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Relative effects of phenolic constituents from *Yucca schidigera* Roezl. bark on Kaposi's sarcoma cell proliferation, migration, and PAF synthesis

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ABSTRACT

Yuccaols (A, B, C) are phenolic constituents isolated from *Yucca schidigera* bark characterized by unusual spirostructures made up of a C_{15} unit and a stilbenic portion closely related to resveratrol. These novel compounds are of particular interest for their antioxidant and anti-inflammatory properties. However, their effects on cell proliferation, migration, and platelet-activating factor (PAF) biosynthesis remain unknown. PAF, a potent mediator of inflammation, is known to promote angiogenesis and in vitro migration of endothelial cells and Kaposi's sarcoma (KS) cells. The objective of our study was to determine the effect of Yuccaols and resveratrol on the vascular endothelial growth factor (VEGF)-induced proliferation, migration, and PAF biosynthesis in KS cells. The results indicated that Yuccaols (25 μM) were more effective than resveratrol (25 μM) in inhibiting the VEGF-induced KS cell proliferation. Western blot analysis revealed that Yuccaols reduced the VEGF-induced phosphorylation of p38 and p42/44, thus indicating a possible interference with the mechanism underlying the VEGF-stimulated cell proliferation. Furthermore, Yuccaols completely inhibited the VEGF-stimulated PAF biosynthesis catalyzed by the acetyl-CoA:lyso-PAF acetyltransferase and enhanced its degradation through the PAF-dependent CoA-independent transacetylase (250% of control). In addition, Yuccaol C abrogated the PAF-induced cell motility whereas Yuccaol A and Yuccaol B reduced the cell migration from 7.6 μm/h to 6.1 μm/h and 5.6 μm/h, respectively. These results indicate that the anti-inflammatory properties attributed to *Yucca schidigera* can be ascribed to both resveratrol and Yuccaols and provide the first evidences of the anti-tumor and anti-invasive properties of these novel phenolic compounds.

1. Introduction

Resveratrol (*trans*-3,4′,5-trihidroxylstilbene), a natural polyphenolic phytoalexin found in seeds, grapes (*Vitis vinifera*) and in some medicinal plants is a potent dietary antioxidant which plays an important role in protecting against pathological events of oxidative diseases, such as cardiovascular diseases, cancer, inflammation, and brain dysfunction [1].

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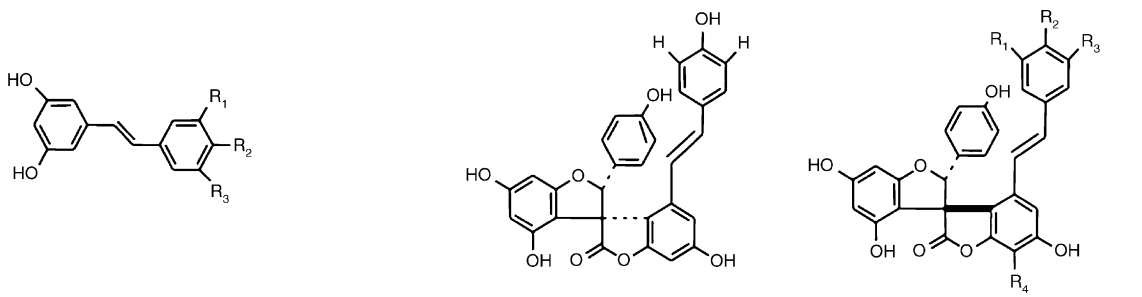
Multiple lines of compelling evidence indicate its striking inhibitory effect on various cellular events associated with the multi-step process of carcinogenesis, i.e., tumor initiation, promotion and progression [2].

The mechanisms for the cardioprotective and cancer-chemopreventive activities of resveratrol include induction of apoptosis, interference with extracellular signal-regulated kinases (ERKs) and p38 phosphorylation, scavenging or inhibition of oxygen-derived radicals, and down-regulation of pro-inflammatory mediators [3,4]. In addition, the mechanism(s) by which resveratrol exerts its anti-inflammatory activity has been ascribed to its ability to inhibit both cyclooxygenase, 5-lipoxygenase and inducible nitric oxide synthase (iNOS) activity [5,6], and to attenuate the inflammatory effects of platelet-activating factor (PAF) [7]. PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), belongs to the related family of acetylated alkyl phosphoglycerides and is produced by a broad range of cells including neutrophils, macrophages, endothelial cells, and Kaposi's sarcoma (KS) cells [8]. It elicits diverse and potent biological properties relevant for the development of inflammatory reaction, embryogenesis, cell differentiation, and cell migration [9,10]. KS, a strongly angiogenic and inflammatory tumor associated with infection by human immunodeficiency virus-1 (HIV-1), is characterized by the presence of three main populations of spindle cells, having the features of lymphatic-vascular endothelial cells, macrophagic-dendritic cells, and a mixed macrophage-endothelial phenotype.

