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Antiproliferative effects of essential oils and their major constituents in human renal adenocarcinoma and amelanotic melanoma cells

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Abstract. *Objectives*: The purpose of this study was to evaluate cytotoxic activity of *Platycladus orientalis*, *Prangos asperula* and *Cupressus sempervirens* ssp. *pyramidalis* essential oils and to identify active components involved in inhibition of population growth of human cancer cell lines. *Materials and methods*: Essential oils were obtained by hydrodistillation and were analysed by gas chromatography and gas chromatography coupled to mass spectrometry. Antiproliferative activity was tested on amelanotic melanoma C32 cells and on renal cell adenocarcinoma cells, using the sulphorhodamine B assay. *Results*: *Cupressus sempervirens* ssp. *pyramidalis* leaf oil exerted the highest cytotoxic activity with an IC_{50} value of 104.90 μg/mL against C32, followed by activity of *P. orientalis* and *P. asperula* on the renal adenocarcinoma cell line (IC_{50} of 121.93 and 139.17 μg/mL, respectively). *P. orientalis* essential oil was also active against amelanotic melanoma with an IC_{50} of 330.04 μg/mL. Three identified terpenes, linalool, β-caryophyllene and α-cedrol, were found to be active on both cell lines tested. *Conclusions*: Our findings provide novel insights into the field of cytotoxic properties of essential oils. This study provided evidence on how cytotoxic activity of the oils is not always related to their major constituents, except for lower activity found in both cell lines for α-cedrol. Interestingly, β-caryophyllene and linalool exhibited comparable IC_{50} values to the commercial drug vinblastine on the ACHN cell line. This opens a new field of investigation to discover mechanisms responsible for the observed activity.

INTRODUCTION

Cancer is the second largest single cause of death claiming over six million lives every year worldwide. There has been a recent upsurge in the use of natural products to supersede current treatment in patients that develop multidrug resistance. Scientific studies of plants used in various

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types of ethnic medicine has led to the discovery of many valuable drugs, including taxol, camptothecin, vincristine and vinblastine (Heinrich & Bremner 2006; Newman & Cragg 2007).

Malignant melanoma is an aggressive, therapy-resistant malignancy of melanocytes. The incidence of melanoma has been steadily increasing worldwide, resulting in an increasing public health problem. Exposure to solar ultraviolet radiation, fair skin, dysplastic nevus syndrome, and a family history of melanoma are major risk factors for melanoma development. Avoidance of ultraviolet radiation and surveillance of high-risk patients have the potential to reduce the population burden of melanoma. Several clinically relevant pathological subtypes have been identified and need to be recognized (Markovic *et al*. 2007).

Incidence of renal cell cancer (RCC) is increasing. Historically, RCC has been divided into subtypes on the basis of histopathological findings alone. According to the current World Health Organization classification system, the only histological distinguishing factor between papillary renal cell carcinoma and papillary adenoma is size, although this size requirement seems somewhat arbitrary. The epithelial lesions with a tubulopapillary architecture measuring more than 5 mm have been classified as papillary renal cell carcinomas, that represent the 10–15% of RCC subtypes (Zambrano *et al*. 1999; Wang *et al*. 2007).

Essential oils are fragrant volatile oils found in specialized plant cells or structures and several common therapeutically used oils have been obtained from Cupressaceae and Apiaceae families. Certain essential oils have been reported to influence carcinogen-metabolizing enzymes (Banerjee *et al*. 1994). Furthermore, essential oils have shown chemopreventive potential (Salim & Fukushima 2003), antitumour activity (de Sousa *et al*. 2004; Prashar *et al*. 2004, 2006; Sigurdsson *et al*. 2005; Lampronti *et al*. 2006) and the ability to induce apoptosis in various cancer cell lines (Cavalieri *et al*. 2004; Yoo *et al*. 2005).

