Biomedicine & Pharmacotherapy 103 (2018) 443–452

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

The chemotherapeutic effect of β-2-himachalen-6-ol in chemically induced skin tumorigenesis

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ARTICLE INFO

Keywords:
Daucus carota
Skin cancer
β-2-himachalen-6-ol
Wild carrot
DMBA/TPA carcinogenesis

ABSTRACT

β-2-himachalen-6-ol (HC), a novel sesquiterpene derived from Lebanese wild carrot, was shown to possess a remarkable anticancer activity. The present study investigates the in vitro anticancer activity of HC and its effect on papillomas induced using a DMBA/TPA skin carcinogenesis mouse model. HaCaT-ras II-4 epidermal squamous cell viability was assessed using WST-1 kit. Cell cycle was analyzed by flow cytometry, and pro/anti-apoptotic proteins were measured using western blot. Mice papillomas were induced by DMBA and promoted with TPA for 18 weeks. At week 12, animals were divided into four groups: HC topically treated (5%Top), HC intraperitoneally treated (25 mg/kg; HC25), Cisplatin treated (2.5 mg/kg), and control (DMSO treated). Papilloma yield, volume, histology, and mice weight and liver function were assessed. HC treatment decreased significantly cell survival (IC_{50} = 7 and IC_{90} = 40 μg/ml) and increased significantly cells undergoing late apoptosis and necrosis. It also significantly decreased the levels of pro-caspase-3, p53, Bcl-2, p-Erk/Erk and p-Akt/Akt and increased p21 and Bax proteins. Treatment with HC25, HC5%Top or Cisplatin showed a significant decrease in papilloma yield and volume. Only Cisplatin treatment caused a significant decrease in body weight and increase in serum ALT. In conclusion, β-2-himachalen-6-ol induced significant tumor shrinkage, an effect partly mediated via promoting apoptosis through inhibition of the MAPK/ERK and PI3K/AKT pathways, with no significant toxicity to laboratory mice.

1. Introduction

Cancer is considered one of the major causes of death worldwide and it is ranked second after cardiovascular diseases in frequency of morbidity. In 2013, 55% of the 14.9 million newly diagnosed cancers worldwide were lost of cancer [1]. The global burden of cancer is continuously rising, with an estimation of 13 million deaths and 21.7 million new cancer cases by 2030 [2]. In the United States, one out of four deaths is attributed to cancer [3]. Skin cancer, one of the most common types of cancer (40% of global cases), is mostly prevalent among Caucasians due to their relative lack of skin pigmentation [4]. The incidence of skin cancer has been increasing over the past decades [5]. In the United States, about 700,000 new cases of cutaneous squamous cell carcinoma are diagnosed annually [6], and one in every five persons will develop skin cancer in their lifetime [7]. Skin cancer can be divided into two kinds: melanoma and non-melanoma. Melanoma is usually less frequent in occurrence, but more aggressive in terms of metastasis and resistance to treatment. Non-melanoma comprises mainly basal and squamous cell carcinomas, and less frequently the neuro-endocrine type (Merkel cell carcinoma), angiosarcomas, cutaneous B- and T-cell lymphomas, dermatofibrosarcoma protuberans, sebaceous gland carcinomas and skin metastasis from other cancers [8]. Basal cell carcinoma, the most common form of skin cancers, is known to be the least aggressive. Squamous cell carcinoma is the second most frequently occurring skin cancer. It is more invasive and has the ability to metastasize to distant organs and tissues [9]. Skin cancer risk factors include ultraviolet radiation, thinning and depletion of the ozone layer, immunosuppression, increased life longevity, mutation of the tumor suppressor gene PTCH1 (Protein patched homolog 1), fair freckled skin, radiation therapy, phototherapy, viral infections, in addition to dermatoses, keratosis and chronically injured or nonhealing wounds and scars [9,10].

Skin cancer treatment includes different therapeutical approaches such as surgical excision, radiation therapy, electrodessication and

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https://doi.org/10.1016/j.biopha.2018.04.027
Received 2 October 2017; Received in revised form 3 April 2018; Accepted 3 April 2018

curettage, cryosurgery, photodynamic therapy, laser surgery, topical chemotherapy, systemic chemotherapy and targeted therapy [11,12]. Unfortunately, most of these procedures are accompanied with serious side effects. Cancer phytotherapy has recently acquired extensive attention and succeeded globally to be used as alternative medicine mainly due to its low cost and limited side effects. Among the currently used phytotherapeutic agents are the taxanes, which are diterpenes derived from *Taxus brevifolia*. Taxanes are part of chemotherapeutic regimens to treat breast, ovarian, lung, prostate and many other cancers [13].