The PAF produced by KS cells is sufficient to activate the PAF receptor (PAF-R) and to elicit biological responses, such as the events involved in angiogenesis and cell migration [11,10]. KS cells newly synthesize PAF when treated with HIV-1 Tat protein or inflammatory cytokines, as shown by the incorporation of labelled acetate in the lyso-PAF molecule to form PAF [10]. The acetylation of the lyso-PAF intermediate occurs during the second step of remodeling pathway *via* the acetyl-CoA:lyso-PAF acetyltransferase (AT). This membrane-bound enzyme catalyses the transfer of the acetyl moiety from acetyl-CoA to the free hydroxyl group at the *sn*-2 position of the lyso-PAF molecule produced by the deacylation of membrane glycerolipids catalyzed by a phospholipase A_2 (PLA_2). We have recently demonstrated that natural antioxidants, such as flavonoids and lycopene, modulate the PAF metabolism in endothelial cells during oxidative stress by blocking its production and, concomitantly, by enhancing the formation of less biologically active lipid mediators through the PAF-dependent CoA independent transacetylase (TA) [12,13]. The TA, a multifunctional enzyme with three catalytic activities, transfers the acetyl group from PAF to lysophospholipids (TA_L activity), sphingosine (TA_S activity), or hydrolyse PAF (AH activity). This enzyme, reported to be identical to PAF-AH (II), plays a key role in the PAF metabolism by modifying the cellular function of PAF through the generation of diverse lipid mediators [14]. In light of the numerous beneficial effects of resveratrol, there has been an unprecedented interest in natural products structurally related to this remarkable compound. Consequently, a wide variety of natural products are under investigation for their potential application in disease prevention and treatment. *Yucca schidigera* (*Y. schidigera*) Roezl, Agavaceae, a plant native of South-Western United States, North Baja California and Mexico deserts, has been used for centuries in folk medicine to treat a variety of inflammatory disorders, especially gonorrea, arthritis, and rheumatism [15]. From the bark of this plant, along with resveratrol (resv) and *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (Y1), three novel phenolic compounds namely Yuccaol A (Yol A), Yuccaol B (Yol B), and Yuccaol C (Yol C) were isolated. Yols (A–C) are characterized by unusual spirostructures made up of a C_{15} unit, probably derived from a flavonoid skeleton, and a stilbenic portion, linked via a γ-lactone ring (Fig. 1). They represent a unique example in nature of spirostructures including C_{15} and C_{14} units condensed to form a γ-lactone ring. Yols (A, B, C) differ for the stilbenic portion and for the stereochemistry at C-3. The stilbenic portion is resveratrol in Yols A–B and *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene in Yol C. The stereochemistry at C-3 is the same in Yols B–C and opposite in Yol A [16]. These compounds with antioxidant properties have recently been shown to reduce enzymatic platelet lipid peroxidation and to inhibit blood platelet oxidative stress [17,18]. Moreover, the anti-inflammatory properties of *Y. schidigera* have been shown to be due to the capability of Yol C to modulate inducible nitric oxide synthase (iNOS) expression via NF-kB [19]. In light of the importance of the potential application of Yols as new anti-inflammatory and anti-cancer agents, in the present study we sought to investigate their effect on cell proliferation, cell migration, and on the modulation of PAF biosynthesis in KS cells.



resveratrol R_1 = H R_2 = OH R_3 = H
trans-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (Y1) R_1 = OH R_2 = OMe R_3 = OH

yuccaol A

yuccaol B R_1 = H R_2 = OH R_3 = H R_4 = H
yuccaol C R_1 = OH R_2 = OMe R_3 = OH R_4 = H

Fig. 1 – Structures of Yuccaols and resveratrol.

2. Materials and methods

2.1. Materials

PAF, acetyl-CoA, lysophosphatidylcholine (lyso-GPC or lyso-PAF), dimethylsulphoxide (DMSO), bovine serum albumin (BSA), leupeptin, sodium orthovanadate, dithiothreitol (DTT), and common laboratory chemicals were purchased from Sigma Chemical Co. VEGF was obtained from Prepotec. MAP kinase inhibitors, SB203580 and PD98059, were from Alexis Biochemicals. Alkenyl-lyso-glycero-phosphoethanolamine (LPE) was a product from Serdary Research Lab. [^{3}H]acetyl-PAF (13.5 Ci/mmol), [^{3}H]acetate (1.9 Ci/mmol), and [^{3}H]acetyl-CoA (1.54 Ci/mmol) were purchased from NEN Life Science Products. XTT Cell Proliferation Kit II was from Roche. Rabbit polyclonal antibodies anti p-p38 and anti p-p42/44, anti-p38, anti p42/44, Cruz Marker Molecular Weight standards, and the horseradish peroxidase-linked secondary antibody were from Santa Cruz Biotechnology Inc. All culture reagents were from Invitrogen, Inc. Silica gel plates were from Analtech Inc. Phospholipids were tested for purity by thin-layer chromatography (TLC), and only >95% pure phospholipids were used in the experiments.

2.2. Isolation of Y. schidigera compounds

Pure resveratrol (resv) and compounds Y1, Yols A–C were obtained as previously reported by Oleszek et al. [16]. Part of the phenolic fraction (50 mg) obtained from Y. *schidigera* bark was chromatographed on HPLC on a Waters μ-Bondapack RP-18) column (30 cm × 7.6 mm i.d.) applying a linear gradient of H_2O/CH_3CN (4:1) for 5 min, then a linear gradient of H_2O/CH_3CN (7:3) for 30 min, followed by isocratic elution for 10 min and a linear gradient of H_2O/CH_3CN (3:2) for 30 min (flow rate 3 mL/min), affording *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (5.1 mg, t_R = 18.8 min), resveratrol (2.5 mg, t_R = 29.9 min), Yuccaol C (8.0 mg, t_R = 43.9 min), Yuccaol A (3.1 mg, t_R = 57.8 min), and Yuccaol B (1.8 mg, t_R = 62.4 min).

