Cachrys pungens Jan inhibits human melanoma cell proliferation through photo-induced cytotoxic activity

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Abstract

Objective: To date, plants belonging to the genus *Cachrys* have not been amply studied. In the present study, aerial components of *Cachrys pungens* Jan from Italy, were examined to assess their free radical-scavenging and antioxidant activity, and their phototoxicity on A375 melanoma cells. In view of potential pharmaceutical applications, a relationship between antioxidant, phototoxic activities and polyphenolic composition has also been investigated.

Materials and methods: Content of sterols, terpenes, fatty acids and coumarins was assessed by gas chromatography–mass spectrometry and GC. Total phenolic content was also determined. Antioxidant activity of the methanol extract and fractions of *C. pungens* Jan was assessed using DPPH scavenging assay and β -carotene bleaching test. Plant phototoxicity was also investigated in this human tumour cell line (amelanotic melanoma).

Results: Analysis of the chloroform extract was particularly interesting, as it led to identification of many coumarins, of which five were linear and one angular furanocoumarins. Methanol and ethyl acetate fractions exhibited substantial antioxidant activity. Moreover, chloroform extract and isolated coumarin fraction had strong phototoxic activity on UVA-induced A375 cells after irradiation at UVA dose of 1.08 J/cm.

Conclusions: Plant-derived natural compounds are an important source for development of cancer-fighting drugs. This study has demonstrated strong photo-

toxic activity of the coumarin fraction of *C. pungens*, a plant which, to our knowledge, has never been studied before. This investigation offers a new perspective for developing other formulations potentially useful in photodynamic therapy for treatment of non-melanoma skin cancers as well as melanomas.

Introduction

Species of the genus *Cachrys* (Apiaceae) are widely present in Europe and Asia (1,2). Turkish traditional medicine has utilized these species as a tonic for treatment of intestinal worms (3).

There are few studies in literature on chemical composition and biological activity of these plants; however, of those studied, the most frequent appear to be *C. ferulacea* and *C. sicula*. The first species has commonly been utilized in the Caucasus as a salad ingredient, as well as a remedy for digestive disorders (3). Many furanocoumarins have been isolated from this plant, such as bergapten, imperatorin and isoimperatorin (4,5). Doković and coworkers isolated the compound 3,5-nonadyne from its roots and demonstrated that this compound was able to inhibit endogenous nitric oxide release in rat peritoneal macrophages (6). Phytochemical investigation of *C. sicula* has also dealt with identification of various furanocoumarins, among which are isoimperatorin, imperatorin, bergapten, xanthotoxin, isopimpinellin, tert-*o*-methylheraclenol, (-)-prantschimgin, (-)-sprengelianin, ulopterol and saxalin (7). Palà-Paúl *et al.* (8) also investigated chemical composition of essential oils of the aerial parts of *C. sicula*, and identified many terpenes, while Pinar (9) isolated diamine *N*-*N'*-di-*o*-tolylethylenediamine. Phytochemical composition of *C. libanotis* L. was also investigated; 5-methoxypsoralen, 8-methoxypsoralen, 5,8-dimethoxypsoralen from the alcoholic extract (10), and various terpenes from the essential oil (11) were identified. Abad and coworkers (12) evaluated biological potential of some constituents

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isolated from *C. trifida* Miller, and demonstrated that furanocoumarins imperatorin, isoimperatorin and prantschimgin have anti-inflammatory effects. Various furano coumarins have also been isolated from *C. pubescens* (Pall) Schischnk and *C. odontalgica* Pall (13,14). In Italy, there are five species, all with distribution in the southern part of the country: *C. sicula* L., *C. libanotis* L., *C. cristata* DC., *C. ferulacea* (L.) Calestani and *C. pungens* Jan. The latter is endemic of the three southern regions Puglia, Calabria and Sicily (15).