Cupressus sempervirens L. (Cupressaceae) cones and young branches have been used in traditional medicine as anthelminthic, antipyretic, antirheumatic, antiseptic, astringent, balsamic and vasoconstrictive agents (Chiej 1984; Chevallier 1996) and oils obtained from leaves and cones are used in aromatherapy (Westwood 1993). *Platycladus orientalis* L. (formerly called *Thuja orientalis* L.), is also a member of Cupressacae family. Kumar *et al*. (2007) reported how administration of a homeopathic formulation of *P. orientalis* in rats retarded tumour growth and significantly reduced elevated marker enzyme levels as revealed by morphological, biochemical and histopathological evaluation. A related species *T. occidentalis* L. is known in homeopathy for its immunopharmacological potential, such as stimulatory and co-stimulatory effects on cytokine and antibody production and activation of macrophages and other immunocompetent cells (Naser *et al*. 2005). *Prangos asperula* Boiss. belongs to the Apiaceae family and an interesting compound, osthol, and has been isolated from *P. pabularia*. This coumarin was able to inhibit P-388 D1 cells *in vivo* and to induce apoptosis in HeLa and HL-60 cells *in vitro*, demonstrating it to be a good lead compound for developing antitumour drugs (Jamwal *et al*. 1962; Yang *et al*. 2003; Chou *et al*. 2007). *P. pabularia* and *P. ferulaceae* have been found to possess antioxidant properties, while anti-HIV activity was reported for *P. tschimganica* (Shikishima *et al*. 2001; Tada *et al*. 2002; Kogure *et al*. 2004).

Cytotoxic screening models provide important preliminary data to select plants with potential anticancer compounds and the sulphorhodamine B assay, used in this study, is commonly employed. The purpose of this investigation was to evaluate any cytotoxicity of *C. sempervirens* ssp. *pyramidalis*, *P. orientalis* and *P. asperula* essential oils to tumour cells and to determine any component responsible for its activity. To this end, each oil, their major components and some selected identified terpenes, determined by gas chromatography (GC) and GC/mass spectrometry (MS) analysis, were tested against human cancer cells, including amelanotic melanoma, and renal adenocarcinoma.

MATERIALS AND METHODS

Materials

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. Ltd. (Milan, Italy) and VWR International (Milan, Italy). Cell culture materials and identified components α-cedrol, α-terpinene, δ-3-carene, α-terpinyl acetate, terpinen-4-ol, α-phellandrene, α-pinene, sabinene, limonene, eucalyptol, linalool, β-caryophyllene and α-humulene were obtained from Sigma-Aldrich Chemical Co. Ltd. (Milan, Italy). Essential oils were obtained by hydrodistillation using a Clevenger-type apparatus, from *Cupressus sempervirens* ssp. *pyramidalis* L., *Platycladus orientalis* L., *Prangos asperula* Boiss. leaves and *Cupressus sempervirens* ssp. *pyramidalis* L. cones (Clevenger 1928).

Cell culture

All media, buffers, trypsin and dyes were filter-sterilized prior to use and were warmed to 37 °C.

The human amelanotic melanoma cell line C32 (C32) [American Type Culture Collection (ATCC), Rockville, MD, USA] (ATCC no. CRL-1585), and renal cell adenocarcinoma ACHN (ACHN) (ATCC no. CRL-1611), were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin. The cell lines were maintained at 37 °C in 5% CO_2 atmosphere and 95% humidity. Cultures were passaged once a week by trypsinization using a 1 : 30 dilution of standard trypsin-ethylenediaminetetraacetic acid solution.

Identification of essential oil constituents

The essential oils, the object of this study, were analysed by GC coupled to GC/MS, using a Hewlett-Packard 6890 gas chromatograph equipped with an SE-30 capillary column (30 m length, 0.25 mm i.d., 0.25 μ film thickness) and interfaced with a Hewlett-Packard 5973 mass-selective device. Ionization of sample components was performed in electron impact mode (EI; 70 eV), with helium as the carrier gas. The analytical conditions were: oven temperature 5 min isothermal at 50 °C, then 50–250 °C at a rate of 13 °C/min, and then held isothermal for 10 min. Injector and detector were maintained at 250 °C and 280 °C, respectively. Analyses were also run by a HP-Innowax capillary column (30 m length, 0.25 mm i.d., 0.25 μ film thickness). Gas chromatographic conditions were as given. Essential oils were analysed by a Shimadzu GC17A gas chromatograph system. An SE-30 capillary column (30 m with an internal diameter of 0.25 mm and a film thickness of 0.25 μ) was used with nitrogen as the carrier gas. GC oven temperature and conditions were as described above. Quantification of the components was performed on the basis of their GC peak areas and percentages of the characterized essential oil components were as given in Table 1. Identification of the compounds was achieved through retention indices (*I*), relative to a homologous series of *n*-alkanes, and mass spectrometry by comparing mass spectra of unknown peaks with those stored in the Wiley GC/MS library, with those reported in the literature and with those of authentic compounds available in our laboratory (Adams 1995; Tranchant 1995).