Daucus carota (Linnaeus) ssp. carota, known as wild carrot, belongs to the family Umbelliferae (Apiacae). It spreads out worldwide, mostly in the moderate regions of Europe, Asia, Africa and America [14]. In Lebanon, it is plentifully found in the coastal areas and regions with moderate altitudes [15]. In the European folk medicine, the essential oil of the plant has been used as an antiseptic and anti-inflammatory agent for the treatment of urinary tract infections, cystitis and prostatitis [16]. In Lebanese folk medicine, it has been used in the treatment of gastric ulcer, diabetes, muscle pain, cancer and in the prevention of liver diseases (http://wildedibleplants.info). Moreover, it has been reported that the plant possesses diuretic and antilithic [17], anti-ulcer and anti-inflammatory [18], antioxidant [19,20], antibacterial and antifungal effects [21–23]. Furthermore, *Daucus carota* oil extract (DCOE) has been shown to be cytotoxic against a panel of cancer cells including colon, breast [19] and acute myeloid leukemia cells [24]. The pentane/diethyl ether fraction of DCOE was shown to possess similar in vitro anticancer activity [25–27], and this led to further analysis of the fraction and isolation of its major compound β-2-himachalen-6-ol (HC). The cytotoxic effect of HC was confirmed against the previously tested cancer cells [28–30]. DCOE, its pentane diethyl ether fraction and HC all showed a protective effect against DMBA/TPA skin induced carcinogenesis in murine model [29,31,32]. The aim of the present study is to confirm the cytotoxic activity of HC against HaCaT-ras II-4 epidermal squamous cell carcinoma, and investigate its anticancer effect from a therapeutic perspective in a DMBA/TPA skin carcinogenesis mice model.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), Dimethyl sulphoxide (DMSO), 7,12-Dimethylbenz(a)anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), trypan blue, trypsin, fetal bovine serum, penicillin-streptomycin, glycine, lysis buffer solution, phosphate buffer saline (PBS), bovine serum albumin (BSA), fetal bovine serum (FBS), tris-buffered saline with Tween 20® (TBST), sodium dodecyl sulfate (SDS), well plates, pentane, diethyl ether, hexane, ethyl acetate, anisaldehyde and ethanol were purchased from Sigma-Aldrich (St. Louis, USA). WST-1 reagent was purchased from Roche (Mannheim, Germany). The fluorescein Isothiocyanate (FITC)-conjugated Annexin V/PI apoptosis detection kit was purchased from Abcam plc (Cambridge, UK). Silica gel 60 was purchased from ACROS organics (New Jersey, USA). Primary mouse monoclonal antibodies against Erk, p-Erk, Bax, Bcl-2, p-53, PARP proteins and HRP-conjugated secondary antibodies were obtained from Abcam (Cambridge, UK). Rabbit polyclonal antibodies to Actin, Pro-Caspase-3, Akt and p-Akt proteins were obtained from Santa Cruz (California, USA). And all other chemicals used in the experiments were of analytical grade.

2.2. Plant collection and HC isolation

Daucus carota (Linnaeus) ssp. carota mature umbels were collected from Byblos, Lebanon, between July and August 2012 at the post flowering season. The plant was identified according to the characteristics mentioned in the "Handbook of Medicinal Herbs" [17] and confirmed by Dr. A. Houri, a Lebanese plant expert at the Lebanese American University. A specimen, (ID 2012-0031) was deposited in the School of Arts and Sciences, Lebanese American University, Lebanon. The oil was then extracted as previously reported [31] and then chromatographed on a normal-phase silica gel column to obtain four fractions: F1 (pentane; 100%), F2 (pentane: diethyl ether; 50:50), F3 (diethyl ether; 100%) and F4 (chloroform: methanol; 93:7) as previously described [25,26]. The F2 fraction was subjected to further chromatographic separation and led to the isolation of β-2-himachalen-6-ol (HC) that was identified using GC–MS and Nuclear Magnetic Resonance (NMR) spectroscopy [28].

2.3. Cell culture and treatment

HaCaT-ras II-4cells (10^5 cells/ml) were plated in 96-well plates in RPMI medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics and maintained at 37 °C in 5% CO_2 humidified incubator for 24 h. Cells were then treated with HC (1, 5, 10, 25 and 50 μg/ml) and incubated for 48 h, and Cisplatin (2.5 μg/ml) was used as a positive control. Cisplatin (2.5 μg/ml) and HC (10 μg/ml) were also used concomitantly to assess the presence of potential synergism.

2.4. Cell viability

WST-1 cell proliferation assay was used to determine cell viability. The assay is based on the enzymatic cleavage of the tetrazolium salt (Roche Applied Science, Penzberg, Germany) to formazan by mitochondrial dehydrogenases present in metabolically active and viable cells. The absorbance of detected formazan was read at 450 nm by a Multiskan FC ELISA Microplate Photometer (Thermo Fisher Scientific, Rockford, IL, USA).

2.5. Cell cycle analysis

The effect of HC on cell cycle distribution was performed using flow cytometry. Briefly, 10^5 HaCaT-ras II-4 cells/ml were cultured in 6-well plates for 48 h, treated with HC (10 or 25 μg/ml) and incubated in 5% CO_2 humidified incubator for 48 h. Cells were harvested, washed with phosphate buffer solution (PBS) and fixed with 70% ethanol on ice. After 24 h, the fixed cells were then washed with ice cold PBS, re-suspended in 100 μl DNase-free RNase, and incubated for 30 min in the dark at room temperature. Subsequently, cells were resuspended in ice-cold PBS, transferred to 6 ml polysterene falcon tubes, and 30 μl propidium iodide (PI) stain (Abcam, Cambridge, UK) was added to them, vortexed and incubated for 10 min in the dark. Cell DNA content was measured using a C6 Flow Cytometer (BD Accuri Cytometers, Ann Arbor, MI USA).

2.6. Apoptosis assay

The apoptotic effect of HC was identified by Annexin V-FITC staining assay and measured using C6 flow cytometer (BD Accuri Cytometers, Ann Arbor, MI USA). Briefly, HaCaT-ras II-4 cells (10^5 cells/ml) were treated with HC (10 or 25 μg/ml), and cultured in 6-well plates for 48 h. Treated cells were then harvested, washed with PBS, centrifuged at 500*g* for 5 min, and the pellet was suspended in 0.5 ml of Annexin V/PI apoptosis detection kit in the dark for 5 min at room temperature. The binding of Annexin V was analyzed using flow cytometry (Ex = 488 nm, Em = 530 nm), FL1 channel to detect Annexin V staining and FL3 channel to detect PI staining.

2.7. Western blot

The protein content of samples from cells was determined using the