In this article, we describe evaluation of phototoxicity of the extract and its fractions obtained from aerial sections of *C. pungens* Jan, on human melanoma cells. Melanoma and non-melanoma skin cancers are among the most prevalent malignancies of human populations. Important findings supporting the free radical hypothesis in skin carcinogenesis are: (i) reactive oxygen species (ROS) are generated in UVA- and UVB-irradiated skin in excessive amounts; (ii) natural cutaneous antioxidant defence is impaired on UV-exposure; (iii) free radicals are involved in all phases of carcinogenesis; (iv) supplementation with antioxidants can inhibit skin carcinogenesis; (v) conditions that increase ROS generation enhance photo-carcinogenesis (16). Over the last number of decades, natural compounds have attracted considerable attention as cancer chemopreventive agents as well as in cancer therapeutics. Research on natural products has recently regained prominence due to greater understanding of their biological significance and growing recognition of origin and functions of their structural diversity. It has been estimated by the World Health Organization that approximately 75–80% of the world's population uses, either in part or solely, plant medicines, either in part or entirely. For many people, this is out of necessity, as they cannot afford high costs of pharmaceutical drugs, or their living areas are remote and pharmaceuticals are not available to them. In the present study, aerial parts of *C. pungens* Jan from Italy were examined to assess their free radical-scavenging activity with 1,1-diphenyl-2-picrylhydrazyl (DPPH), and their phototoxicity, on A375 melanoma cells. In view of potential pharmaceutical applications, relationship between antioxidant, phototoxic activities and polyphenolic composition has also been investigated.

Materials and methods

Reagents

Chlorogenic acid, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, DMEM medium, foetal bovine serum, L-glutamine, penicillin/streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hanks'

balanced salt solution, were obtained from Sigma-Aldrich S.p.a. (Milano, Italy). Melanoma A375 cells were purchased from ATCC No.: CRL-1619, UK. All other reagents, of analytical grade, were supplied by VWR International s.r.l. (Milan, Italy).

Plant materials

Aerial parts of *Cachrys pungens* Jan were collected in August 2008 in the province of Cosenza (Calopezzati), Calabria, Italy. Plants were identified by Dr Carmen Gangale from the Botanical Garden, University of Calabria, Italy, and a voucher specimen was deposited at the Herbarium of the Natural History Museum of Calabria.

Preparation of samples

Air-dried aerial components of *C. pungens* (682 g) were extracted with methanol through maceration (72 h $\times$ 3 times) at room temperature. Obtained total extract (84.36 g) was successively suspended in methanol/water (9:1) mixture and fractioned with *n*-hexane (7.5 g), chloroform (23.9 g) and ethyl acetate (3.4 g). Chloroform obtained the most abundant fraction, with 3.5% in comparison with *n*-hexane (1.1%) and ethyl acetate (0.5%) fractions. Hexane and chloroform fractions were analysed by gas chromatography–mass spectrometry (GC–MS). Content and composition of sterols, terpenes, fatty acids and coumarins were assessed. Total phenolic content was also determined.

Chromatographic separation

Aliquots of the chloroform fraction (2 g) were analysed by column chromatography, the column being eluted with *n*-hexane-ethyl acetate (7:3 to 3:7). Obtained fractions were analysed by TLC on Merck Silica gel 60 F254 plates, developed in hexane/ethyl acetate and visualized with sulphuric acid reagent; they were then pooled according to their TLC-profile. The first 120 fractions from this column (705 mg) were loaded on to a column eluted with chloroform–methanol 97:3. The first 37 fractions (455 mg) were collected together and the obtained fraction (CF) was analysed by GC–MS and tested for biological activity. During phytochemical investigation, formation of a precipitate was observed, from the total extract; structure of this molecule was determined by NMR spectroscopy. NMR spectra were recorded by Bruker AC 300 (Milano, Italy) using DMSO as solvent. ^1H NMR and ^{13}C NMR signals were unambiguously attributed by 2D NMR experiments.

Gas chromatography–mass spectrometry analysis

n-hexane and chloroform fractions were analysed by GC–MS. Qualitative GC–MS analyses were carried out using

a Hewlett-Packard 6890 gas chromatograph (Milano, Italy) equipped with an SE-30 capillary column (100% dimethylpolysiloxane, 30 m length, 0.25 mm in diameter, 0.25 µm film thickness) directly coupled to a selective mass detector (model 5973; Hewlett Packard). Electron impact ionization was carried out in Electron Impact mode (EI, 70 eV). Helium was utilized as carrier gas and analysis was conducted using programmed temperature from 60 to 280 °C (rate 16°/min). Injector and detector were set at temperatures of 250 and 280 °C, respectively. Identification of compounds was based on comparison of GC retention factors with those of standards, and comparison of mass spectra with those present in the Wiley 138 library data of the GC–MS system.

