H), 3.30 (d, *J* = 6.6 Hz, 2H), 3.45 (s, 3H), 3.51 (s, 3H), 4.95 (s, 2H), 5.12-5.14 (m, 1H), 5.21 (s, 2H), 6.47 (s, 1H).

4.5. 2′,4′-Dimethoxymethyl-6′-methoxy-3′-(3-methylbut-2-en-1-yloxy)-acetophenone (19).

To a stirred solution of **18** (270 mg, 0.83 mmol) in 17 mL of acetone, K_2CO_3 (230 mg, 1.66 mmol) was slowly added and then dimethyl sulfate (0.15 mL, 1.66 mmol). The reaction mixture was refluxed for 6 h, allowed to cool to room temperature, and quenched with 16 mL of NH_4OH. Then, it was extracted with CH_2Cl_2/H_2O (1:1) 3 × 30 mL. The organic layers were combined, dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (*n*-hexane/EtOAc 8:1) using a Isolute Flash Si II cartridge to afford **19** [20] as yellow oil (183 mg, 65% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.66 (s, 3H), 1.74 (s, 3H), 2.49 (s, 3H), 3.30 (d, *J* = 6.6 Hz, 2H), 3.47 (s, 3H), 3.48 (s, 3H), 3.79 (s, 3H), 4.90 (s, 2H), 5.12-5.14 (m, 1H), 5.20 (s, 2H), 6.54 (s, 1H).

4.6. General Procedure to Synthesize Chalcones 8, 11, 20-23.

To a stirred solution of **19** (1 eq) and the properly substituted benzaldehyde (1 eq) in MeOH (40 mL/mmol), 10% NaOH aqueous solution (2 mL/mmol) was added and the reaction mixture was refluxed for 4 h. The solution was then extracted with EtOAc/H_2O (1:1). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated. The residue was purified by flash chromatography affording the desired compounds **8, 11, 20-23**.

4.7. (E)-3-(3-fluoro-4-methoxyphenyl)-1-(6-methoxy-2,4-bis(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (8).

The title compound was prepared according the general procedure, using 3,4 difluorobenzaldehyde (42 mg, 0.29 mmol). The crude was purified by flash chromatography (*n*-hexane/EtOAc 10:1) using a Isolute Flash Si II cartridge to afford **8** as yellow oil (57 mg, 41% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.70 (s, 3H), 1.78 (s, 3H), 3.37 (d, J = 6.8 Hz, 2H), 3.45 (s, 3H), 3.52 (s, 3H), 3.79 (s, 3H), 3.94 (s, 3H), 4.93 (s, 2H), 5.15-5.22 (m, 1H), 5.26 (s, 2H), 6.61 (s, 1H), 6.89 (d, J = 15.7 Hz, 1H), 7.01 (d, J = 8.4 Hz, 1H), 7.25-7.38 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.9, 22.1, 25.8, 55.9, 56.2, 56.2, 57.4, 89.3, 94.2, 101.0, 108.3, 109.0, 113.9, 114.3, 123.6, 126.4, 126.8, 129.0, 131.4, 141.0, 150.2, 153.6 (d, $^1J_{C\text{-}F}$ = 246.6 Hz)), 161.5, 162.0, 164.3, 193.0. Elemental Analysis for $C_{26}H_{31}FO_7$ calculated: % C, 65.81; % H, 6.58; found: % C, 65.93; % H, 6.76.

4.8. (E)-3-(2-fluoro-4-methoxyphenyl)-1-(6-methoxy-2,4-bis(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (11).

The title compound was prepared according the general procedure, using 2,4 difluorobenzaldehyde (114 mg, 0.80 mmol). The crude was purified by flash chromatography (*n*-hexane/EtOAc 8:1) using a Isolute Flash Si II cartridge to afford **11** as yellow oil (118 mg, 31%

yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.68 (s, 3H), 1.77 (s, 3H), 3.36 (d, J = 6.6 Hz, 2H), 3.44 (s, 3H), 3.51 (s, 3H), 3.77 (s, 3H), 3.83 (s, 3H), 4.92 (s, 2H), 5.14-5.21 (m, 1H), 5.24 (s, 2H), 6.60 (s, 1H), 6.65-6.74 (m, 2H), 6.98 (d, J = 16.0 Hz, 1H), 7.46-7.56 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.0, 23.1, 25.8, 55.8, 56.1, 56.2, 57.6, 89.2, 94.5, 100.9, 101.9, 110.8, 111.1, 115.8, 122.8, 128.7, 130.0, 131.3, 136.7, 161.0, 162.6 (d, $^{1}J_{C\text{-}F}$ = 254.0 Hz), 162.6, 162.7, 164.3, 194.1. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -111.32. Elemental Analysis for $C_{26}H_{31}FO_7$ calculated: % C, 65.81; % H, 6.58; found: % C, 66.17; % H, 6.63.

