Numerous bioactive chemical compounds of plant origin may influence the angiogenic activity of various cell types and may thus affect the formation of blood vessels. Here we present the angiogenic effects of extracts of edible plants collected in Crete, Southern Italy and Southern Spain.

Extracts have been applied to cultured human microvascular endothelial cells (HMEC-1), human umbilical vein endothelial cells (HUVEC) and human keratinocytes (HaCaT). About half out of 96 extracts exerted an inhibitory effect on HMEC-1 proliferation. Additionally, we have noted the inhibitory effects of extracts on HUVEC differentiation on a Matrigel layer. None of the extracts showed a stimulatory activity. The extract of *Thymus piperella* exerted moderate inhibitory effect on cobalt-chloride induced VEGF synthesis, however, CoCl$_2$-induced activation of hypoxia responsive element of VEGF promoter was significantly attenuated only by extract of *Origanum heracleoticum*.

Our study indicates that extracts of local food plants, of potential value as nutraceuticals, contain chemical compounds which may inhibit angiogenesis. Demonstration of their real influence on human health requires, however, extensive animal studies and controlled clinical investigations.

**Key words:** angiogenesis, vascular endothelial growth factor, hypoxia, HIF-1, endothelium

* Those authors contributed equally to this work
INTRODUCTION

Angiogenesis, the process of the formation of new blood vessels from preexisting capillaries is a feature of numerous pathological conditions (1). Particularly, the growth of capillaries into the tumors leads to their enlargement and helps the tumor cells to metastasize (2). Similarly, new blood vessels are formed in the enlarging atherosclerotic plaque (3) or in the inflammatory synovium of the rheumatoid arthritic joints (4). Interestingly, also some incurable skin diseases, particularly psoriasis (5), are very much dependent on the vascular network.

The search for different compounds affecting the properties of endothelial cells and influencing the synthesis of vascular mediators is extensive and comprises different strategies (see accompanying review paper by Dulak). One of the most popular approaches, which is also quite easily acceptable by the society, is based on utilization of the natural compounds derived from edible plants. There is a widespread belief that components of such plants may protect from diseases or inhibit their progression. Here we determined whether such anti-angiogenic effects can be exerted by extracts of plants collected in several Mediterranean regions.

MATERIALS AND METHODS

Extracts

Dry extracts of 96 plants collected in Crete (No. 1001-1027), Spain (2001-2050) and Southern Italy (3000-3022) have been used. Extracts have been prepared in the following way. 50 g of air dried material were extracted by reflux with ethanol (90%) for 30 min. The plant material was then pressed and the resultant liquids were pooled and cooled at room temperature as well as filtered through filter paper Whatman No. 1 (Whatman, Maidstone, England). The extracts were then concentrated with a rotary evaporator (40°C) to facilitate its further freeze-drying process. Finally, the extracts were freeze-dried at -50 °C and stored. Dry extracts were stored at 4°C and the day before experiments they were dissolved in DMSO at the stock concentration of 1 mg/ml. Solutions of extracts have been aliquoted and stored frozen at -70°C. Each thawed sample was used only once. Unless stated otherwise, cell cultures have been treated with extracts at the concentrations ranging from 10 - 100 µg/ml. Maximal concentration of DMSO added to cells was 0.1%, and the solvent was always used as control.

Cells

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical veins by collagenase digestion. Cells were cultured in M199 medium supplemented with foetal calf serum (FCS) (10%), endothelial cell grow supplement (ECGS), heparin, L-glutamine (2 mM), hydrocortisone (1 µg/ml), and antibiotics. Experiments were performed on cell cultures of the second to fourth passages. Effects of plant extracts on proliferation and differentiation of HUVEC were determined as described below.

Human microvascular endothelial cells (HMEC-1) were provided by Dr. Candal from Center for Disease Control and Prevention (Atlanta) (6) and cultured in MCDB medium containing 10%
FCS, L-glutamine (2 mM), EGF (10 ng/ml), hydrocortisone (1 µg/ml), penicillin (100 U/ml) and streptomycin (10 µg/ml). Cells were treated with extracts for 24 hours. Effect of plant extracts on proliferation of HMEC-1 has been determined as described below. Additionally, production of VEGF by HMEC-1 was assessed by ELISA.