Gas chromatography analysis

Quantitative GC analyses were performed on a Shimadzu GC17A gas chromatograph equipped with a capillary column (30 m length; 0.25 mm i.d.; 0.25 mm film thickness; static phase methylsilicone SE-30) and a flame ionization detector controlled by Borwin Software; carrier gas was nitrogen. Percentage utilized for composition of samples analysed was computed by the normalization method from GC peak areas related to GC peak area of external standards, injected into GC equipment in isothermal conditions at 180 °C. Percentage of their total area was obtained by their addition. All determinations were performed in triplicate and averaged.

Determination of total phenolics content

Total phenolic content of methanolic extract was assessed using Folin-Ciocalteu reagent with chlorogenic acid as standard (17). A total of 50 mg of extract was weighed in 50-ml plastic tubes, vortexed with 25.0 ml of extraction solvent (acetone: methanol: water: acetic acid, 40:40:20: 0.1), heated at 60 °C for 1 h, allowed to cool to room temperature and homogenized for 30 s by sonication. Total volume of 200 µl of samples (three replicates), 1.0 ml of Folin-Ciocalteu's reagent and 1.0 ml of sodium carbonate (7.5%) were introduced into test tubes, vortexed and allowed to remain for 2 h. Absorbance was measured at 726 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer (Milano, Italy) and total phenolic content was expressed as mg of chlorogenic acid equivalent per g of dried material.

Free radical scavenging activity assay

Free radical scavenging activity was determined using a rapid TLC screening method based on reduction of methanolic solution of the coloured free radical DPPH

(1,1-diphenyl-2-picrylhydrazyl). After application of methanolic extract and fractions of *C. pungens*, TLC plates were developed, dried and sprayed with a 0.2% DPPH solution in MeOH (18). Plates were examined 30 min after spraying and antioxidant samples appeared as yellow spots against purple background.

To determine radical scavenging potency, samples were also investigated using an experimental procedure adapted from Wang *et al.* (19). A volume of 200 µl of test sample solution (2.5–1000 µg/ml) was added to 800 µl of 10–4 Methanolic solution of DPPH; reaction mixtures were vigorously shaken and kept in the dark for 30 min. Absorbance of resulting solutions was measured in 1 cm cuvettes using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm, against blank (EtOH). All tests were run in triplicate. Ascorbic acid was used as positive control.

β-carotene bleaching-linoleic acid assay

Antioxidant activity was determined using the β-carotene bleaching test (20) with modifications (21). One millimeter chloroform β-carotene solution (0.2 mg/ml) was added to 0.02 ml linoleic acid and 0.2 ml 100% Tween 20. After evaporation of chloroform, 100 ml water was added and 5 ml emulsion was transferred to different test tubes containing 0.2 ml of samples in methanol, at different concentrations. Approximately 0.2 ml methanol in 5 ml of the above emulsion was used as control. Tubes were then gently shaken and placed in a water bath at 45 °C for 60 min. Absorbances of samples, standard and control were measured at 470 nm using the Perkin Elmer Lambda 40 UV/VIS spectrophotometer against blank, consisting of emulsion without β-carotene. Measurements were carried out at initial time ($t = 0$) and successively at 30 and 60 min. All samples were assayed in triplicate. Propyl gallate at the same concentration was used as positive control. Antioxidant activity was measured in terms of the successful prevention of β-carotene bleaching by using the following equation:

$$\text{Antioxidant activity} = [1 - (A_0 - A_t)/(A_0^\circ - A_t^\circ)] \times 100$$

where A_0 and A_0° are absorbance values measured at initial incubation time for samples/standard and control, respectively, while A_t and A_t° are absorbance values measured in samples/standard and control respectively at $t = 30$ min and $t = 60$ min.

Cell phototoxicity

The phototoxicity test was adapted from an experimental procedure described by Barraja *et al.* (22) with modifications. Melanoma cells (A375) were grown in DMEM medium (Sigma-Aldrich) supplemented with 1% L-gluta-

mine, 1% of penicillin/streptomycin and 10% foetal bovine serum. Volumes of 100 µl of medium containing 1×10^4 cells were introduced in each well of a 96-well tissue culture microtitre plate (Sigma-Aldrich, Mialno, Italy). Plates were incubated at 37 °C in humidified 5% CO₂ incubator for 24 h. After medium removal, 100 µl of samples, dissolved in DMSO and diluted with Hanks' balanced salt solution (HBSS; pH 7.2), was added to each well and incubated at 37 °C for 30 min, then irradiated. HPW 125 Philips lamps, mainly emitting at 365 nm, were used for irradiation experiments. Spectral irradiance of the source was 0.3 mW/cm as measured, at sample level, by a Cole-Parmer Instrument Company radiometer (Niles, IL, USA), equipped with a 365-CX sensor. Cells were irradiated for 1 h at a dose of 1.08 J/cm. After irradiation, solutions were replaced with medium, and plates were incubated for 48 h. Cell viability was assayed by MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test, as previously described (23).