Cytotoxicity studies

The sulphorodamine B assay was used for measurement of cell proliferation as described previously (Loizzo *et al*. 2005). C32 and ACHN cells were placed in 96-well plates at optimal plating density for each cell line ($5–15 \times 10^4$) and were incubated to allow for cell attachment. After 24 h, the cells were treated with 100 μL/well of serial dilutions of samples to obtain final

Table 1. Composition of essential oils obtained from *P. orientalis*, *P. asperula* and *C. sempervirens* ssp. *pyramidalis* cones and leaves

Compound	GC area (%)[b]				
	I[a]	*P. orientalis* leaves	*P. asperula* leaves	*C. pyramidalis* cones	*C. pyramidalis* leaves
α-Thujene	926 (1035)	0.3	0.1	0.2	0.2
α-Pinene	936 (1032)	35.7	8.4	53.6	24.4
α-Fenchene	951 (1096)	1.2	tr	0.7	0.4
Camphene	953 (1076)	0.2	1.1	0.2	1.2
Sabinene	973 (1138)	0.9	20.6	1.0	3.1
β-Pinene	978 (1118)	0.8	0.1	1.8	0.7
Myrcene	986 (1174)	2.6	1.5	1.9	1.3
α-Phellandrene	1005 (1186)	0.2	6.1	–	0.1
δ-3-Carene	1012 (1159)	9.5	3.0	0.3	16.2
m-Cymene	1013	0.2	1.4	0.1	0.1
α-Terpinene	1016 (1188)	0.1	1.4	18.9	0.2
o-Cymene	1020			0.7	0.7
p-Cymene	1025 (1280)	1.2	4.2	0.8	2.0
β-Phellandrene	1030 (1218)	–	19.0	0.3	0.3
Limonene	1032 (1203)	3.6	0.7	1.9	0.8
Eucaliptol	1035 (1221)	–	0.4	–	–
(*E*)-β-Ocimene	1047 (1266)	0.2	0.1	–	–
γ-Terpinene	1059 (1255)	0.9	9.0	0.3	0.4
Terpinolene	1089 (1290)	2.9	1.0	3.1	4.4
Linalool	1098 (1553)	–	0.1	–	1.0
endo-Fenchol	1113	0.3	–	–	–
α-Campholene aldehyde	1128 (1499)	0.2	–	tr	0.9
trans-Pinocarveol	1138 (1665)	0.6	–	–	0.5
Camphor	1147 (1532)	0.8	–	tr	tr
Isopinocamphone	1174 (1562)	0.4	–	–	–
Terpinen-4-ol	1178 (1611)	–	1.0	1.8	2.1
α-Terpineol	1189 (1683)	0.2	–	1.1	0.8
Myrtenol	1196 (1493)	0.2	–	–	0.8
Isoborneol	1203	0.1	–	–	0.6
Verbenone	1216 (1725)	0.2	–	tr	1.3
α-Fenchyl acetate	1218	–	–	0.7	1.1
trans-Carveol	1217 (1845)	–	0.3	–	0.4
Eucarvone	1223 (1465)	–	–	–	1.4
Thymol methyl ether	1265 (1604)	–	–	0.1	1.0
Thymol	1278 (2113)	–	–	3.8	0.4
Bornyl acetate	1288 (1597)	0.9	–	–	0.8
α-Terpinyl acetate	1358	–	–	–	2.3
α-Cubebene	1351 (1466)	–	0.9	–	2.2
α-Ylangene	1373 (1493)	0.5	0.1	–	0.1
α-Copaene	1377 (1497)	0.4	0.3	–	0.8
β-Elemene	1379 (1584)	0.5	0.1	–	0.1
Isolongifolene	1389	–	–	1.3	–
α-Bergamotene	1403	0.1	–	–	–
Methyl eugenol	1408 (2030)	–	0.7	–	2.5
α-Gurjunene	1410 (1544)	–	1.0	–	–
β-Caryophyllene	1418 (1612)	3.4	0.6	0.1	2.7
Widdrene	(1645)	0.8	–	–	0.1
β-Gurjunene	1432 (1610)	–	–	0.3	–
Aromadendrene	1439 (1628)	–	–	–	–