2.3. Cell culture and treatment

KS cells, derived from HIV-1 patient and spontaneously immortalized, were a kind gift of Dr. Giovanni Camussi (Univ. of Torino, Italy). Cells were cultured in 75 cm^2 flasks in 20 ml RPMI 1640 with 10% FBS at 37 °C in 5% CO_2 in air. Subconfluent monolayers were prepared by seeding KS in 24-well plates at the density of 5×10^3 cells/well.

The day before the experiments, subconfluent monolayers of KS cells were starved for 12 h in serum free media. Rested cells were then washed twice with 5 ml of HBSS–10 mM HEPES and incubated for the indicated times with or without VEGF (1 nM) in serum-free media. VEGF was used at a concentration of 1 nM, previously reported by others to be the optimal concentration for the activation of cell proliferation, migration, and PAF biosynthesis in endothelial cells and KS cells [20,21]. When phenolic constituents from Y. *schidigera* were tested before VEGF treatment, cells were pre-incubated at 37 °C for 2 h with resv, Y1, Yol A, Yol B, or Yol C prepared in DMSO. At the end of the pre-incubation, the media were removed and the cells were washed twice with 5 ml of HBSS–10 mM HEPES. The final DMSO concentration in the media was less than 0.1% and the cells viability was >95%, as assessed by trypan blue dye exclusion

2.4. Mitogenic assay

To assay mitogenic activity, KS cells were seeded in 24-well plates (5×10^3 cells/well) and allowed to attach for 24 h. Cells were then washed twice with 2 ml HBSS–10 mM HEPES and rested for 12 h in serum-free media. Rested cells were pre-incubated at 37 °C for 2 h in fresh serum-free media with or without different concentration of resv, Y1, Yol A, Yol B, or Yol C. At the end of preincubation, the media were removed and the cells were washed twice with 2 ml HBSS–10 mM HEPES before stimulation with VEGF (1 nM) for 24 h in media containing 1% FBS. The cell number was determined both by XTT colorimetric assay and a Coulter counter. The optical density (OD) of each well was measured with a microplate spectrophotometer (Biorad) equipped with 490 nm filter.

2.5. Western blot analysis of p38 and p42/44 phosphorylation

Experiments to determine p38 and p42/44 phosphorylation during treatment of KS cells with VEGF in the presence or absence of pre-treatment with Yol C were performed by treating KS cells as described above for the mitogenic assay.

Briefly, MAP kinase inhibitors, SB 203580 and PD 98059, were employed to block selectively the activation of p38 and p42/44 MAP kinase pathways, respectively. Cells were pre-treated with SB 203580 (25 μM) for 30 min at 37 °C in 5% CO_2 in air or for 60 min with PD 98059 (50 μM) (20 min at 4 °C, 20 min at room temperature, and 20 min at 37 °C in 5% CO_2 in air) before stimulation with VEGF (1 nM) for various times. DMSO was used as inhibitor vehicle and its final concentration in the media was less than 0.1%.

After treatments, cells were lysed at 4 °C for 1 h in lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml Aprotinin, 5 μg/ml Leupeptin, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) and centrifuged at 10,000 × *g* for 10 min. The protein content of the supernatants was measured by Lowry method [22]. Aliquots containing 50 μg of cell lysate proteins were subjected to 12.5% SDS-PAGE under reducing conditions and blotted onto nitrocellulose membrane filters. The blots were blocked for 2 h at 37 °C in 2% BSA, 1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, 0.05 Tween-20 (PBS-T). The nitrocellulose membranes were washed once with PBS-T for 15 min, three times for 5 min with 1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl (PBS) and immunoblotted for 2 h at room temperature with rabbit polyclonal anti-p-p38 antibodies or with rabbit anti-p-p42/44 antibodies. After one washing with PBS-T and three washing with PBS, the blots were incubated for 1 h at room temperature with HRP labelled anti-rabbit IgG, washed with PBS, developed for 2 min with ECL reagent (Pierce), and exposed to Biomax film (Kodak Co.).

2.6. In vitro KS cell migration

Subconfluent monolayers were prepared the day before the experiment by seeding KS cells in 35-mm Petri dishes.

Cells were rested for 12 h in serum-free media and pre-incubated for 2 h with 25 μM of each compound. After pre-incubation, KS were washed twice with 2 ml HBSS–10 mM HEPES and treated for 24 h with PAF (40 ng/ml) in serum-free medium. Cell migration was studied under a Nikon Diaphot inverted microscope in a plexiglass Nikon NP-2 incubator at 37 °C. Migration tracks were generated by marking the position of nucleus of individual cells on each image. Image analysis of at least 30 cells/sample was performed by digital saving of images at 30 min of interval with a Micro-Image analysis system (Casti Imaging s.r.l., Venice, Italy).