Cell morphology microscopy

Changes in cell morphology were visualized and assessed after irradiation, and without irradiation, using an inverted microscope (AE20 Motic; Motic Instruments, Inc., VWR, Milano, Italy). Images were captured on a VWR digital camera (VisiCam 3.0 USB camera, Milano, Italy).

Statistical analysis

All experiments were carried out in triplicate; data were expressed as mean value $\pm$ SEM. Concentration providing 50% inhibition (IC₅₀) was calculated by non-linear regression using GraphPad Prism Software (San Diego, CA, USA). Statistical significance was assessed with one-way analysis of variance (ANOVA) using SigmaStat Software (Jantel scientific software, San Rafael, CA, USA). Significant differences among means were analysed using Tukey's multiple comparisons test. Differences of $P < 0.05$ were considered significant.

Results

Chemical composition

Total phenolic content of total extract was 18 mg/g, expressed as mg chlorogenic acid equivalents *per g* dried material. To identify main non-polar components of *C. pungens* *n*-hexane and chloroform extracts, GC-MS and GC analyses were carried out. Table 1 summarizes chemical composition of the *n*-hexane fraction. Thirty-nine compounds were identified: 5 monoterpenes, 9 sesquiterpenes and 1 sesquiterpenic alcohol, 1 diterpene, 1 triterpene,

Table 1. Chemical composition of the *n*-hexane fraction from *Cachrys pungen*s

Component	<i>R</i> _t	RAP
β-Myrcene	8.054	Tr
α-Phellandrene	8.343	Tr
β-Phellandrene	8.797	0.1
β-Ocimene	9.117	Tr
Carvacrol	12.701	0.1
α-Cubebene	13.221	Tr
α-Copaene	13.535	Tr
<i>trans</i> -caryophyllene	14.038	0.1
Clovene	14.118	Tr
β-Farnesene	14.267	0.1
β-Selinene	14.393	Tr
α-Longipinene	14.776	0.1
β-Bisabolene	14.827	0.2
Dodecanoic acid	15.301	Tr
γ-Cadinene	16.153	0.1
Caryophyllenol-II	16.456	0.1
Valeranone	16.513	0.7
Tetradecanoic acid, methyl ester	17.073	0.3
Pentadecanoic acid, methyl ester	17.526	Tr
Neophytadiene	17.645	0.3
Hexadecanoic acid, methyl ester	18.336	1.1
Hexadecanoic acid	18.742	2.5
Hexadecanoic acid, 14-methyl-, methyl ester	19.085	0.2
Heptadecanoic acid	19.394	0.2
8,11-octadecadienoic acid, methyl ester	19.639	2.2
9,12-octadecadienoic acid (Z,Z)-	20.565	0.2
Pentacosane	22.246	0.7
Docosanoic acid, methyl ester	22.474	0.3
Octacosane	24.635	0.2
Nonacosane	25.755	1.5
Docosane	28.647	Tr
Vitamin E	29.710	0.4
Campesterol	31.779	0.1
Stigmasta-5,22-dien-3-ol (3β, 22 <i>E</i>)	32.596	2.3
γ-Sitosterol	34.156	1.2
β-Amyrin	35.088	0.1
Stigmast-7-en-3-ol (3β, 5α, 24 <i>S</i>)	35.982	0.1
Cycloartenol	36.202	0.1
9,19-Ciclolanostan-3-ol,24-methylene (3β)	38.214	0.1

Compounds listed in order of elution from SE30 MS column; *R*_t, Retention time (min); RAP, relative area percentage (peak area relative to total peak area %); Tr, values <0.1% are denoted as traces.

10 fatty acids, 6 phytosterols, 4 alkanes, 1 tetrahydronaphthalene and 1 tocopherol. Terpenes were found to be the most numerous compounds, although they were present in small quantities in the fraction. Among them, the two sesquiterpenes valeranone and β-bisabolene were found to be the major constituents (0.7% and 0.2% of the extract, respectively).