Table 1. *Continued*

Compound	GC area (%)[b]				
	I^a	*P. orientalis* leaves	*P. asperula* leaves	*C. pyramidalis* cones	*C. pyramidalis* leaves
trans-β-Farnesene	1441 (1662)	tr	0.2	–	–
α-Humulene	1454 (1690)	0.4	0.3	tr	0.4
β-Acoradiene	1467	0.6	–	–	–
Ar-curcumene	1482 (1784)	tr	0.3	tr	0.1
Epi-bicyclosesquiphellandrene	1495 (1731)	1.1	0.5	–	–
α-Muurolene	1499 (1740)	–	–	–	0.6
β-Himachalene	1501	–	–	–	–
β-Bisabolene	1508 (1741)	2.2	–	0.2	–
Bicyclo[4.4.0] dec-1-en-2-isopropyl-5-methyl-9-methylene	1512	1.2	–	–	2.1
γ-Cadinene	1515 (1765)	tr	–	–	0.9
Calamenene	1519 (1839)	tr	0.3	–	0.5
δ-Cadinene	1524 (1772)	2.9	1.5	0.2	–
Palustrol	1567 (1953)	1.0	0.4	–	–
Spathulenol	1579 (2144)	–	0.6	–	–
Viridiflorol	1592 (2110)	–	0.3	0.2	–
Longiborneol	1595	–	–	0.1	–
α-Cedrol	1598 (2149)	11.5	–	0.5	5.8
Junipene	1624	–	–	1.3	0.7
α-Bisabolol	1695 (2232)	–	2.0	–	–
Neophytadiene	1790	–	0.1	–	–
Rimuene	1895 (2148)	–	0.2	–	–
Manoyl oxide	1989 (2375)	–	1.3	0.2	0.5
Manool	2054				0.4
Total identified (%)		91.9	92.1	97.7	94.9

I^a: retention indices on SE-30 column and HP-Innowax column in parenthesis; [b]Area percentage (peak area relative to total peak area %); tr: < 0.1%

concentrations, ranging from 5 to 400 μg/mL for essential oils, and up to 100 μg/mL for the commercially available, identified, constituents. By these serial dilutions, the final mixture used for treating cells contained not more than 0.5% of the solvent (dimethyl sulfoxide), as in solvent control wells. After 48 h exposure time, cells were treated with ice-cold 40% trichloroacetic acid and trichloroacetic acid-fixed cells were chromogen prepared with 0.4% (w/v) sulphorhodamine B in 1% acetic acid, were washed and left to dry overnight. On the day of reading the plates, bound dye was solubilized with 100 μL of 10 mM tris base (tris[hydroxymethyl]aminomethane). Absorbance of each well was read on a Molecular Devices SpectraMax Plus Plate Reader at 490 nm. Vinblastine sulfate salt was used as the positive control. All experiments were carried out in triplicate or more alongside control (untreated) cells. Cytotoxicity of the oils and components was expressed in terms of the IC_{50} value calculated by nonlinear regression curve with the use of GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The dose–response curve was obtained by plotting percentage of inhibition versus concentration.