4.9. (E)-3-(3,4-dichlorophenyl)-1-(6-methoxy-2,4-bis(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (20).

The title compound was prepared according the general procedure, using 3,4-dichlorobenzaldehyde (93 mg, 0.53 mmol). The crude was purified by flash chromatography (*n*-hexane/EtOAc 12:1) using a Isolute Flash Si II cartridge to afford **20** as a semisolid yellow oil (163 mg, 62% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.68 (s, 3H), 1.76 (s, 3H), 3.34 (d, J = 6.8 Hz, 2H), 3.41 (s, 3H), 3.50 (s, 3H), 3.77 (s, 3H), 4.90 (s, 2H), 5.14-5.21 (m, 1H), 5.24 (s, 2H), 6.59 (s, 1H), 6.96 (d, J = 15.9 Hz, 1H), 7.32 (d, J = 15.9 Hz, 1H), 7.38-7.47 (m, 2H), 7.60 (d, J = 1.8 Hz, 1H).

4.10. (E)-3-(4-fluorophenyl)-1-(6-methoxy-2,4-bis(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (21).

The title compound was prepared according the general procedure, using 4-fluorobenzaldehyde (47 µL, 0.44 mmol). The crude was purified by flash chromatography (*n*-hexane/EtOAc 12:1) using a Isolute Flash Si II cartridge to afford **21** as a semisolid yellow oil (47 mg, 24% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.68 (s, 3H), 1.76 (s, 3H), 3.34 (d, J = 7.1

Hz, 2H), 3.42 (s, 3H), 3.50 (s, 3H), 3.76 (s, 3H), 4.90 (s, 2H), 5.14-5.22 (m, 1H), 5.24 (s, 2H), 6.59 (s, 1H), 6.92 (d, *J* = 16.0 Hz, 1H), 7.06 (m, 2H), 7.38 (d, *J* = 16.0 Hz, 1H), 7.49-7.56 (m, 2H).

4.11. (E)-3-(2-fluorophenyl)-1-(6-methoxy-2,4-bis(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (22).

The title compound was prepared according the general procedure, using 2-fluorobenzaldehyde (275 mg, 0.81 mmol). The crude was purified by flash chromatography (*n*-hexane/EtOAc 12:1) using a Isolute Flash Si II cartridge to afford **22** as yellow oil (170 mg, 47% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.68 (s, 3H), 1.79 (s, 3H), 3.35 (d, *J* = 6.9 Hz, 2H), 3.43 (s, 3H), 3.49 (s, 3H), 3.82 (s, 3H), 4.93 (s, 2H), 5.19-5.24 (m, 1H), 5.28 (s, 2H), 6.50 (s, 1H), 7.00-7.11 (m, 2H), 7.18 (d, *J* = 16.0 Hz, 1H), 7.29-7.36 (m, 1H), 7.50-7.60 (m, 2H).

4.12. (E)-1-(6-methoxy-2,4-bis(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one (23).

The title compound was prepared according the general procedure, using 4-nitrobenzaldehyde (201 mg, 1.33 mmol). The crude was purified by flash chromatography (*n*-hexane/EtOAc 6:1) using a Isolute Flash Si II cartridge to afford **23** as yellow oil (274 mg, 44% yield). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.69 (s, 3H), 1.77 (s, 3H), 3.36 (d, *J* = 6.6 Hz, 2H), 3.42 (s, 3H), 3.48 (s, 3H), 3.79 (s, 3H), 4.92 (s, 2H), 5.16-5.24 (m, 1H), 5.26 (s, 2H), 6.61 (s, 1H), 7.11 (d, *J* = 16.2 Hz, 1H), 7.48 (d, *J* = 16.2 Hz, 1H), 7.66-7.71 (m, 2H), 8.20-8.26 (m, 2H).

4.13. General Procedure to Synthesize Xanthohumol analogues 2-7, 9, 10, 12-14.

To a stirred solution of chalcone in MeOH/H_2O, concentrated HCl was added until pH 1 and the mixture was heated at 45 °C for 45 min. The reaction mixture was then extracted with EtOAc/H_2O, the organic layers were combined, dried over Na_2SO_4, filtered, and evaporated at 20 °C. The residue was purified by flash chromatography affording the desired compounds **2-7**, **9**, **10**, **12-14.**

4.14. (E)-3-(3,4-dichlorophenyl)-1-(2-hydroxy-6-methoxy-4-(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (2) and (E)-3-(3,4-dichlorophenyl)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (3).