HaCaT keratinocytes were kindly provided by Dr. Robert Fusenig (Heidelberg University, Germany) (7) and were cultured in DMEM containing 5.5 mM glucose and 10% FCS. Cells were seeded into plates and grown to full confluence, then starved 24 hours before stimulation in the fresh medium containing 0.5% FCS. After this time media were changed and cells were treated with CoCl₂ (200 µM), a known hypoxia mimic (8) to induce VEGF. The tested extracts (10-100 µg/ml, unless otherwise indicated) have been added just before CoCl₂. Concentration of VEGF in culture media was determined 24 hours after stimulation.

**NIH 3T3 cells stably transfected with HRE-luc plasmid**

The construct containing a hypoxia responsive element (HRE) fragment of human VEGF promoter (-1014 to -903) cloned into reporter plasmids pT81, was kindly provided by Dr. Hideo Kimura (Chiba, Japan) (9). This plasmid contains luciferase cDNA, which was used as a reporter gene. NIH 3T3 fibroblasts stably transfected with HRE-luc were obtained by co-transfection of HRE-luc with pcDNA3.1 plasmid, containing neomycin resistance gene, according to previously described method (10). Transfected cells were cultured in the medium containing G418 antibiotic (400 µg/ml) and surviving clones were selected. After 3-4 weeks of culture the clones were tested for the inducibility of HRE. To this end cells were treated with CoCl₂, which activates HIF-1 transcription factor (11). Significantly (i.e. 3 fold) enhanced activity of luciferase in the cell extract was taken as a measure of a stable transfection with HRE-luc. A clone of cells with the lowest basal level of luciferase and highest induced expression was used for further experiments.

**Luciferase activity assay**

Determination of enzyme activity was done according to manufacturer’s protocol (Promega, Madison, USA) using plate reader luminometer.

**Proliferation assay**

Experiments were performed on HUVEC cultured in media with 10% FCS plus basic fibroblast growth factor (bFGF) (10 ng/ml) in the absence or in the presence of plant extract (10-100 µg/ml). HMEC-1 were cultured in media with FCS but without bFGF. After 48 h incubation period, bromodeoxyuridine (BrdU) was added for 2 h and proliferation was measured by ELISA for BrdU (Roche).

**Matrigel assay**

The effect of extracts on angiogenic activity was determined by measuring endothelial tube formation in a Matrigel matrix (12). HUVEC seeded on Matrigel were treated for 12 hours with 10-100 µg/ml of the extracts and the formation of vascular tubes has been determined by microscopic observations.

**Cell viability assay**

Cell viability was assessed by colorimetric measurement of LDH release according to vendor’s protocol (Promega, Madison, USA).
Statistical analysis

Experiments were performed in duplicates or triplicates and were repeated at least two times. Data are presented as mean (±SD). Statistical evaluation was done with Student’s t-test. Differences were accepted as statistically significant at p < 0.05.

RESULTS

Effect of plant extracts on endothelial cell proliferation

Altogether 96 extracts were used in the screening. The effect of plant extracts ranging from 10-100 µg/ml was studied. About half of the extracts exerted an inhibitory effect on HMEC-1 proliferation as demonstrated by BrdU incorporation assay (Table 1). Twelve extracts decreased it for more than 50% at the highest concentration tested with more or less-pronounced concentration-dependent effect, whereas 40 extracts demonstrated inhibitory effect in the range between 20-50% (Table 1).

Seven extracts were tested in Matrigel assay for HUVEC differentiation, production of VEGF by HMEC-1 and HaCaT cells and activation of hypoxia-responsive element of VEGF promoter in NIH 3T3 cell line. These were extracts No. 1006 - *Daucus carota* L., 1012 - *Papaver rhoeas* L., 1016 - *Scandix pecten-veneris* L., 2025 - *Thymus piperella* L., 2032 - *Nasturtium officinale* L., 3007 - *Cynara cardunculus* L. and 3022- *Origanum heracleoticum* L.. Selection of just those extracts was based not only on the high activity exerted in the screening assay but also on their activities in other tests (see accompanying papers).