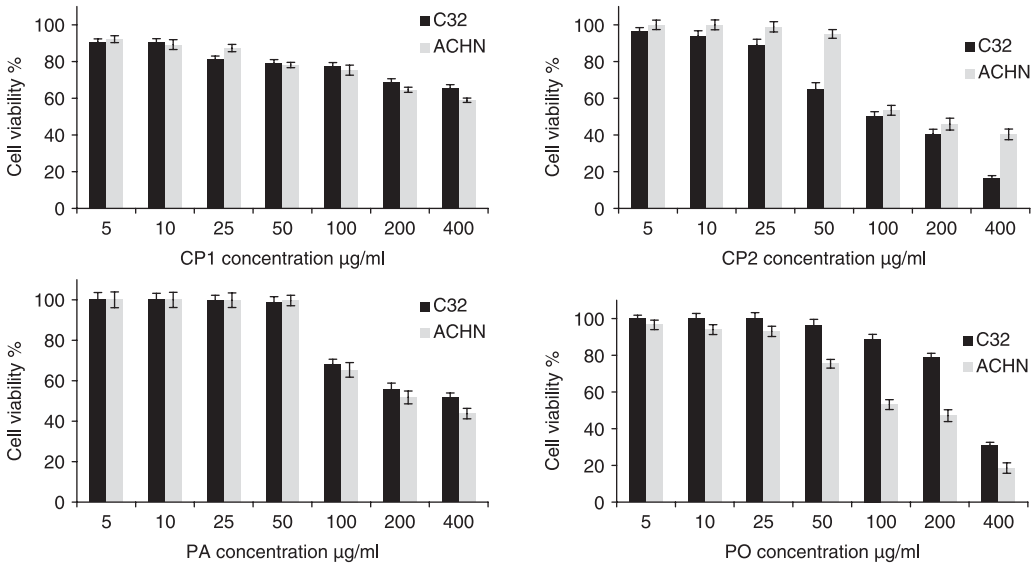

Figure 1. Dose-dependent cytotoxicity of oil (48 h exposure) to C32 and ACHN cells as determined by the sulphorhodamine B (SRB) assay. *P. orientalis* (PO), *P. asperula* (PA), and *C. sempervirens* ssp. *pyramidalis* cones (CP1) and leaves (CP2). Cells were seeded and after 24 h treated with serial dilution of oils. After 48 h, cells were treated with trichloroacetic acid 40% and successively with SRB dye. Absorbance was read at 490 nm. Errors bars indicate the standard deviation ($n = 3$).

Statistical analysis

Data were expressed as means ± SD and statistical analysis was carried out using GraphPad Prism version 4.0. Differences were evaluated by one-way analysis of variance (ANOVA) to compare group means. To correlate activity of the components with corresponding essential oils, non-linear regression was carried out; r^2 values thus obtained were used to predict such relationships. To complete the statistical analysis, a Bonferroni's multicomparison test was performed.

RESULTS

Cytotoxicity of the oils and identified compounds on human cancer cell lines is shown in Figs 1 and 2 and IC_{50} values are given in Table 2. Chemical profile of tested oils is reported in Table 1. One-way ANOVA and Bonferroni's multicomparison test were performed to evaluate differences among oils and identified constituents. In, both cell models applied $P < 0.0001$ and $r^2 = 0.99$.

Cupressus sempervirens ssp. *pyramidalis* oils

Viability of the ACHN cell type dropped by 95% to 53% when the concentration of leaf oil increased from 50 to 100 µg/mL. Leaf oil activity was almost constant across the same cell type in concentrations ranging from 100 (53%) to 400 µg/mL (40%). *C. sempervirens* ssp. *pyramidalis* leaf oil exerted the highest cytotoxic activity with IC_{50} value of 104.90 µg/mL against amelanotic melanoma cells, while it was inactive against renal adenocarcinoma cells. Interestingly, cone oil