The title compounds were prepared according the general procedure, starting from compound **20** (62 mg, 0.12 mmol). The residue was purified by flash chromatography (*n*-hexane/EtOAc 7:1) using a Isolute Flash Si II cartridge to afford **2** (31 mg, 57% yield) as orange solid and **3** (9 mg, 18% yield) as yellow solid. **(2)** mp 105-107 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (s, 3H), 1.79 (s, 3H), 3.32 (d, J = 7.1 Hz, 2H), 3.49 (s, 3H), 3.92 (s, 3H), 5.19-5.24 (m, 1H), 5.27 (s, 2H), 6.24 (s, 1H), 7.40 (dd, J_1 = 8.3 Hz, J_2 = 2.0 Hz, 1H), 7.46 (d, J = 8.3 Hz, 1H), 7.61 (d, J = 15.6 Hz, 1H), 7.65 (d, J = 2.0 Hz, 1H), 7.81 (d, J = 15.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.7, 21.6, 25.8, 55.9, 56.2, 89.1, 93.9, 106.9, 111.0, 122.5, 127.3, 129.6, 129.6, 130.8, 131.4, 133.1, 133.6, 135.7, 138.9, 160.9, 161.3, 164.3, 192.5. Elemental Analysis for $C_{23}H_{24}Cl_2O_5$ calculated: % C, 61.21; % H, 5.36; found: % C, 61.49; % H, 5.48. **(3)** mp 62-64 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.78 (s, 3H), 1.83 (s, 3H), 3.40 (d, J = 6.3 Hz, 2H), 3.90 (s, 3H), 5.26-5.32 (m, 1H), 5.95 (s, 1H), 6.26 (br, 1H), 7.39-7.48 (m, 2H), 7.60-7.66 (m, 2H), 7.84 (d, J = 15.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.0, 21.7, 25.9, 56.0, 91.4, 106.2, 106.4, 121.7, 127.4, 129.7, 129.8, 130.9, 133.2, 133.8, 135.9, 136.3, 139.1, 161.3, 162.5, 165.3, 192.3. Elemental Analysis for $C_{21}H_{20}Cl_2O_4$ calculated: % C, 61.93; % H, 4.95; found: % C, 62.24; % H, 5.12.

4.15. (E)-3-(4-fluorophenyl)-1-(2-hydroxy-6-methoxy-4-(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (4) and (E)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)-3-(4-fluorophenyl)prop-2-en-1-one (5).

The title compounds were prepared according the general procedure, starting from compound **21** (91 mg, 0.20 mmol). The residue was purified by flash chromatography (*n*-hexane/EtOAc 7:1) using a Isolute Flash Si II cartridge to afford **4** (6 mg, 7% yield) as orange solid and **5** (29 mg, 41% yield) as yellow solid. **(4)** mp 80-82 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (s, 3H), 1.79 (s, 3H), 3.33 (d, J = 7.1 Hz, 2H), 3.50 (s, 3H), 3.92 (s, 3H), 5.20-5.24 (m, 1H), 5.27 (s, 2H), 6.24 (s, 1H), 7.07-7.11 (m, 2H), 7.56-7.60 (m, 2H), 7.72 (d, J = 15.6 Hz, 1H), 7.79 (d, J = 15.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.9, 21.8, 25.9, 56.0, 56.3, 89.3, 94.0, 107.2, 111.1, 116.1 (d, $^{2}J_{C\text{-}F}$ = 21.8 Hz), 122.7, 127.7, 130.2, 131.4, 132.0, 140.8, 161.0, 161.2, 163.8 (d, $^{1}J_{C\text{-}F}$ = 250.9 Hz), 164.4, 193.1. Elemental Analysis for $C_{23}H_{25}FO_5$ calculated: % C, 68.99; % H, 6.29; found: % C, 69.13; % H, 6.13. **(5)** mp 167-169 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.77 (s, 3H), 1.83 (s, 3H), 3.40 (d, J = 7.2 Hz, 2H), 3.89 (s, 3H), 5.27-5.31 (m, 1H), 5.95 (s, 1H), 6.30 (br, 1H), 7.07-7.11 (m, 2H), 7.56-7.60 (m, 2H), 7.73 (d, J = 15.6 Hz, 1H), 7.82 (d, J = 15.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.0, 21.7, 25.9, 55.9, 91.3, 106.2, 106.5, 116.1 (d, $^{2}J_{C\text{-}F}$ = 21.9 Hz), 121.8, 127.7, 130.2, 132.0, 136.0, 140.9, 161.3, 162.2, 163.9 (d, $^{1}J_{C\text{-}F}$ = 250.0 Hz), 165.3, 192.8. Elemental Analysis for $C_{21}H_{21}FO_4$ calculated: % C, 70.77; % H, 5.94; found: % C, 70.99; % H, 5.70.

4.16. (E)-3-(2-fluorophenyl)-1-(2-hydroxy-6-methoxy-4-(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (6) and (E)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)-3-(2-fluorophenyl)prop-2-en-1-one (7).