Six extracts, i.e. *D. carota*, *P. rhoeas*, *S. pecten-veneris*, *T. piperella*, *N. officinale* and *O. heracleoticum* have been always tested at the concentration 10-100 µg/ml, while *C. cardunculus* was assayed additionally at 1-10 µg/ml, as in the screening phase the toxic effect of higher concentration on BrdU incorporation has been observed (Table 1). Three independently performed experiments confirmed some inhibitory effect of *N. officinale* and *C. cardunculus* on HMEC-1 proliferation (Fig. 1), observed in the screening assays (Table 1). In contrast, neither *T. piperella* nor *O. heracleoticum* significantly affected HMEC-1 proliferation. Similar influence has been observed on HUVEC proliferation (not shown).

Table 1. Effect of plant extracts on BrdU incorporation by HMEC-1 cells.

<table>
<thead>
<tr>
<th>Extract</th>
<th>BrdU Incorporation (%)</th>
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<tbody>
<tr>
<td><em>Daucus carota</em> L.</td>
<td>50%</td>
</tr>
<tr>
<td><em>Papaver rhoeas</em> L.</td>
<td>60%</td>
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<tr>
<td><em>Scandix pecten-veneris</em> L.</td>
<td>70%</td>
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<tr>
<td><em>Thymus piperella</em> L.</td>
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<tr>
<td><em>Nasturtium officinale</em> L.</td>
<td>90%</td>
</tr>
<tr>
<td><em>Cynara cardunculus</em> L.</td>
<td>95%</td>
</tr>
<tr>
<td><em>Origanum heracleoticum</em> L.</td>
<td>98%</td>
</tr>
</tbody>
</table>

Extracts which exerted potent, i.e. at least 50% inhibition at the highest concentration are written in italics and bolded. Some extracts, marked with a star (*) affected strongly cell proliferation, but were concomitantly toxic.
<table>
<thead>
<tr>
<th>Extract No</th>
<th>Concentration</th>
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<tr>
<td>2042*</td>
<td>31.84</td>
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</table>
Table 2. Effect of selected plant extracts on hypoxia-induced HRE activity

Values in table show the % of HRE activity in cells with extracts in comparison to HRE activity in cells treated with CoCl₂, a hypoxia mimic, which was calculated as 100%. Altogether 28 extracts were tested. All but one (no. 1012) extracts were tested in two-three separate experiments, each done in triplicates. Bolded extracts (no. 2032 and 3022) exerted concentration-dependent inhibitory effect (n=3 independent experiments, each done in triplicate). For further analysis, shown on Fig. 5, extracts 2032 (*N. officinale*), 3022 (*O. heracleoticum*) as well as additionally *T. piperella* (2025) and *C. cardunculus* (3007) have been used.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration</th>
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<td></td>
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<tr>
<td>1002</td>
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<tr>
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<td>93.8 ± 17.73</td>
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<tr>
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<td>102.48 ± 14.37</td>
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<td>2012</td>
<td>104.25 ± 19.15</td>
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<tr>
<td>2027</td>
<td>86.51 ± 1.63</td>
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<td>3019</td>
<td>73.72 ± 18.17</td>
</tr>
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<td>3022</td>
<td>97.93 ± 16.39</td>
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</table>
N. officinale, C. cardunculus, O. heracleoticum, S. pecten-veneris and D. carota demonstrated the inhibitory effect on HUVEC differentiation on Matrigel, as shown for N. officinale and C. cardunculus (Fig. 2). In turn, neither T. piperella nor O. heracleoticum affected the migratory properties of endothelial cells (Fig. 2). However, C. cardunculus appears to be very toxic at concentrations above 10 µg/ml, thus the lower concentrations have been tested in further studies.