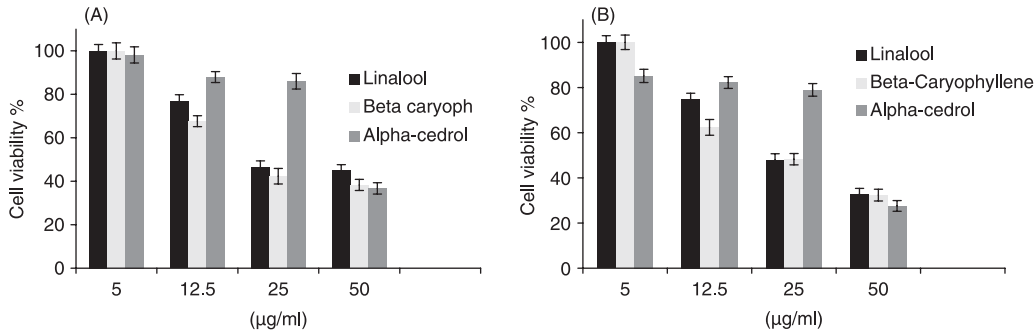

Figure 2 Dose-dependent cytotoxicity of oil (48 h exposure) to C32 (A) and ACHN (B) cells as determined by the sulphorhodamine B (SRB) assay. Cells were seeded and after 24 h treated with terpenes at different concentration. After 48 h, cells were treated with trichloroacetic acid 40% and successively with SRB dye. Absorbance was read at 490 nm. Errors bars indicate the standard deviation ($n = 3$).

did not exhibit any activity on either cell line ($IC_{50} > 400$ µg/mL). Cell viability across the cell types did not fall below 60%. *C. sempervirens* ssp. *pyramidalis* leaf oil was characterized by 53 compounds (94.9% of the total oil) in which α-pinene (24.4%), δ-3-carene (16.16%), α-cedrol (5.8%), terpinolene (4.4%) and sabinene (3.1%) were the main abundant compounds. With a percentage of 53.6%, α-pinene was the major constituent of cone oil, together with α-terpinene (18.9%), thymol (3.8%) and terpinolene (3.15%).

Platycladus orientalis oil

A notable activity against ACHN cells was found when *P. orientalis* was assayed (IC_{50} of 121.93 µg/mL). These renal adenocarcinoma cells' viability dropped by 47% to 18% when concentration of leaf oil increased from 200 to 400 µg/mL. The effect of the oil on viability of ACHN cells was not significantly different across concentrations ranging from 5 (96%) to 25 µg/mL (93%).

The same cell viability pattern was observed for amelanotic melanoma cells. *P. orientalis* essential oil exerted easily detectable activity also against C32 cells with IC_{50} of 330.04 µg/mL. Oil inhibited cell population growth in a dose-dependent manner (Fig. 1). GC/MS analysis of *P. orientalis* essential oil revealed the presence of 45 compounds, α-pinene (35.7%), δ-3-carene (9.5%), α-cedrol (11.5%), β-caryophyllene (3.4%) and terpinolene (2.9%) being the major components.

Prangos asperula oil

An interesting result for renal cell adenocarcinoma was observed when *P. asperula* oil was applied in cell culture (IC_{50} of 139.17 µg/mL). Viability of analysed cell types dropped by 99% to 65% when concentration of oil increased from 50 to 100 µg/mL, in spite of the IC_{50} value for C32 cells being > 400 µg/mL. Forty-two terpenes were identified in *P. asperula* essential oil, representing 92.1% total oil. Sabinene (20.6%), β-phellandrene (19.0%), γ-terpinene (9.0%) and α-pinene (8.4%) were the most representative constituents. Other interesting oil components were α-phellandrene, δ-3-carene, *p*-cymene and α-bisabolol.

Cytotoxic terpenes

In order to identify putative active oil constituents, the commercially available identified compounds (α-cedrol, α-terpinene, δ-3-carene, α-terpinyl acetate, terpinen-4-ol, α-phellandrene,

Table 2. Cytotoxic activity of oils and identified compounds on C32 and ACHN cell lines