2.7. Enzyme assays

The AT and TA_L activities were assayed on total cell homogenates as previously described [23]. Briefly, the incubation mixtures for the AT assay contained 500 μM [^{3}H]acetyl-CoA (0.2 μCi), 50 μM lyso-PAF suspended in 3.3% BSA-saline, 100 mM Tris–HCl (pH 7.2) and 100 μg of cell homogenate protein in a final volume of 0.5 ml. The samples were incubated at 37 °C for 30 min and the lipids were extracted by the method of Bligh and Dyer [24] except that 2% of acetic acid was included in the methanol. The extracted lipids were separated by thin layer chromatography (TLC) using a solvent system of $CHCl_3/CH_3OH/NH_4OH/H_2O$ (60:35:8:2.3, v/v/v/v). The radioactivity of the areas corresponding to PAF was determined by liquid scintillation counting. The TA_L activity was determined by incubating at 37 °C for 30 min [^{3}H]acetyl-PAF (50 μM, 0.5 μCi in 0.1% BSA-saline) as acetyl donor and LPE (300 μM in 0.1% BSA-saline) as substrate acceptor in a mixture containing 100 μg of cell homogenate protein, 100 mM Tris–HCl (pH 7.4), 5 mM EDTA, 1 mM sodium acetate in a final volume of 0.25 ml. The extracted lipids were separated by TLC using a solvent system of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50/25/8/4, v/v/v/v) and the radioactivity of the areas corresponding to [^{3}H]acetyl-PAF and [^{3}H]acetyl-PE was determined by liquid scintillation counting.

2.8. PAF accumulation assay

To measure PAF accumulation, a metabolic labelling was performed as previously described [23]. Briefly, KS cells were cultured in 100 mm culture dish at cell density of 5×10^6 cells/dish and incubated at 37 °C with [^{3}H]acetate (25 μCi) in the presence of 10 ml of HBSS–10 mM HEPES with or without agonists for various times. At the end of the incubations, cells were washed twice with 5 ml of HBSS–10 mM HEPES to remove excess of radiolabel before scraping into 3 ml of methanol. The cellular lipids were extracted by the method of Bligh and Dyer [24] and the 1-radyl-[^{3}H]acetyl-GPC fraction was isolated by TLC with a solvent system of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (50:25:8:4, v/v/v/v/). Radioactivity of the lipid fractions was determined by area scraping of the silica gel into vials for liquid scintillation counting.

2.9. Statistical analysis

Data are expressed as means ± S.E.M. from at least three independent experiments in duplicate. Statistical analysis was performed with Student's *t*-test. Probability values were considered significant at $P < 0.05$.

3. Results

3.1. Effects of *Y. schidigera* compounds on KS cell proliferation

VEGF, a regulator of lymphatic and vascular endothelial development has been shown to be involved not only in KS-associated angiogenesis but also in the control of KS cell growth and migration [25]. To determine the effect of the phenolic constituents isolated from *Y. schidigera* bark on the VEGF-induced KS cell proliferation, cells were pre-incubated for 2 h with each compound, i.e., resv, Y1, Yol A, Yol B, or Yol C (Fig. 1).

It has been reported that resveratrol markedly inhibits mitogen-, cytokine-, and alloantigen-induced proliferation of splenic lymphocytes at concentrations of 25–50 μM [26]. Therefore, 25 μM was initially chosen as the concentration to study the anti-proliferative effect of *Y. schidigera* compounds after testing the absence of in vitro cytotoxic activity by XTT and trypan blue dye assays.

Results shown in Fig. 2 indicated that treatment with VEGF (1 nM) was highly mitogenic for KS cells reaching a maximal induction after 48 h (from 0.9 OD in control cells to 2.3 OD in VEGF-treated cells). After 48 h of treatment, KS cell number was 45×10^3 in control and 154×10^3 in VEGF-treated cells compared to control cells at time zero, i.e., 5×10^3 cells/well

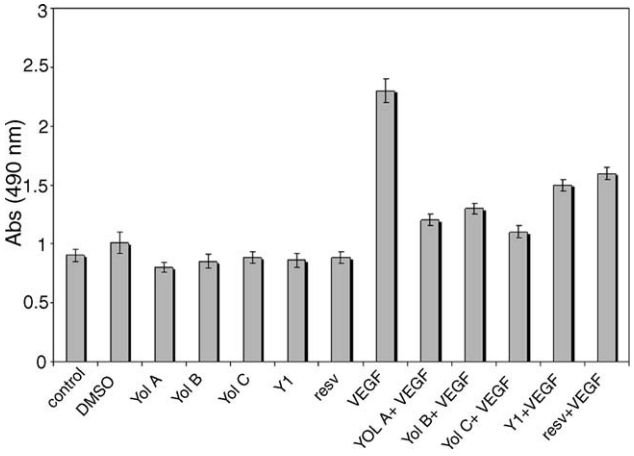


Fig. 2 – Growth inhibition in Yol-treated KS cells. Rested KS cells were pre-treated at 37 °C for 2 h with or without 25 μM resveratrol (resv), *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (Y1), and phenolic compounds, Yuccaol A (Yol A), Yuccaol B (Yol B), and Yuccaol C (Yol C) followed by stimulation with VEGF (1 nM) for 24 h in media containing 1% FBS. The cell number was determined both by XTT colorimetric assay and Coulter counter as described under Methods. Data represents the means ± S.E.M. from at least three independent experiments in duplicates with $P < 0.01$ for cells treated with resv, Y1, and Yols vs. cells pre-treated with DMSO before VEGF stimulation.

(data not shown). Pre-treatment with the compounds at a concentration of 25 μM considerably inhibited the VEGF-induced KS cell growth. A significant difference was observed between the abilities of the compounds to inhibit cell growth. In particular, Yols A–C were more efficient in inhibiting KS cell proliferation compared to resv and Y1. Moreover, among them, Yol C almost completely blocked the VEGF-induced cell growth. Compounds alone did not significantly inhibit the basal cell proliferation.