The title compounds were prepared according the general procedure, starting from compound **22** (170 mg, 0.39 mmol). The residue was purified by flash chromatography (*n*-hexane/EtOAc 10:1) using a Isolute Flash Si II cartridge to afford **6** (86 mg, 54% yield) and **7** (13 mg, 9% yield) as yellow-orange solids. **(6)** mp 91-93 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (s, 3H), 1.79 (s, 3H), 3.33 (d, J = 7.1 Hz, 2H), 3.49 (s, 3H), 3.91 (s, 3H), 5.20-5.25 (m, 1H), 5.27 (s, 2H), 6.24 (s, 1H), 7.08-7.19 (m, 2H), 7.31-7.37 (m, 1H), 7.55-7.60 (m, 1H), 7.83 (d, J = 15.8 Hz, 1H), 7.97 (d, J = 15.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.9, 21.7, 25.9, 55.9, 56.3, 89.2, 94.0, 107.2, 111.1, 116.3 (d, $^{2}J_{C\text{-}F}$ = 22.0 Hz), 122.8, 123.8, 124.5, 129.8, 130.7, 131.3, 131.4, 134.6, 161.1, 161.3, 161.8 (d, $^{1}J_{C\text{-}F}$ = 254.0 Hz), 164.4, 193.3. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -113.85. Elemental Analysis for $C_{23}H_{25}FO_5$ calculated: % C, 68.99; % H, 6.29; found: % C, 69.28; % H, 6.62. **(7)** mp 157-159 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.77 (s, 3H), 1.83 (s, 3H), 3.39 (d, J = 7.2 Hz, 2H), 3.88 (s, 3H), 5.26-5.30 (m, 1H), 5.94 (s, 1H), 6.40 (br, 1H), 7.08-7.19 (m, 2H), 7.31-7.37 (m, 1H), 7.56 -7.60 (m, 1H), 7.84 (d, J = 15.8 Hz, 1H), 8.00 (d, J = 15.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 18.0, 21.7, 25.9, 55.8, 91.3, 106.3, 106.5, 116.3 (d, $^{2}J_{C\text{-}F}$ = 22.0 Hz), 121.8, 123.8, 124.5, 129.8, 130.6, 131.3, 134.7, 135.8, 161.4, 161.7 (d, $^{1}J_{C\text{-}F}$ = 253.9 Hz), 162.3, 165.3, 192.9. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -113.89. Elemental Analysis for $C_{21}H_{21}FO_4$ calculated: % C, 70.77; % H, 5.94; found: % C, 70.54; % H, 5.73.

4.17. (E)-3-(3-fluoro-4-methoxyphenyl)-1-(2-hydroxy-6-methoxy-4-(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (9) (E)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)-3-(3-fluoro-4-methoxyphenyl)prop-2-en-1-one (10).

The title compounds were prepared according the general procedure, starting from compound **8** (275 mg, 0.58 mmol). The residue was purified by silica gel flash chromatography (*n*-hexane/EtOAc 4:1) to afford **9** (24 mg, 10% yield) and **10** (111 mg, 50% yield) as yellow-orange

solids. **(9)** mp 89-91 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (s, 3H), 1.79 (s, 3H), 3.32 (d, J = 7.1 Hz, 2H), 3.49 (s, 3H), 3.93 (s, 6H), 5.21-5.23 (m, 1H), 5.27 (s, 2H), 6.24 (s, 1H), 6.97 (dd, $J_{H\text{-}F}$ = 8,5 Hz, $J_{H\text{-}H}$ = 8,5 Hz, 1H), 7.28-7.31 (m, 1H), 7.36 (dd $J_{H\text{-}F}$ = 12.2 Hz, $J_{H\text{-}H}$ = 2.0 Hz, 1H), 7.68 (d, J = 15.6 Hz, 1H), 7.74 (d, J = 15.6 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.9, 21.8, 25.9, 56.0, 56.4, 56.4, 89.3, 94.1, 107.2, 111.1, 113.3, 114.6 (d, $^{2}J_{C\text{-}F}$ = 18.5 Hz), 122.8, 126.1, 126.9, 129.1, 131.4, 140.9, 149.4, 152.6 (d, $^{1}J_{C\text{-}F}$ = 246.6 Hz), 161.0, 161.2, 164.4, 193.0. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -134.56. Elemental Analysis for $C_{24}H_{27}FO_6$ calculated: % C, 66.96; % H, 6.32; found: % C, 66.71; % H, 6.04. **(10)** mp 163-165 °C. ^{1}H NMR (400 MHz, Acetone-d_6) δ: 1.63 (s, 3H), 1.76 (s, 3H), 3.28 (d, J = 7.2 Hz, 2H), 3.93 (s, 3H), 3.95 (s, 3H), 5.23-5.26 (m, 1H), 6.15 (s, 1H), 7.20 (dd $J_{H\text{-}F}$ = 8.6 Hz, $J_{H\text{-}H}$ = 8.5 Hz, 1H), 7.48-7.51 (m, 1H), 7.54 (dd $J_{H\text{-}F}$ = 12.1 Hz, $J_{H\text{-}H}$ = 2.1 Hz, 1H), 7.69 (d, J = 15.5 Hz, 1H), 7.90 (d, J = 15.5 Hz, 1H), 9.23 (s, 1H). ^{13}C NMR (100 MHz, Acetone-d_6) δ: 17.8, 22.0, 25.8, 56.2, 56.6, 91.6, 106.3, 108.8, 114.5, 115.3, 115.5, 123.9, 126.9, 127.6, 129.7, 131.0, 141.4, 154.4, 161.9, 163.0, 166.3, 193.0. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -135.91. Elemental Analysis for $C_{22}H_{23}FO_5$ calculated: % C, 68.38; % H, 6.00; found: % C, 68.44; % H, 5.85.