On the whole, most abundant compounds were the three fatty acids hexadecanoic acid (2.5%), hexadecanoic acid methyl ester (1.1%) and 8,11-octadecadienoic acid methyl ester (2.2%), the alkane nonacosane (1.5%) and the phytosterol γ-sitosterol (1.2%).

Table 2. Chemical composition of the chloroform fraction from *Cachrys pungens*

Component	R_t	RAP
Carvacrol	12.615	0.1
Psoralene	17.782	0.2
Tetradecanoic acid	18.114	0.1
Xanthotoxin	19.399	6.4
Bergapten	19.662	5.2
3-Methylsuberosine	20.097	25.2
Isopimpinellin	20.868	8.7
Columbianetin	21.360	1.2
9,12-octadecadienoic acid	21.731	0.5
Isooxypeucedanin	22.611	0.4
Jatamansin	24.652	5.7
Vitamin E	29.333	0.4

Compounds listed in order of elution from SE30 MS column; R_t , retention time (min); RAP, relative area percentage (peak area relative to total peak area %).

Analysis of the chloroform extract was particularly interesting, as it led to identification of many coumarins, as shown in Table 2. Twelve compounds were identified, among which eight were coumarins: one dimethyl-pyrancoumarin (jatamansin), five linear furanocoumarins (psoralen, xanthotoxin, bergapten, isopimpinellin and isoxypeucedanin), one angular furanocoumarin (columbianetin) and one coumarin (3-methylsuberosine). The most abundant molecule was 3-methylsuberosin, corresponding to 25.2% of the chloroform extract. Among other compounds, a significant percentage was also observed of linear furanocoumarins isopimpinellin (8.7%), xanthotoxin (6.4%) and bergapten (5.2%) and pyranocoumarin jatamansin (5.7%).

The chloroform fraction was also analysed by column chromatography, and an interesting fraction (455 mg) (CF) was analysed by GC-MS (Fig. 1) and GC. Xanthotoxin and isopimpinellin were the most abundant compounds (2.64 ± 0.04 and 1.27 ± 0.01 mg/g of fraction, respectively). Presence of bergapten was also relevant (0.52 ± 0.005 mg/g of fraction), while 3-methylsuberosine, isoxypeucedanin and xanthotoxin were less abundant (0.016 ± 0.002 , 0.012 ± 0.001 and 0.006 ± 0.001 mg/g, respectively). Moreover, during the phytochemical investigation, formation of precipitate from the methanolic extract was observed. Structure of this molecule was determined by NMR spectroscopy and was identified as the flavonone glycoside hesperidin (hesperetin-7-O-rutinoside) by comparison with literature data (24) (Fig. 2).

Antioxidant activity

Antioxidant activity of the methanol extract and fractions of *C. pungens* Jan was investigated using DPPH scaveng-

Figure 1. Chromatograms of the coumarin and concentration (mg per g of dried material) of the major components. **Figure 2.** Chemical structure of hesperidin (hesperetin-7-O-rutinoside).

ing assay and by β -carotene bleaching test. Both have proven the effectiveness of the extracts compared to reference antioxidant standards: ascorbic acid and propyl galate. Free radical-scavenging activity exerted by total extract and fractions of *C. pungens* is shown in Table 3. The relatively stable organic radical, DPPH, has been widely used in determination of antioxidant activity of single compounds, as well as that of many plant extracts. The effect of antioxidants on DPPH radical-scavenging is thought to be due to their hydrogen-donating ability (25). Extracts were able to reduce the stable free radical DPPH in a concentration-dependent way. Ethyl acetate extract was the most active fraction, with IC_{50} value of 12.15 ± 0.02 μ g/ml (Fig. 3). The methanol extract had an IC_{50} value of 145.6 ± 0.02 μ g/ml. Lower activity was observed for chloroform and *n*-hexane extracts (IC_{50} of 281.0 ± 0.04 and 391.0 ± 0.15 μ g/ml, respectively). Antioxidant capacity could be attributed to phenolic compounds whose amount was measured using Folin-Ciocalteu reagent and expressed as mg of chlorogenic acid equivalents *per g* of dried material. Phenolic content of total extract was 18 mg/g of dried material.