As shown in Fig. 3 (panel A) pre-treatment with Yol C reduced KS cell proliferation dose-dependently with a maximal effect starting at 10 μM (OD = 1.05), as compared to KS cells pre-treated only with DMSO (OD = 2.41). Moreover, micrographs of KS cells during treatment with Yol C further demonstrated that this compound did not show any cytotoxic effect up to a concentration of 25 μM (Fig. 3 panel B).

These data indicated that phenolic compounds from *Y. schidigera* bark, in particular the three novel resveratrol related compounds, Yols A–C, markedly inhibit mitogen-induced proliferation of KS cells.

3.2. *Yol effect on p38 and p42/44 phosphorylation*

The activation of p42/44 MAP kinase has been shown to be important for VEGF-induced cell proliferation in different cell types [27]. In KS cells growth factors, oncostatin M, sIL, 6R/IL-6, TNFα, and IL-1β have been shown to induce tyrosine phosphorylation of several proteins and to activate Flk-1/KDR, c-Src kinase, p38 and p42/44 MAP kinase with a clearly recognizable p-p42/44 immunoreactive bands observed between 5 and 30 min. [21,28]. Moreover, resveratrol is known to inhibit p38 and p42/44 phosphorylation induced by fibroblast growth factor 2 (FGF-2) and by endothelial growth factor (EGF) [29–31]. Therefore, we sought to determine the signalling components that regulate the anti-proliferative action of Yols by investigating the p38 and p42/44 activation pattern in KS cells challenged with Yols and then stimulated with VEGF. Data shown in Fig. 4 confirmed that VEGF lead to simultaneous activation of p38 (panel A) and p42/44 (panel B) MAP kinase signalling pathways. Specifically, we observed a transient time-dependent phosphorylation of both p38 (p-p38) and p42/44 (p-p42/44) with a peak within 10–30 min following VEGF stimulation. The phosphorylation of p38 and p42/44 was completely inhibited by pre-treatment with SB 203580 and PD 98059, respectively. Interestingly, as shown in Fig. 5, Western blot analysis of lysates from KS cells pre-treated for 2 h with 25 μM Yols before VEGF stimulation revealed a considerable reduction of the p-p42/44 and p-p38 immunoreactive bands. These results indicated that Yols (A, B, C) interfere with p42/44 and p38 MAP kinase signalling pathways activated by VEGF.

3.3. *Effect of Y. schidigera compounds on PAF metabolism*

The multiple actions of VEGF include the stimulation of cell migration and PAF synthesis [20]. PAF synthesis has been shown to play a critical role in triggering the motogenic activity of both KS and endothelial cells [25,10]. Specifically, it has been shown that KS cells synthesize PAF upon stimulation with HIV-1 Tat protein and that cell motility induced by this protein is dependent on PAF synthesis [10]. However, it is still unknown whether VEGF acts as agonist for the PAF biosynthesis in KS cells.

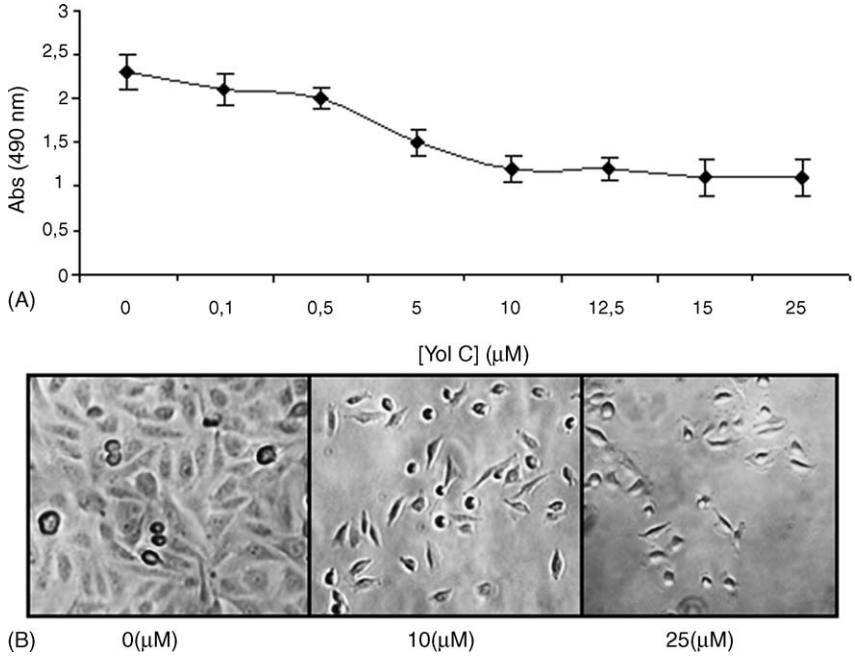


Fig. 3 – Dose-dependent effect of Yol C on KS cell growth. Rested KS cells were pre-treated at 37 °C for 2 h with or without various concentration of Yol C and then stimulated with VEGF (1 nM) for 24 h in media containing 1% FBS. (A) The cell number was determined by XTT colorimetric assay, as described under Methods. (B) Micrographs representative of control KS cells and KS cells pre-treated with 10 μM or 25 μM Yol C. Results shown are the means ± S.E.M. from four independent experiments in duplicates ($n = 8$) with $P < 0.01$ for cells pre-treated with 10 μM up to 25 μM Yol C vs. cells pre-treated with DMSO.