4.18. (E)-3-(2-fluoro-4-methoxyphenyl)-1-(2-hydroxy-6-methoxy-4-(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)prop-2-en-1-one (12) and (E)-1-(2,4-dihydroxy-6-methoxy-3-(3-methylbut-2-en-1-yl)phenyl)-3-(2-fluoro-4-methoxyphenyl)prop-2-en-1-one (13).

The title compounds were prepared according the general procedure, starting from compound **11** (100 mg, 0.21 mmol). The residue was purified by silica gel flash chromatography (*n*-hexane/EtOAc 7:1) to afford **12** (41 mg, 45% yield) as orange solid and **13** (15 mg, 18% yield) as yellow-orange amorphous solid. **(12)** mp 94-96 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.66 (s, 3H), 1.78 (s, 3H), 3.31 (d, J = 7.1 Hz, 2H), 3.48 (s, 3H), 3.83 (s, 3H), 3.89 (s, 3H), 5.19-5.23 (m,

1H), 5.25 (s, 2H), 6.22 (s, 1H), 6.64 (dd, $J_{H\text{-}F}$ = 12.5 Hz, $J_{H\text{-}H}$ = 2.5 Hz, 1H), 6.72 (dd, J_1 = 8.6 Hz, J_2 = 2.5 Hz, 1H), 7.49 (dd, $J_{H\text{-}F}$ = 8.7 Hz, $J_{H\text{-}H}$ = 8.6 Hz, 1H), 7.80 (d, J = 15.8 Hz, 1H), 7.87 (d, J = 15.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.9, 21.8, 25.9, 55.8, 55.9, 56.3, 89.3, 94.1, 102.1 (d, $^2J_{C\text{-}F}$ = 26.0 Hz), 107.3, 110.8, 111.1, 116.6, 122.9, 128.1, 130.8, 131.4, 135.2, 161.0, 162.4, 162.5, 162.9 (d, $^1J_{C\text{-}F}$ = 254.4 Hz), 164.4, 193.3. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -110.93. Elemental Analysis for $C_{24}H_{27}FO_6$ calculated: % C, 66.96; % H, 6.32; found: % C, 67.15; % H, 6.06. **(13)** ^{1}H NMR (400 MHz, Acetone-d_6) δ: 1.65 (s, 3H), 1.77 (s, 3H), 3.30 (d, J = 7.1 Hz, 2H), 3.91 (s, 3H), 3.93 (s, 3H), 5.23-5.26 (m, 1H), 6.83 (dd, $J_{H\text{-}F}$ = 12.8 Hz, $J_{H\text{-}H}$ = 2.5 Hz, 1H), 6.88 (dd, J_1 = 8.4 Hz, J_2 = 2.5 Hz, 1H), 7.74-7.78 (m, 1H), 7.85 (d, J = 15.7 Hz, 1H), 8.02 (d, J = 15.7 Hz, 1H) 9.03 (br, 1H). ^{13}C NMR (100 MHz, Acetone-d_6) δ: 16.9, 21.1, 24.9, 55.2, 55.4, 90.8, 101.6 (d, $^2J_{C\text{-}F}$ = 26.0 Hz), 105.4, 108.0, 111.2, 115.8, 122.9, 127.5, 130.1, 130.2, 133.8, 161.0, 162.5 (d, $^1J_{C\text{-}F}$ = 252.0 Hz), 162.7, 162.8, 165.5, 192.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ: -114.07. Elemental Analysis for $C_{22}H_{23}FO_5$ calculated: % C, 68.38; % H, 6.00; found: % C, 68.49; % H, 6.31.

4.19. (E)-1-(2-hydroxy-6-methoxy-4-(methoxymethoxy)-3-(3-methylbut-2-en-1-yl)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one (14)

Starting from compound **23** (274 mg, 0.58 mmol) and following the general procedure, MOM mono-protected derivative **14** was obtained as the only reaction product. The residue was purified by flash chromatography (*n*-hexane/EtOAc 7:1) using a Isolute Flash Si II cartridge to afford **14** (191mg, 77% yield) as yellow-orange solids, mp 125-127 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.67 (s, 3H), 1.79 (s, 3H), 3.33 (d, J = 7.1 Hz, 2H), 3.50 (s, 3H), 3.94 (s, 3H), 5.19-5.25 (m, 1H), 5.28 (s, 2H), 6.26 (s, 1H), 7.70-7.74 (m, 3H), 7.94 (d, J = 15.7 Hz, 1H), 8.24-8.27 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 17.9, 21.7, 25.9, 56.1, 56.4, 89.3, 94.1, 107.1, 111.3,

122.6, 124.3, 128.8, 131.6, 132.1, 138.5, 142.1, 148.3, 161.1, 161.7, 164.5, 192.5. Elemental Analysis for $C_{23}H_{25}NO_7$ calculated: % C, 64.63; % H, 5.90; % N, 3.28; found: % C, 64.47; % H, 5.77; % N, 3.44.