Table 3. IC₅₀ values (µg/ml) of antioxidant and photodynamic activities of extracts from *Cachrys pungens* Jan

Extract	DPPH	MTT test (A375 cells)		β-carotene bleaching test	
		Irradiated cells	Unirradiated cells	30 min of incubation	60 min of incubation
Methanol	145.60 ± 0.02 ^c	0.487 ± 0.037 ^d	49.950 ± 0.018 ^a	9.16 ± 0.27 ^b	21.53 ± 0.34 ^d
<i>n</i> -Hexane	391.00 ± 0.15 ^e	N.D.	N.D.	N.D.	N.D.
Chloroform	281.00 ± 0.04 ^d	0.286 ± 0.067 ^e	34.280 ± 0.022 ^b	19.73 ± 0.61 ^c	29.82 ± 0.05 ^e
Ethyl acetate	12.15 ± 0.02 ^b	N.D.	N.D.	8.33 ± 0.03 ^b	19.48 ± 0.05 ^c
Coumarin fraction	N.D.	0.209 ± 0.033 ^e	31.620 ± 0.018 ^c	N.D.	N.D.
Bergaptene*	–	0.0416 ± 0.008 ^f	N.D.	N.D.	N.D.
Ascorbic acid*	2 ± 0.01 ^a	–	–	–	–
Propyl gallate*	–	–	–	1 ± 0.02 ^a	1 ± 0.02 ^a

For antioxidant activities, data are expressed as means ± SEM ($n = 3$). For photodynamic activity, cells were pre-treated (30 min) with samples (0.010–100 µg/ml). For irradiation, the dose of 1.08 J/cm² was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) under the same conditions. Data are expressed as mean ± SEM ($n = 6$). Different letters along column (DPPH test), or within the two columns (MTT and β-carotene test) indicate statistically significant differences at $P < 0.05$ (Tukey's test).

N.D., not detectable.

*Positive control.

Figure 3. Free radical scavenging activity on DPPH of *C. pungens*. (a) methanol extract; (b) *n*-hexane fraction; (c) chloroform fraction; (d) ethyl acetate fraction. Data represent mean ± SEM ($n = 3$). Ascorbic acid (IC₅₀ value of 2 ± 0.01 µg/ml) was used as positive control.

Antioxidant activity of *C. pungens* was also determined using β-carotene bleaching (Table 3). Ethyl acetate fraction and methanol extract had best activities, with IC₅₀ values of 8.33 ± 0.03 and 9.16 ± 0.27 µg/ml, respectively, after 30 min incubation (Fig. 4). As determined by Tukey testing, there was no statistically significant difference between activities of the two extracts. Lower antioxidant potential was observed for chloroform extract, with IC₅₀ value of 19.73 ± 0.61 µg/ml. No significant activity was observed for hexane fraction (IC₅₀ > 100 µg/ml). After 60 min incubation, a statistically significant increase in IC₅₀ value was observed, indicating a decrease in anti-

oxidant activity of extracts over reaction time. After 60 min incubation, IC₅₀ value of ethyl acetate fractions was 19.48 ± 0.05 µg/ml, and activity of total (methanol) extract was significantly different (IC₅₀ of 21.53 ± 0.34 µg/ml) (Fig. 4). As reference, IC₅₀ of propyl gallate was 1 ± 0.02 µg/ml, after both 30 and 60 min incubation.

Cell phototoxicity

Phototoxicity of *C. pungens* Jan was investigated on our human tumour cell line A375 (amelanotic melanoma). Cells were irradiated at UVA dose of 1.08 J/cm. Control

Figure 4. Lipid peroxidation inhibition activity using β -carotene-linoleic acid system after 30 and 60 min of incubation of *C. pungens*. ●: Methanol extract; ■: chloroform fraction; ▲: ethyl acetate fraction; (a) 30 min; (b) 60 min; Data represent mean $\pm$ SEM ($n = 3$). Propyl gallate ($IC_{50} = 1 \pm 0.02 \mu\text{g/ml}$) was used as positive control.