	IC_{50} (μg/mL)	
	C32	ACHN
Essential oils		
CP1	>400	>400
CP2	104.90 ± 1.2	>400
PO	330.04 ± 2.7	121.93 ± 1.4
PA	>400	139.17 ± 1.8
Identified compounds		
α-Pinene	>100	>100
Sabinene	>100	>100
Limonene	>100	>100
Eucalyptol	>100	>100
Linalool	23.16 ± 0.8	23.77 ± 1.2
β-Caryophyllene	20.10 ± 0.4	21.81 ± 1.1
α-Humulene	>100	>100
α-Cedrol	44.36 ± 0.9	41.06 ± 0.7
α-Terpinene	>100	>100
δ-3-carene	>100	>100
α-Terpinyl acetate	>100	>100
Terpinen-4-ol	>100	>100
α-Phellandrene	>100	>100
VN	3.0 ± 0.08	22.70 ± 0.14

C. sempervirens ssp. *pyramidalis* cones (CP1) and leaves (CP2), *P. orientalis* (PO), and *P. asperula* (PA). Vinblastine (VN) was used as positive control. Data are given as the mean of at least three independent experiments ± SD. C32 cell line: one-way ANOVA, ***$P < 0.0001$ ($F = 13250$, $r^2 = 0.99$). Bonferroni's multiple comparison test, **$P < 0.001$, except °$P > 0.05$ (CP1 versus PA; CP2 versus α-cedrol, δ-3-carene, α-terpinil acetate, α-terpinen-4-ol, α-phellandrene; linalool versus β-caryophyllene; a-terpinen-4-ol versus δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; δ-3-carene versus α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-terpinil acetate versus terpinen-4-ol, α-phellandrene; terpinen-4-ol versus α-phellandrene; eucalyptol versus CP2, α-humulene, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; limonene versus CP2, α-humulene, eucalyptol, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; sabinene versus CP2, α-humulene, eucalyptol, limonene, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-pinene versus CP2, α-humulene, eucalyptol, limonene, sabinene, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-humulene versus CP2, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene). ACHN cell line: one-way ANOVA, ***$P < 0.0001$ ($F = 9312$, $r^2 = 0.99$). Bonferroni's multiple comparison test, **$P < 0.001$, except °$P > 0.05$ (VN versus linalool, β-caryophyllene; CP1 versus CP2; linalool versus β-caryophyllene; eucalyptol versus α-humulene, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; limonene versus α-humulene, eucalyptol, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; sabinene versus α-humulene, eucalyptol, limonene, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-pinene versus α-humulene, β-caryophyllene, eucalyptol, limonene, sabinene, α-cedrol, α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-humulene versus α-terpinene, δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-terpinene versus δ-3-carene, α-terpinil acetate, terpinen-4-ol, α-phellandrene; δ-3-carene versus α-terpinil acetate, terpinen-4-ol, α-phellandrene; α-terpinil acetate versus terpinen-4-ol, α-phellandrene; terpinen-4-ol versus α-phellandrene).

α-pinene, sabinene, limonene, eucalyptol, linalool, β-caryophyllene and α-humulene) were tested for their cytotoxic activity on a human tumour cell *in vitro* models.

The monoterpene α-pinene, the most abundant compound in *P. orientalis* and *C. pyramidalis* seeds and leaf oils, were inactive on tumour cell population growth and proliferation together

with sabinene, the most abundant terpene in *P. asperula* oil. Another id cells (IC_{50} of 23.16 μg/mL) and renal cell adenocarcinoma cells (IC_{50} of 23.77 μg/mL). Viability of the C32 cell type dropped by 45–80%, when concentration of linalool increased from 12.5 to 25 μg/mL. At the same time, the monoterpene did not exhibit any effect with significant difference in cell viability comparing results obtained at 25 and 50 μg/mL. The results also provided evidence that sesquiterpene β-caryophyllene was active in a similar manner against both ACHN and C32 cell lines, with IC_{50} values of 21.81 μg/mL and 20.10 μg/mL, respectively. A linear dose–response relationship was observed when β-caryophyllene was applied to each cell culture. Viability of C32 cells dropped by 67% to 42% when concentration of sesquiterpene increased from 12.5 to 25 μg/mL. The same pattern was observed in renal adenocarcinoma cells (62% to 48%). With a lower potency, α-cedrol, with an IC_{50} of 44.36 and 41.06 μg/mL acted against C32 and ACHN cells, respectively. Viability of C32 cells dropped by 86% to 36% when concentration of sesquiterpene increased from 25 to 50 μg/mL. The same pattern was observed in ACHN cells (78% to 27%).