4.20. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased by PromoCell (Heidelberg, Germany) and cultured in endothelial cell growth medium-2 (*EGM-2*) (Lonza, Basel, Switzerland) containing 10% FBS, 1% L-glutamine 2 mM and 1% Penicillin/Streptomycin (Sigma-Aldrich, Milan, Italy). Cells were treated with XN (Sigma-Aldrich, Milan, Italy) and its derivatives dissolved in DMSO in a stock solution of 20 mM and used at indicated concentrations for different time periods. In all biological assays XN was used as positive control.

4.21. Cell Proliferation assay

The effect of XN and its derivatives on HUVE cell proliferation was determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich) assay. Briefly, 1 x 10^3 cells were seeded into 96-well plates and incubated at 37 °C overnight. Cells were then treated with XN and its derivative with different concentrations ranging from 1 μM to 20 μM and incubated for 24, 48, 72 or 96 h. Control cells (CTRL) received EGM-2 medium containing 10% FBS, 1% L-glutamine 2 mM and 1% Penicillin/Streptomycin with dissolved vehicle (DMSO). MTT reagent 5 (mg/mL) was added to cell cultures and incubated for 3 hours at 37 °C. After incubation, medium was replaced with 100 μL of DMSO and the amount of solubilized formazan was quantified at 540 nm in a SpectraMax M2 (Molecular Devices, Sunnyvale CA).

4.22. Determination of apoptosis by Flow Cytometry

HUVECs (2 x 10^5 cells/well) were treated with increasing concentrations of XN and its synthetic derivatives (ranging from 5 μM to 20 μM) for 24h. Control cells received EGM-2 medium containing 10% FBS, 1% L-glutamine 2 mM and 1% Penicillin/Streptomycin with dissolved vehicle (DMSO). Cells were then collected and stained with FITC-conjugated Annexin V (BD: Becton Dickinson Bioscences, San Jose, CA) and 7-AAD (BD). Untreated cells and those treated with DMSO (vehicle) were used as control. This method allows discrimination between viable cells (Annexin V negative, 7-AAD negative) and apoptotic cells (Anexin V positive, 7-AAD positive/negative). Analysis was performed on 20000 gated cells, excluding cell debris, using a FACSCanto II (BD), with excitation set at 488 nm and emission at 518 nm (FITC detector) or 620 nm (PE fluorescence detector).

4.23. Cell adhesion assay in vitro

HUVECs (3x10^3 cells) were treated with XN and its derivatives (10 μM) and seeded in a 48 wells plate pre-coated for 45 min with fibronectin (2 μg/mL). Control cells received EGM-2 medium containing 10% FBS, 1% L-glutamine 2 mM and 1% Penicillin/Streptomycin with dissolved vehicle (DMSO). After 90 min incubation, the supernatants were removed and cells were washed with PBS. Cells were then fixed with 4% paraformaldehyde and stained with DAPI. Six different replicates for each condition were considered. Eighteen microscope fields were randomly selected from the six wells for each treatment to count the number of adherent cells.

4.24. HUVEC migration and invasion in vitro assays

Chemoinvasion and chemotaxis assays were performed in modified Boyden chambers, as previously described [25, 26]. Briefly, viable HUVE cells (5 x 10^4) were seeded in the upper compartment of the Boyden chamber and treated with XN and its derivatives (10 μM) for 6 h

and 24 h respectively for migration and invasion *in vitro* assays. Control cells received EGM-2 medium containing 10% FBS, 1% L-glutamine 2 mM and 1% Penicillin/Streptomycin with dissolved vehicle (DMSO). Chemoattractant (FBS 10%) in EGM-2 was added in the lower compartment. 10 µm pore-size polycarbonate filters were pre-coated with collagen IV (50 µg/mL, Sigma Aldrich) for the chemotaxis assay and matrigel (1 mg/mL, BD) for the chemoinvasion assay. After 6 h (chemotaxis) or 24 h (chemoinvasion) of incubation, filters were recovered and migrated or invaded cells on the lower filter surface were fixed with absolute ethanol and stained with DAPI. Viable cells were counted in a double-blind manner in 5 consecutive fields each with a Zeiss Microscope associated with a Nikon camera. Experiments were performed in triplicate.