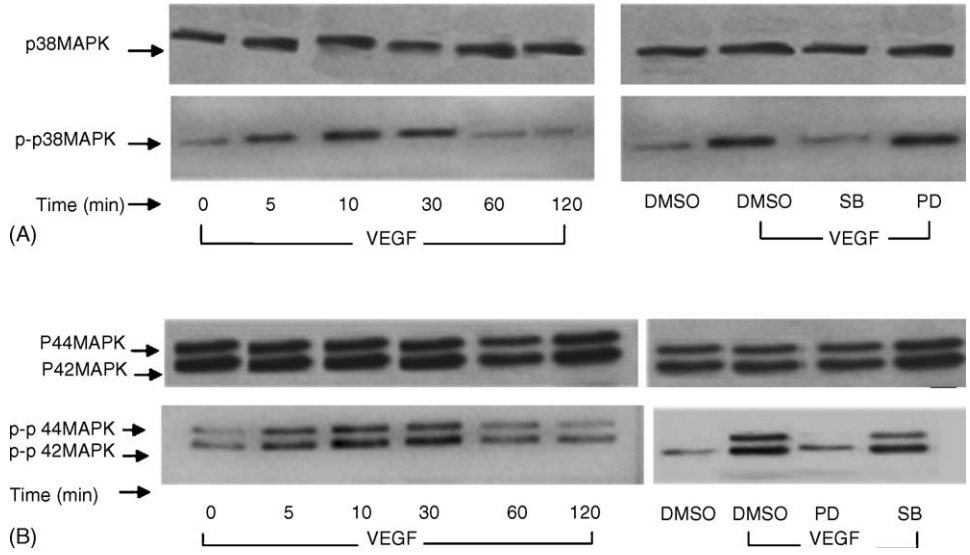


Fig. 4 – Time-dependent effect of VEGF on the MAPK phosphorylation in KS cells. Rested KS cells were incubated for various times with VEGF (1 nM) or were pre-treated for 60 min with 50 μM PD 98059 or for 30 min with 25 μM SB 203580 before stimulation with VEGF (1 nM). Both total and phosphorylated p38 (A) and p 42/44 (B) in cell homogenates were determined by Western blot analysis as described under Methods. The immunoblots shown are representative of four experiments.

Therefore, before analyzing the effect of *Y. schidigera* phenolic compounds on the in vitro KS cells migration induced by PAF, we first asked whether VEGF activates the production of this potent lipid mediator in KS cells. As reported in Fig. 6, KS cells stimulated with VEGF (1 nM) showed a rapid increase of the PAF synthesis catalysed by the AT. Both AT and TA_L activities peaked between 5 and 10 min after VEGF addition. Specifically, at 7 min the AT activity increased about 300% (% of the control) whereas the TA_L activity was slightly induced (179% of the control). On the basis of these data we determined the PAF accumulation in KS cells by quantifying the [^{3}H]acetate incorporation into 1-radyl-2-lyso-GPC during stimulation with VEGF (1 nM) for 7 min. Results indicated that the 1-radyl-2-[^{3}H]acetyl-GPC production was increased about four-fold in VEGF treated cells, specifically from 1530 ± 26 cpm/dish in control cells to about 6275 ± 44 cpm in VEGF treated cells (data not shown).

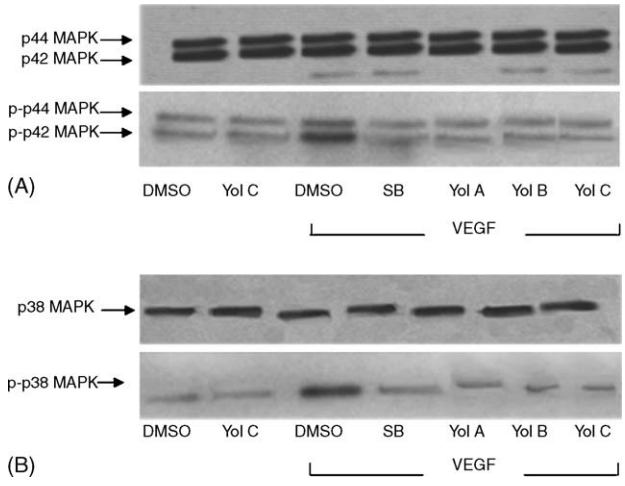


Fig. 5 – Effect of Yols on MAPK phosphorylation in KS cells. Rested KS cells were pre-treated at 37 °C with or without (25 μM) Yol A, (25 μM) Yol B, (25 μM) Yol C (50 μM) PD 98059 or (25 μM) SB 203580 and then stimulated with VEGF (1 nM) for 30 min, as described under Methods. Cell lysates were used for Western blot analysis to determine both total and phosphorylated p38 and p 42/44. The immunoblots shown are representative of five experiments.