DISCUSSION

The first objective of our investigation was to evaluate tumour cell growth inhibitory activity of *P. orientalis*, *P. asperula* and *C. sempervirens* ssp. *pyramidalis* essential oils on amelanotic melanoma and renal adenocarcinoma cell lines *in vitro*. The second objective was to characterize the oils in respect of their chemical composition. Finally, the main constituents and other identified compounds were screened for cytotoxic activity against the same panel of human tumour cell lines, in order to correlate biological activity of essential oils with specific identified terpenes.

C. sempervirens ssp. *pyramidalis* leaf oil exhibited the most interesting biological activity on ACHN and C32 cell population growth inhibition, while cone oils were inactive against the same cell lines. This is an interesting result. It may be that α-pinene, while inactive alone, may act in synergy with the other cytotoxic components of the essential oils and one study has recently appeared that addresses possible synergy of essential oil components (Wright *et al*. 2007). Nevertheless, this monoterpene was able to inhibit human erytroleukaemia K562 cells with an IC_{50} of 117.3 μM (Lampronti *et al*. 2006). High amounts of α-pinene were also found in *P. orientalis* oil that exerted an interesting activity on renal adenocarcinoma cells.

Previous studies have reported anticancer activity of some *Thuja* genus members and some isolated constituents (Iwamoto *et al*. 2003; Sunila & Kuttan 2006). Among sesquiterpenes identified in *P. orientalis*, we have tested β-caryophyllene that exerted strong inhibitory activity against both tested cell lines. Previously, Sibanda *et al*. (2004) reported notable cytotoxic activity of caryophyllene oxide with LC_{50} values ranging from 147 to 351 μM against SK-MEL-28, MDA-MB-231, Hs 578T, 5637, MCF-7 and PC-3 cells. Some β-caryophyllene derivatives exhibited interesting activity ranging from 4.6 to 19.5 μg/mL against KB, Hepa59T/VGH, NCI-H661, HeLa and DLD-1 cells (Ahmed *et al*. 2004). β-Caryophyllene was shown to be inactive when it was applied to A-549 and DLD-1 cell cultures (Sylvestre *et al*. 2007). Neither limonene, linalool nor β-caryophyllene previously tested on MDA-MB-231, MCF7, Hs 578T, HepG2 and PC-3 human tumour cells turned out to be appreciably cytotoxic (Schmidt *et al*. 2006). On the basis of these bibliographic data, our results on C32 and ACHN cells are interesting and open a new field of investigation to discover the mechanisms which may be responsible for the observed activity. Renal adenocarcinoma cell proliferation was inhibited when *P. asperula* essential oil was tested in cell culture. Among terpenes identified by GC/MS analysis, only

linalool in a monoterpene fraction, and β-caryophyllene in a sesquiterpene fraction were found to possess cell population growth inhibitory activity. Interestingly, both identified compounds exhibited IC_{50} values comparable to the commercial drug vinblastine (IC_{50} of 22.70 μg/mL). A previous study has reported that linalool showed the strongest activity against U937 and P3 H1 cells (Chiang *et al*. 2003). In contrast to Mikus *et al*. (2000) who reported lower cytotoxic activity against human promyelocytic leukaemia HL-60 of terpinen-4-ol, this study has demonstrated inability of this monoterpene to exert cytotoxic activity against our panel of cell lines.

In conclusion, we found that *P. orientalis* and *P. asperula* oils exerted an interesting activity on renal adenocarcinoma cells while *C. sempervirens* ssp. *pyramidalis* leaf oils showed the highest cytotoxicity on melanoma cells. This activity could not be related to the main compounds but to some identified terpenes, such as linalool, β-caryophyllene and α-cedrol. It is of interest that different components, present in small amounts, could synergistically contribute to the cytotoxic effect. The data obtained from this study may be used as a starting point for further research that is clearly required to establish the mechanism of action of these kinds of activity.

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