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Cachrys libanotis L. Extracts: Photocytotoxic Effects on UVA-Irradiated Human Melanoma Cells †

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Abstract: Melanoma is the most aggressive form of skin cancer. Photochemotherapy, combining the action of a light source and a chemical photosensitizer, is one of the most interesting current therapeutic approaches. Plants represent a rich source of photoactive compounds, and furanocoumarins are some of the most important naturally occurring phytoconstituents. The aim of this study was to evaluate the photocytotoxic potential of *Cachrys libanotis* L. (Apiaceae) from Southern Italy. This species belongs to a genus rich in furanocoumarins and widely distributed in Europe. The aerial parts were extracted through both traditional maceration and pressurized cyclic solid-liquid (PCSL) extraction using Naviglio extractor®. Qualitative and quantitative analyses were performed to detect the coumarins content using GC-MS, and the photocytotoxic effects of the extracts were assessed on UVA-irradiated C32 melanoma cells. The apoptotic responses were also evaluated. Furthermore, phenolic content and the in vitro antioxidant potential were also estimated. Xanthotoxin, bergapten and isopimpinellin were identified and quantified. Both extracts affected cell viability in a concentration-dependent manner after irradiation for 1 h at a dose of 1.08 J/cm^2. Sample obtained through PCSL extraction was the most effective, with an IC_{50} equal to 3.16 μg/mL, a very interesting value if compared with the positive control bergapten. This extract induced up-regulation of apoptotic signals such as BAX and PARP cleavage and, in the presence of UVA radiation, it caused a greater upregulation of p21 protein. Obtained results suggest that investigated species could be a good candidate for further studies aimed to find new drugs with photocytotoxic potential.

Keywords: apiaceae; furanocoumarins; plant extracts; photochemotherapy; skin cancer

1. Introduction

Melanoma is the most aggressive type of skin cancer [1]. Beside the earliest treatment options such as surgery, chemotherapy and radiation, more recent therapeutic approaches include photochemotherapy, immunotherapy, nanodrugs and molecular-targeted therapy [2,3]. Several natural compounds with photosensitizing properties have been identified, and some of these molecules are commercially available [4].

In our previous studies focusing on the search for photoactive phytocompounds, we highlighted the biological properties of *C. pungens* Jan species [5]. The aerial parts methanolic extract, together with its cloroform fraction and isolated coumarins fraction induced strong photocytotoxic effects on UVA-irradiated A375 melanoma cells, with IC_{50} values equal to 0.487 ± 0.037, 0.286 ± 0.067 and 0.209 ± 0.033 μg/mL, respectively.

Based on these promising previous results, we decided to investigate other species belonging to this interesting genus. The aim of the work was to investigate the photobiological properties of *Cachrys libanotis* L. (Apiaceae). This species is widely distributed around the Mediterranean basin [6].

The phytochemical composition and biological properties of aerial parts extracts were investigated. The photocytotoxic properties were assessed on melanoma C32 cells. We also compared two different methods of extraction, traditional maceration (TM) and pressurised cyclic solid-liquid (PCSL) extraction.

2. Experiments

C. libanotis aerial parts from Southern Italy were extracted with methanol (plant to solvent ratio 1:10 g/mL) through both traditional maceration (TM) and pressurised cyclic solid-liquid (PCSL) extraction technique using Naviglio extractor® (Atlas Filtri SrL, Limena, PD, Italy).

The apolar compounds, coumarins, fatty acids and terpenes, were identified by means of gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett Packard model 5973 selective mass detector. The operating conditions were as previously reported [7].

The total phenolic and flavonoid contents were determined using Folin-Ciocalteau method and the aluminium chloride colorimetric method [8], respectively.

The antioxidant activity of *C. libanotis* extracts was assessed through the well-established DDPH assay [9] and the β-carotene-linoleate bleaching test [10].

The photocytotoxic activity of samples was determined on human melanoma cancer cells C32 (ATCC no. CRL-1585). Cells were grown in RPMI-1640 medium supplemented with penicillin/streptomycin, L-glutamine and fetal bovine serum (1%, 1% and 10%, respectively). For the experiments, 100 μL of medium (3.8×10^4 cells) were introduced in each well of a 96-well microtiter plate. Medium was removed 24 h later, and replaced by 100 μL of sample dissolved in MeOH and diluted with Hanks' Balanced Salt Solution (concentrations ranging from 0.63 to 100 μg/mL). Plates were irradiated 30 min later with an HPW 125 Philips lamp, mainly emitting at 365 nm. Cells were irradiated for 1 h at a dose of 1.08 J/cm^2 [11]. Then, the solution was replaced with fresh medium, and the cytotoxicity was evaluated 48 h later using the 3-[4,5-dimethyl-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, as previously reported [12]. The known photocytotoxic compound bergaptene was used as positive control and experiments were carried out in quadruplicate.

To assess the apoptotic responses, immunoblotting analysis was also performed. C32 cells were lysed for total protein extraction at the end of each treatment. Equal amounts of proteins were resolved on 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and probed with p21, Bax, PARP and GAPDH antibodies (Santa Cruz Biotechnology). Finally, the antigen-antibody complex was detected by incubation of the membranes with peroxidase-coupled goat anti-mouse or goat anti-rabbit antibodies and shown using the ECL System (Amersham Pharmacia) [13].