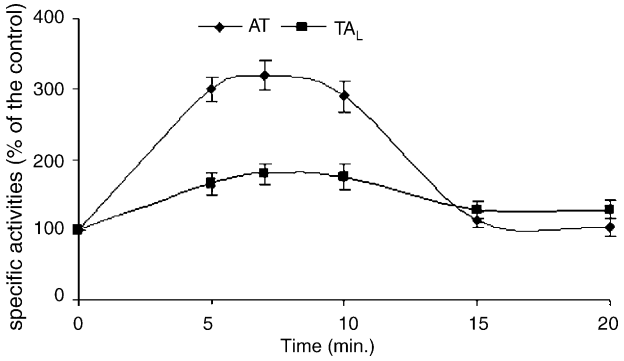


Fig. 6 – Time-dependent activation of AT and TA_L in VEGF-treated KS cells. Monolayers of subconfluent KS cells were stimulated with VEGF (1 nM) at the indicated times. At the end of the incubations the total cell homogenates were prepared and the AT and TA_L activities were determined as described in Methods. The AT and TA_L specific activities were 11.5 nmol/min/mg prot. and 276 pmol/min/mg prot., respectively. Data are mean ± S.E.M. of three separate experiments in duplicates ($n = 6$) with $P < 0.001$ for the AT activity at 7 min (VEGF vs. untreated).

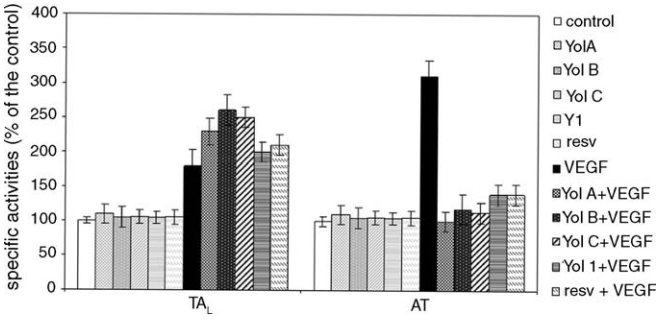


Fig. 7 – Effect of *Y. schidigera* phenolic constituents on AT and TA$_L$ activities. AT and TA$_L$ activities were determined on the total cell homogenates from untreated KS cells, and KS cells pre-treated for 2 h with or without 25 μM resveratrol (resv), *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (Y1), Yuccaol A (Yol A), Yuccaol B (Yol B), or Yuccaol C (Yol C) followed by stimulation VEGF (1 nM) for 7 min. The AT and TA$_L$ specific activities were 13.2 nmol/min/mg prot. and 254 pmol/min/mg prot., respectively. Data are expressed as means ± S.E.M. (n = 4) with P < 0.001 for the AT activity (VEGF vs. untreated) and TA$_L$ activity (Yols A–C vs. untreated).

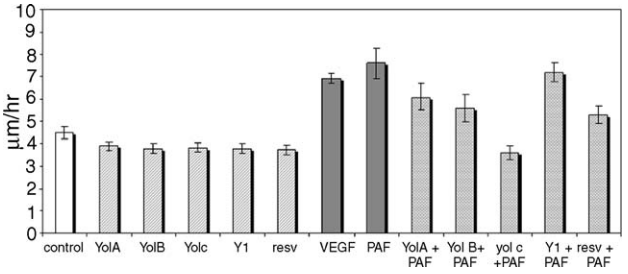


Fig. 8 – Effect of *Y. schidigera* phenolic constituents on PAF-induced KS cell motility. KS cells were pre-incubated for 2 h with vehicle alone or with 25 μM resveratrol (resv), *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (Y1), Yuccaol A (Yol A), Yuccaol B (Yol B), or Yuccaol C (Yol C) before treatment with PAF (40 ng/ml) in serum-free medium for 24 h. Four experiments were performed with similar results. Results shown are the means ± S.E.M. (n = 8) with P < 0.01 for cells pre-treated with 25 μM Yol C (Yol + PAF) vs. cells pre-treated with vehicle alone before stimulation with PAF (PAF).

When we tested the effect of Yols A–C, Y1, and resv on the VEGF-activated PAF metabolism in KS cells, results shown in Fig. 7 indicated that each compound (25 μM) completely blocked the VEGF-induced activation of the AT. Concomitantly, the Yols A–C, Y1, and resv determined an increase of the TA$_L$ activity compared to cells pre-treated with DMSO. Yols A–C showed the highest effect in increasing the TA$_L$ activity (about 250% of the control versus 180% of the control in cells treated pre-treated with DMSO) compared to resv and Y1 (about 200% of the control versus 180% of the control in cells treated pre-treated with DMSO). Compounds alone had no effect on the AT and TA$_L$ activities compared to control cells indicating that these molecules inhibit the VEGF-stimulated PAF synthesis and do not interfere with the basal enzyme activities. These results indicated that phenolic compounds from *Y. schidigera*, in particular Yols A–C, are able to modulate the VEGF-activated production of PAF by either blocking its synthesis and by enhancing its degradation, thus, they can prevent the pathological effects of this potent inflammatory lipid mediator in KS cells like angiogenesis and cellular motility [10,11].

3.4. *Effect of Y. schidigera compounds on in vitro KS cell migration*

In order to test the effect of *Y. schidigera* compounds on in vitro KS cells migration, cells were treated with VEGF or PAF to enhance cell motility, as previously described [10,20]. The baseline migration of KS cells corresponding to spontaneous motility of rested, unstimulated cells, i.e., control cells, was 4.5 μm/h. As shown in Fig. 8 results indicated that both VEGF and PAF determined a marked acceleration of cell motility compared to unstimulated cells (about 7.6 μm/h versus 4.5 μm/h of control cells) (Fig. 8). However, when we tested the effect of *Y. schidigera* compounds on in vitro PAF-induced KS cell migration results indicated that pre-incubation of KS cells with Yols A–C, or resv reduced the cell motility from 7.6 μm/h (cells treated only with PAF) to 6.1 μm/h, 5.6 μm/h, and 5.3 μm/h, respectively. In particular, Yol C, the most effective in inhibiting KS cell motility among Yol compounds, constantly maintained cell motility near to control values up to 24 h (Fig. 9). Compounds alone had no significant effect on the basal cell migration indicating that these molecules do not interfere with inherent cell motility. As shown in Fig. 8, these data indicated that that Yols, in particular Yol C, are able to block cancer cell migration known to be responsible for cancer cell invasion.