Effect of plant extracts on CoCl₂-induced VEGF synthesis

Effect of 32 extracts on CoCl₂-induced VEGF synthesis in HaCaT keratinocytes has been tested in a preliminary screening. Majority of extracts did not influence VEGF synthesis, only some showed a slight inhibitory effect (not shown). Four extracts have been selected for more detailed analysis.

None of the analyzed extracts influenced basal production of VEGF (data not shown), whereas only extract of T. piperella showed some effects on CoCl₂-induced VEGF synthesis when applied at 50 or 100 µg/ml together with CoCl₂ (Fig. 3). The extract of C. cardunculus at concentrations below 10 µg/ml did not affect VEGF production (Fig. 3) while at high concentrations it was toxic (not shown).
Fig. 2. Effect of selected plant extracts on tube formation by HUVEC laid onto a Matrigel. Note some sort of concentration-dependent effect of *N. officinale* extracts and strong toxic effect of *C. cardunculus* extract. Neither *T. piperella* nor *O. heracleoticum* affected HUVEC differentiation. Extracts from *D. carota*, *P. rhoes* and *S. pecten-veneris* exerted similar inhibitory effect as *N. officinale* and *C. cardunculus* (not shown).

Effect of three extracts on HMEC-1 has been also determined. A tendency towards an inhibitory effect of *T. piperella* extract on basal VEGF synthesis in HMEC-1 has been observed (*Fig. 4*). However, due to the variability between experiments the differences were not statistically significant. *N. officinale* and *O. heracleoticum* extracts did not affect VEGF production in HMEC-1 (*Fig. 4*).

Effects of plant extracts on HRE activity

In the preliminary screening 28 extracts have been tested (*Table 2*). Only two of those extracts - i.e. *N. officinale* (2032) and *O. heracleoticum* (3022) demonstrated some concentration-dependent inhibitory effect on CoCl$_2$-induced HRE activation. In further studies the effect of those two extracts (at concentrations 10-100 µg/ml) and, additionally, extracts from *T. piperella* (10-100 µg/ml) and *C. cardunculus* (1-10 µg/ml) have been evaluated. Again, *T. piperella* and *C. cardunculus* have been chosen because of their activity exerted in other assays (Grande S et al, submitted and see accompanying papers in this issue). For each extract 4-8 independent experiments have been performed. Extract of *O. heracleoticum* exerted modest, although significant inhibitory effect on CoCl$_2$-induced HRE activity (*Fig. 5*). A similar tendency has
Fig. 3. Effect of selected plant extracts on cobalt-chloride induced VEGF synthesis in HaCaT keratinocytes.
Data are shown as % of VEGF produced after CoCl$_2$, which was normalized to 100% for each experiment. Mean of three-five independent experiments each performed in duplicates or triplicates. * p<0.02 vs control (i.e. CoCl$_2$-stimulated cells).

Fig. 4. Effect of selected plant extracts on VEGF synthesis in HMEC-1.
Note a tendency towards an inhibitory effect of *T. piperella* extract. However, the differences are not statistically significant. Mean of three independent experiments each performed in duplicates.
been observed in the case of *N. officinale* extract. Thus the effect of *N. officinale* on HRE to some extent parallels the inhibition of VEGF synthesis observed in HaCaT cells.

Extracts of *T. piperella* and *C. cardunculus* showed some tendency toward stimulatory effect on HRE activation (*Fig. 5*), but the results are not statistically significant.

**DISCUSSION**

In the present study we have determined the effect of plant extracts on angiogenic potential of endothelial cells. We have also determined the effect on the synthesis of VEGF, the major angiogenic mediator, by microvascular endothelial cells and keratinocytes. We found that many of the extracts can inhibit the proliferation of endothelial cells. Additionally, we found that extract of *T. piperella* influenced also the synthesis of VEGF, while extract from *O. heracleoticum* affected HRE activity.