Biological data were fitted through nonlinear regression in order to calculate the IC_{50} values using GraphPad Prism Software (San Diego, CA, USA) and statistical differences were tested by one-way analysis of variance (ANOVA).

3. Results and Discussion

The aim of this study was to investigate the phytochemical composition and the photocytotoxic effects of *C. libanotis* aerial parts subjected to different extraction processes on C32 human melanoma cell line.

TM technique allowed to obtain a higher yield (17.8%) than PCSL extraction (12.6%). Moreover, TM extract also showed higher amounts of total phenolic and total flavonoid contents (25.0 ± 0.2 and 1.29 ± 0.04 mg/g, expressed as chlorogenic acid and quercetin equivalents per g of dry plant material, respectively) compared to the second sample (12.8 ± 0.1 and 0.09 ± 0.01 mg/g). Consistently, the sample obtained with traditional maceration showed a better radical scavenging potency (IC_{50} = 102.13 ± 0.79 μg/mL) and a better antioxidant activity in the β-carotene bleaching test compared to the second sample (IC_{50} = 19.22 ± 1.07 μg/mL after 30 min of incubation).

The coumarin content was assessed by means of GC-MS. Three furanocoumarins were detected in both *C. libanotis* extracts: xanthotoxin, bergapten and isopimpinellin. Unlike polar compounds, the most abundant component xanthotoxin was detected in higher percentage in the extract obtained

with PCSL extraction (14.8%) compared to TM extract (9.1%). Consistently, the same trend was observed for the pyranocoumarin compound 2-methyl-2-butenoic acid 9,10-dihydro-8,8-dimethyl-2-oxo-2H,8H-benzo[1,2-b:3,4-b']dipyran-9-yl ester, which was detected only in the Naviglio® extract (9.7%), and for the coumarin isogeijerin (5.6% and 1.2% for PCSL and TM samples, respectively). Percentages equal to 2.5% and 2.8% were observed for bergaptene while isopimpinellin was detected at percentages of 3.0% and 3.4%. The only exceptions were the two compunds osthol and suberosin, only identified in *C. libanotis* macerate.

Furthermore, three fatty acids and a terpene were also identified in *C. libanotis* extracts: myristic, palmitic and α-linolenic acids and estragole.

The phoyocytotoxic properties of investigated samples were evaluated on melanoma C32 cell line. Cell cultures were irradiated with UVA light for 1 h at a dose of 1.08 J/cm² in the presence of different concentration of each sample. Both *C. libanotis* extracts affected cell viability in a concentration-dependent manner (Figure 1).

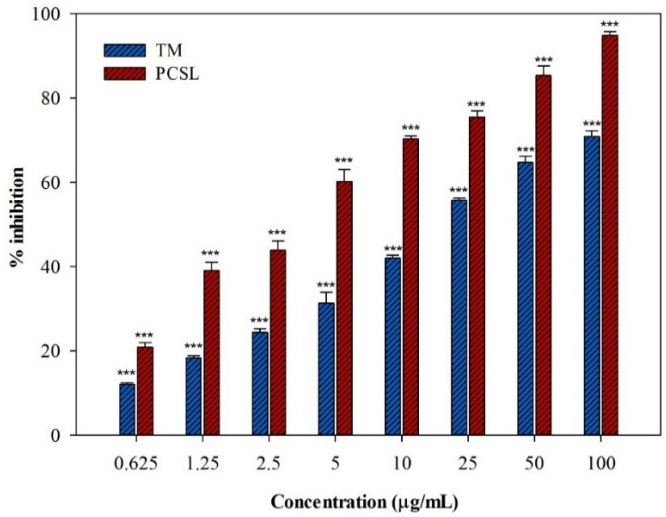

Figure 1. Concentration-dependent photocytotoxic effects induced by *C. libanotis* L. extracts: TM, traditional maceration; PCSL: pressurized cyclic solid-liquid extraction. Data were expressed as means ± S.E.M. (n = 4). *** $p < 0.001$ compared to control (Dunnett's test).

PCSL extraction allowed a better phytochemical composition for the antiproliferative activity than TM: the raw extract obtained with Naviglio® extractor showed the best activity, with an IC_{50} value equal to 3.16 μg/mL. This sample induced also some cytotoxic effects in the dark at the highest concentration tested, but the IC_{50} value observed for unirradiated cells (55.20 ± 1.65 μg/mL) was significantly higher than that referred to irradiated plates. The extract obtained through traditional maceration was also effective, even if to a lesser extent (IC_{50} value equal to 18.18 ± 1.33 μg/mL), without affecting cell viability in the dark.

Furthermore, the apoptotic responses on C32 cells were also assessed. The PCSL extract was able to increase the cyclin-dependent kinase inhibitor p21 protein, with respect to control, and a greater up-regulation was observed under the combination with UV. Moreover, this sample induced up-regulation of apoptotic signals such as BAX and PARP cleavage. Differently, sample obtained with TM did not cause an increase of p21 protein levels.

4. Conclusions

Obtained results demonstrated the photocytotoxic activity of *C. libanotis* species. Moreover, by comparing two different extraction techniques, it was observed that PCSL extraction allowed a better phytochemical composition for the anticancer activity compared to TM, inducing significant apoptotic effects on human melanoma cell line. This species could be a promising candidate for

further studies with the aim to find new potential drugs useful in the photochemotherapy of skin cancer.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PCSL	pressurized cyclic solid-liquid extraction
TM	traditional maceration

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