4.25. Morphogenesis assay

$2{,}5x10^4$ HUVE cells were seeded on a 10 mg/mL matrigel pre-coated 96-well plate and treated with XN and its derivatives (10 µM) for 6 h at 37 °C and 5% CO_2. Control cells received EGM-2 medium containing 10% FBS, 1% L-glutamine 2 mM and 1% Penicillin/Streptomycin with dissolved vehicle (DMSO). After 6 h, the network formation was detected with an inverted microscope (Zeiss). Master segment numbers and length, meshes numbers and area were quantified using ImageJ software and the Angiogenesis Analyzer tool [27]. Four to five different replicates were used for each experiment. Cells treated with Serum free medium (SFM), media containing DMSO (vehicle) and media containing 10% FBS (FBS 10%) were used as controls.

4.26. Statistical Analyses

Flow cytometry analyses were performed using BD FACSDiva (v6.1.2) software. For the morphogenesis analysis, master segment length and, mesh numbers and areas were quantified

using the ImageJ software and the Angiogenesis Analyzer tool. Statistical analyses were performed using the GraphPad Prism 5 statistics and graphing program (GraphPad Software, San Diego, CA). One-way ANOVA with Dunnett's Multiple Comparison Test were used to compare multiple data sets.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at:

References

[1] J. Folkman, Angiogenesis: an organizing principle for drug discovery?, Nat. Rev. Drug Discov. 6 (2007) 273-286.

[2] D.M. Noonan, R. Benelli and A. Albini, Angiogenesis and cancer prevention: a vision, Recent Results Cancer Res. 174 (2007) 219-224.

[3] Z. Wang, C. Dabrosin, X. Yin, M.M. Fuster, A. Arreola, W.K. Rathmell, D. Generali, G.P. Nagaraju, B. El-Rayes, D. Ribatti, Y.C. Chen, K. Honoki, H. Fujii, A.G. Georgakilas, S. Nowsheen, A. Amedei, E. Niccolai, A. Amin, S.S. Ashraf, B. Helferich, X. Yang, G. Guha, D. Bhakta, M.R. Ciriolo, K. Aquilano, S. Chen, D. Halicka, S.I. Mohammed, A.S. Azmi, A. Bilsland, W.N. Keith and L.D. Jensen, Broad targeting of angiogenesis for cancer prevention and therapy, Semin. Cancer Biol. 35, Supplement (2015) S224-S243.

[4] A. Albini and M.B. Sporn, The tumour microenvironment as a target for chemoprevention, Nat. Rev. Cancer 7 (2007) 139-147.

[5] M.B. Sporn and K.T. Liby, Cancer chemoprevention: scientific promise, clinical uncertainty, Nat. Clin. Prac. Oncol. 2 (2005) 518-525.

[6] N. Ferrara and A.P. Adamis, Ten years of anti-vascular endothelial growth factor therapy, Nat. Rev. Drug Discov. 15 (2016) 385-403.

[7] T. Rossi, C. Gallo, B. Bassani, S. Canali, A. Albini and A. Bruno, Drink your prevention: beverages with cancer preventive phytochemicals, Pol. Arch. Med. Wewn. 124 (2014) 713-722.

[8] A. Albini, F. Tosetti, V.W. Li, D.M. Noonan and W.W. Li, Cancer prevention by targeting angiogenesis, Nat. Rev. Clin. Oncol. 9 (2012) 498-509.

[9] A. Albini, D.M. Noonan and N. Ferrari, Molecular Pathways for Cancer Angioprevention, Clin. Cancer Res. 13 (2007) 4320-4325.

[10] D.M. Noonan, R. Benelli and A. Albini, Angiogenesis and Cancer Prevention: A Vision. In: H.J. Senn, U. Kapp, (Eds.), Cancer Prevention, Springer Berlin Heidelberg, Berlin, Heidelberg, 2007; pp. 219-224.

[11] X. Yang, Y. Jiang, J. Yang, J. He, J. Sun, F. Chen, M. Zhang and B. Yang, Prenylated flavonoids, promising nutraceuticals with impressive biological activities, Trends Food Sci. Tech. 44 (2015) 93-104.

[12] C. Gerhauser , A. Alt, E. Heiss, A. Gamal-Eldeen, K. Klimo, J. Knauft, I. Neumann, H.-R. Scherf, N. Frank, H. Bartsch and H. Becker, Cancer Chemopreventive Activity of Xanthohumol, a Natural Product Derived from Hop, Mol. Cancer Ther. 1 (2002) 959-969.

[13] M. Verzele, J. Stockx, F. Fontijn and M. Anteunis, Xanthohumol, a New Natural Chalkone, Bull. Soc. Chim. Belg. 66 (1957) 452-475.

[14] M. Liu, P. Hansen, G. Wang, L. Qiu, J. Dong, H. Yin, Z. Qian, M. Yang and J. Miao, Pharmacological Profile of Xanthohumol, a Prenylated Flavonoid from Hops (Humulus lupulus), Molecules 20 (2015) 754-779.