Figure 5. Phototoxic effects exerted by total extracts and fractions of *C. pungens* Jan on UVA-induced A375 cells. ●: Methanol extract; ■: chloroform fraction; ▲: coumarin fraction; (a) after irradiation; (b) without irradiation. Data represent mean $\pm$ SEM ($n = 6$). Bergaptene ($IC_{50} = 0.0416 \pm 0.008 \mu\text{g/ml}$) was used as positive control.

experiments with non-irradiated cells were carried out without any significant cytotoxic effects (Table 3). Chloroform extract and isolated coumarinic fraction had best anti-proliferative activity, with IC_{50} values of 0.286 ± 0.067 and $0.209 \pm 0.033 \mu\text{g/ml}$, respectively (Fig. 5). Anti-proliferative activity of both fractions may be attributed to presence of furanocoumarins, which represent a novel class of potentially effective natural drugs for treatment of several types of cancer (26). Realization of Tukey's test revealed that chloroform extract and the new coumarins' fraction did not significantly differ from each other. This result confirmed that phototoxicity of chloroform extract was related to furanocoumarins content. Activity of methanol extract was significantly lower (IC_{50} values of $0.487 \pm 0.037 \mu\text{g/ml}$).

Cell morphology microscopy

Figure 6 shows extent of cell survival after irradiation of cells incubated with different concentrations of coumarin fraction of *C. pungens*, and irradiated at UVA dose of 1.08 J/cm^2 . Morphological changes of cell line A375 were assessed after irradiation, and significant changes in cell shape and structure were observed. No morphological changes nor decreases in number of cells were observed within the control group of cells in DMEM 0.5% DMSO, irradiated without plant extracts (a). Figure 6 also shows comparison between cells treated with coumarin fraction ($5 \mu\text{g/ml}$) after irradiation (c) and treated cells without irradiation (d), in which number and morphological appearance were comparable to standard control cell groups (a). Coumarin fraction isolated from chloroform

Figure 6. Morphological changes in A375 cells incubated after 48 h with isolated coumarin fraction. (a) control, irradiated cells in DMEM 0.5% DMSO, without sample; (b) irradiated cells, $100 \mu\text{g/ml}$; (c) irradiated cells, $5 \mu\text{g/ml}$; (d) non-irradiated cells, $5 \mu\text{g/ml}$.

extract of *C. pungens* caused high quantitative decrease in cell number and also considerable changes in morphological shape.

Discussion

Malignant melanoma is one of the more common tumours. Currently, there are limited treatment options

available for this disease due to lack of efficacy of chemotherapy and radiotherapy (27). PUVA and photodynamic therapy are two important emerging strategies against cancer and treatment based on oral or topical administration of psoralens followed by exposure to UVA (320–360 nm) is a type of photochemotherapy called PUVA (28). It is used in treatment of vitiligo, psoriasis and cutaneous T-cell lymphoma (29).

Recent studies suggest that PUVA therapy is important, not only for anti-proliferative effects on skin cells, but also for some biological effects on regulation of the immune system. Accordingly, psoralens are considered not only as photosensibilizing agents, but are now also emerging as photochemoprotective agents against cancer and immune system disorders (30). Furanocoumarins have a furan ring attached at the 6,7 position (psoralen type, linear compounds), or 7,8 (angelicin type, angular compounds). These compounds are present in plants belonging to the Apiaceae (umbrelliferae) family, such as celery and parsnip, Fabaceae, such as *Psoralea corylifolia* L., Moraceae, such as fig, and Rutaceae such as lemon and bergamot.

Over the last few decades, many studies on biological and particularly photobiological activities of furanocoumarins, their spectrum of action, mutagenic and carcinogenic effects, have been published in international journals. Mechanisms of photosensibilization of these compounds is linked to their capacity to interact with DNA. Furanocoumarins have attracted attention of researchers, and several mono-functional derivatives, mostly angelicin analogues (iso-psoralens), have recently been prepared and studied (26).

Plant-derived natural compounds are an important resource for development of anti-cancer drugs and the present study has demonstrated the strong phototoxic activity of chloroform extract of *C. pungens*, a plant which, to our knowledge, has never been studied before.

Here, phototoxicity of *C. pungens* Jan was investigated on the human amelanotic melanoma cell line A375. Chloroform extract and isolated coumarinic fractions showed most notable anti-proliferative activity. These two results do not significantly differ from each other (as determined by Tukey's test). This result confirmed that phototoxicity of chloroform extract was related to furanocoumarins content, when assessed by means of GC–MS.

The genus *Cachrys* represents a potential source of many coumarins; however, as yet there are very few studies present in literature. In this work, chemical composition of *C. pungens* and analysis of biological activities of this plant, have been shown for the first time, as far as we are aware. This study offers a new perspective for developing new formulations potentially useful in PUVA therapy for treatment of malignant melanoma and other diseases.

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