Out of the seven extracts which have been tested in detail, five regularly demonstrated the inhibitory effect on various angiogenic potentials. Thus, the
proliferation of HUVEC and HMEC-1 cells, as determined by BrdU incorporation has been decreased in the presence of extracts from D. carota, P. rhoeas, S. pecten-veneris, N. officinale and C. cardunculus. These extracts also abolished the endothelial cells differentiation and formation of capillaries in a tube formation assay. On the other hand, extracts from O. heracleoticum and T. piperella did not affect the differentiation of HUVEC, although the proliferation of endothelial cells could be moderately inhibited by the highest concentration of the extract (i.e. 100 µg/ml) (Table 1). Interestingly, those effects of T. piperella and O. heracleoticum did not parallel the influence on VEGF synthesis and HRE activity exerted by the same extracts.

Food and medicinal plants are well known to be a rich source of bioactive constituents and it will now be necessary to identify the chemical entities which are responsible for these effects on endothelial cells and keratinocytes. Proliferation and migration of endothelial cells appears to be at least partially dependent on distinct pathways in endothelial cells. For example, we have recently shown that VEGF-induced migration but not proliferation of HUVEC relies on the production of nitric oxide, released by the activated endothelial NO synthase (13). How and to what extent plant-derived compounds may affect NO-signaling pathway remains to be investigated.

Besides affecting directly endothelial cells the compounds may act through influence on the production of angiogenic growth factors. Those are not usually generated by endothelial cells, most probably due to mechanisms which prevents the unwanted autocrine stimulation of endothelial cells (14), (12). However, some endothelial cell types, namely microvascular cells can generate small quantities of VEGF (15), (16), which can be responsible for angiogenic activities of those cells. We have used in our study one such cell line, HMEC-1. In contrast, many other kinds of cells generate high amounts of VEGF, which can affect endothelial cells (for reviews see: (17), (18)). Here we have applied a model HaCaT keratinocytes, a non-tumorigenic human cell line which is often used in the studies investigating the role of angiogenesis in wound healing and cancer (7).

Analysis performed by other members of the Local Food Nutraceutical Consortium showed various concentrations of polyphenols in the extracts of different plants. Thus, respectively, the concentration was 243.7 mg/g of dry mass of D. carota extract, 119.6 mg/g for P. rhoeas, 130.65 mg/g for S. pecten-veneris, 119.43 mg/g for T. piperella, 25.98 mg/g for N. officinale, 448.83 mg/g for C. cardunculus and 65.96 mg/g for O. heracleoticum (full data will be presented in: The Local Food Nutraceutical Consortium - Understanding of the Local Mediterranean Diet: a joined ethnobotanical and phyto-pharmacological approach. Pharmaceutical Research, submitted). Thus, the angiogenic activities as determined in the present study did not show any correlation with the contents of those compounds. Extracts which did not influence HUVEC differentiation, such as T. piperella and O. heracleoticum contained similar amounts of polyphenols as S. pecten-veneris or P. rhoeas, which were very inhibitory. Also,
strong inhibitory activity in Matrigel assay was observed both in case of \textit{N. officinale} extract containing very low amounts of polyphenols and in case of \textit{D. carota} and \textit{C. cardunculus} which contained high amounts. The potent cytotoxic effect of \textit{C. cardunculus} can be due to sesquiterpene lactones, which are known to be highly cytotoxic (19). Further studies are, however, necessary to elucidate what substances are responsible for some inhibitory influence exerted by \textit{T. piperella} and \textit{O. heracleoticum} extracts.

Out of 96 screened extracts those derived from \textit{T. piperella} and \textit{O. heracleoticum} appear to exert the most consistent, although moderate inhibitory effect on the production of VEGF or activation of HRE, respectively. Noteworthy, those extracts do not significantly affect endothelial cell proliferation and differentiation. Thus, they may be considered as potential nutraceuticals which may modulate angiogenic processes. Interestingly, in a recent study of the consortium members (Grande S et al, submitted), the extract of one related species, i.e. \textit{T. pulegioides} has been demonstrated to positively affect the vasculoprotective properties of endothelial cells.

In summary, our study indicates that extracts of some of plants used as local food nutraceuticals contain chemicals which may inhibit angiogenesis. Demonstration of their real effectiveness and influence on human health requires, however, extensive animal studies and controlled clinical investigations.

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