[15] Clinical Trials.gov, Identifier: NCT01367431, NCT02432651, NCT02848430. https://clinicaltrials.gov/ 2017 (accessed March 2017).

[16] C. Gallo, K. Dallaglio, B. Bassani, T. Rossi, A. Rossello, D.M. Noonan, G. D'Uva, A. Bruno and A. Albini, Hop derived flavonoid xanthohumol inhibits endothelial cell functions via AMPK activation, Oncotarget 7 (2016) 59917-59931.

[17] A. Rossello, E. Nuti, E. Orlandini, S. Nencetti, A. Albini, A.R. Cantelmo, D. Bartolini, D.M. Noonan and C. Gallo, Synthetic analogues of xanthohumol, PCT Int. Appl. (2014) WO2014167481.

[18] B. Zhang, D. Duan, C. Ge, J. Yao, Y. Liu, X. Li and J. Fang, Synthesis of Xanthohumol Analogues and Discovery of Potent Thioredoxin Reductase Inhibitor as Potential Anticancer Agent, J. Med. Chem. 58 (2015) 1795-1805.

[19] J.A. Kim, Y. Kang, D. Thapa, J.S. Lee, M.A. Park, K.H. Lee, W.S. Lyoo and Y.R. Lee, Anti-Invasive and Anti-Angiogenic Effects of Xanthohumol and Its Synthetic Derivatives, Biomol. Ther. 17 (2009) 422-429.

[20] R.S. Khupse and P.W. Erhardt, Total Synthesis of Xanthohumol, J. Nat. Prod. 70 (2007) 1507-1509.

[21] S. Vogel, S. Ohmayer, G. Brunner and J. Heilmann, Natural and non-natural prenylated chalcones: Synthesis, cytotoxicity and anti-oxidative activity, Bioorg. Med. Chem. 16 (2008) 4286-4293.

[22] A. Albini, R. Dell'Eva, R. Vené, N. Ferrari, D.R. Buhler, D.M. Noonan and G. Fassina, Mechanisms of the antiangiogenic activity by the hop flavonoid xanthohumol: NF-κB and Akt as targets, FASEB J. 20 (2006) 527-529.

[23] T. Kumazawa, T. Minatogawa, S. Matsuba, S. Sato and J.-i. Onodera, An effective synthesis of isoorientin: the regioselective synthesis of a 6-C-glucosylflavone, Carbohydr. Res. 329 (2000) 507-513.

[24] X. Bu, L. Zhao and Y. Li, A Facile Synthesis of 6-C-Prenylflavanones, Synthesis 1997 (1997) 1246-1248.

[25] A. Albini and R. Benelli, The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation, Nat. Protoc. 2 (2007) 504-511.

[26] A. Albini and D.M. Noonan, The 'chemoinvasion' assay, 25 years and still going strong: the use of reconstituted basement membranes to study cell invasion and angiogenesis, Curr. Opin. Cell Biol. 22 (2010) 677-689.

[27] G. Carpentier, Contribution: Angiogenesis Analyzer. ImageJ News, 2012.

Figure captions

Fig. 1. Natural product Xanthohumol (XN) and its new synthetic analogues.

Figure 2. Effects of XN derivatives on endothelial cell proliferation *in vitro* –XN and XN derivatives interfere with endothelial cell (HUVEC) proliferation, as assessed by MTT assay. Results are showed as Mean ± SEM; *p <0.05, ***p<0.001 compared to XN alone. "Vehicle" indicates cells treated with vehicle (DMSO in the medium).

Figure 3. Effects of XN derivatives on endothelial cell viability *in vitro* - Flow cytometry analysis showed that 24 h treatment of endothelial cells with 20 μM XN derivatives resulted in increased rates of apoptotic (AnnexinV^{+}7-AAD$^{-/+}$) cells. **"**Vehicle" indicates cells treated with vehicle (DMSO in the medium). Results are showed as Mean ± SEM.

Figure 4. Effects of xanthohumol derivatives on HUVE cell adhesion, invasion and migration. XN and XN derivatives at 10 μM concentration can interfere with crucial steps of angiogenesis by decreasing HUVEC A**)** adhesion B) migration and C) invasion, as compared to vehicle-treated cells. **"**Vehicle" indicates cells treated with vehicle (DMSO in the medium). Results are showed as Mean ± SEM. *p <0.05; **p<0.001; ***p<0.001 as indicated by the bars.

Figure 5. Effects of XN derivatives on HUVEC morphogenesis – Following 6 h treatment, XN and all XN derivatives at 10 μM concentration can inhibit HUVEC ability to form capillary-like structures on matrigel compared with vehicle-treated cells. SFM: cells treated with serum-free EGM-2 medium as a negative control. FBS 10%: cells treated with medium containing 10% FBS without vehicle. **"**Vehicle" indicates cells treated with vehicle (DMSO in the medium). Results are showed as Mean ± SEM. *p <0.05; **p<0.001; ***p<0.001 as indicated by the bars.