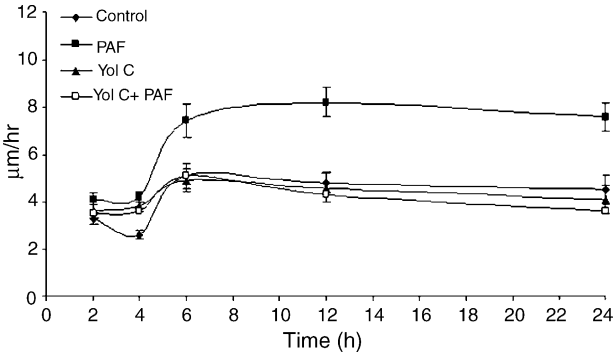


Fig. 9 – Time-dependent effect of Yol C on PAF-induced KS cell motility. KS cells were pre-incubated for 2 h with vehicle alone or with 25 μM Yol C and then treated with PAF (40 ng/ml) in serum-free medium for 24 h. Data are mean ± S.E.M. of three separate experiments in duplicates (n = 6).

4. Discussion

Resveratrol has been the focus of a number of studies investigating the cellular mechanism(s) responsible for its anti-inflammatory and cancer-chemopreventive activities. Recently, it has been demonstrated that this stilbene inhibits platelet aggregation and prostanoid synthesis, regulates lipid metabolism, and blocks tumor initiation, promotion and progression [1]. Among the natural compounds under investigation for their potential application as new therapeutic agents, Yols A–C, isolated from *Y. schidigera* bark have recently been shown to modulate inducible nitric oxide synthase (iNOS) expression via NF-kB [19], to reduce enzymatic platelet lipid peroxidation, adhesion and secretion, and to inhibit blood platelet oxidative stress [17,18]. These compounds, strictly related to resveratrol for the stilbenic origin, are characterized by unusual spirostructures made up of a C-15 unit and a stilbenic portion linked via a γ-lactone ring. In Yols A and B the stilbenic portion is closely related to resveratrol whereas in Yol C derives from *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (Y1). Previous results indicated that Yol C is the most active compound in inhibiting iNOS expression via the transcription factor NF-kB [19].

Results from the present study provide the first evidences that Yols A–C (25 μM), when administered to cultured KS cells before VEGF stimulation, inhibit cell proliferation (Fig. 2) with a potency higher than Y1 and resv. Moreover, similarly to resveratrol, the novel phenolic compounds from *Y. schidigera* target the p38 and p42/44 pathway in KS cells. In fact, pre-treatment of KS cells with Yol C, the more efficient inhibitor of the VEGF-induced cell proliferation (Fig. 2), attenuates p38 and p42/44 signalling (Fig. 5) activated by VEGF (Fig. 4).

The anti-proliferative activity of resveratrol is well known and several studies have determined that the modulation of p38 and p42/44 cascades is a key event in the resveratrol action [29,30]. Here, our results showed that Yols are able to inhibit the VEGF-induced cell proliferation and are effective in attenuating MAPK signalling cascades through the modulation of the p38 and p42/44 phosphorylation. Therefore, these new naturally occurring compounds, able to interfere with the MAPK pathway activated by VEGF, [27,21,28,31] proved to be potential therapeutic tools in disease prevention and treatment.

PAF, newly synthesized by KS cells following stimulation with HIV-Tat-protein, plays a critical role in triggering cell motogenic activity [25,10]. Moreover, PAF produced in vitro by KS cells stimulated with IL-1 or TNF induces and sustaines in vivo angiogenesis in KS cooperating with other angiogenic molecules and chemokines [11]. We observed that the PAF-induced motogenic activity of KS cells was reduced by Yols A and B, and was completely abrogated by Yol C (Figs. 8 and 9) thus, indicating that these novel phenolic compounds are able to prevent cancer cell invasion by blocking cell migration.

Moreover, as the positive effect of Yols on the modulation of PAF biosynthesis may comprise relevant clinical implications for their potential not only as anti-inflammatory but also as cancer-chemopreventive agents, we tested the possible effect of these compounds on the PAF metabolism in KS cells. We found that KS cells produce PAF upon stimulation with VEGF via activation of the AT in the remodeling biosynthetic route (Fig. 6) and that Yols completely blocked the VEGF-induced activation of the AT (Fig. 7). Yols A–C showed the highest effect in enhancing PAF degradation through the TA_L activity. Taken together, these findings indicated that Yols are able to protect against inflammation by inhibiting the PAF biosynthesis (AT activity) and, concomitantly, by activating its degradation (TA_L activity). These results are strongly supported by our previous studies which demonstrated a similar effect of other natural antioxidants, such as flavonoids and lycopene [12,13].

In summary, our study provides the first evidence of the ability of Yols (A, B, C) to inhibit cancer cell growth, migration, and PAF synthesis in KS cells and suggests that the anti-inflammatory properties attributed to *Y. schidigera* can be ascribed to the synergic action of resveratrol and Yols.

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