

Immunomodulatory and anticancer effects of *Pituranthos tortuosus* essential oil

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Abstract Many studies have been performed to assess potential utility of natural products as immunomodulants to enhance antitumor activity in situ. In this study, an essential oil (EO) from the aerial parts of *Pituranthos tortuosus* was prepared using hydrodistillation, its composition was characterized, and its immunomodulatory potential was assessed. The results indicated that the EO contained sabinene, α -pinene, limonene, and terpinen-4-ol as major constituents. EO was also found to be able to significantly promote lipopolysaccharide (LPS)-stimulated splenocyte proliferation, suggestive of a potential for activation of B cells and enhanced humoral immune responses in hosts given this product. Effects of EO on cell proliferation and apoptosis were also investigated in B16F10 melanoma cells. EO-induced tumor cell growth inhibition was associated with characteristic apoptotic changes in the cells, including nuclear condensation. In conclusion, these data suggested to us that an EO of *P. tortuosus* could evolve to be a potential medicinal resource for use in the treatment of cancers.

Keywords EO · Melanoma · Anticancer · Immunomodulator

Introduction

A great number of aromatic, medicinal, and spices plants have interesting biological properties. Evaluation of phytotherapeutic properties, such as antifungal, insecticidal, and antibacterial activity, remains of keen interest in particular for less frequently evaluated or newly discovered plants. These, like many other plants, represent a potential source of active compounds, including those found in the plant materials themselves (roots, leaves, bark) or by products, i.e., extracts and essential oils.

A plant widespread in Tunisia and endemic to North Africa is *Pituranthos tortuosus*. *P. tortuosus* is often used by local citizens to relieve stomach pains and to regulate menstruation [1] and in traditional medicine for treatment of fevers, hepatitis, asthma, diabetes digestive difficulties, and rheumatism [2]. An essential oil has been extracted from the plant, and its composition previously reported [3]. However, there remains very little understanding of its biological, much less its medicinal, properties. Abdelwahed et al. [1] demonstrated the antimicrobial activity of the oil; a follow-on study identified its antimutagenic, cytotoxic, and apoptotic activities [4]. Krifa et al. [2] subsequently demonstrated other effects attributable to this product, including induction antifungal, allelopathic, larvicidal, and insecticidal activities.

Many natural antimicrobial substances have anticancer or anticarcinogenic properties. However, little information is available on the antitumor effects of essential oil, particularly against melanomas. Thus, the purpose of this research is to study the immunomodulatory and anticancer properties, of the essential oil isolated from the aerial parts of *P. tortuosus*.

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Material and methods

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was purchased from Euromedex (Mundolsheim, France) and dimethylsulfoxide (DMSO) from Sigma-Aldrich (St. Suintin Fallavier, France). RPMI 1640, trypsin, and L-glutamine were all purchased from Invitrogen Life Technologies (Cergy Pontoise, France). Fetal bovine serum (FBS) was obtained from Biowhitaker, Lonza, Belgium. Nonessential amino acids (NEA) were bought from Gibco/BRL (Edinburgh, Scotland).

Plant material

P. tortuosus aerial parts were collected from plants found along the coast of Tunisia in March 2014. The aerial parts were washed, air-dried, and then reduced in powder with a pestle.

The essential oils from the materials were extracted by hydrodistillation (100-g sample in 500 ml distilled water) using a Clevenger-type apparatus for 4 h. The oils that were generated were then dried over anhydrous sodium sulfate and stored in sealed glass vials at 45 °C prior to analysis. Yield (based on dry weight of the sample) was calculated; the values were routinely 0.25 %.

Experimental animals

Specific pathogen-free Balb/c mice (6–8 weeks old, male, 18–22 g) were obtained from the Pasteur Institute (Tunis, Tunisia). All mice were housed under standard conditions of temperature (22–28 °C), humidity (30–70 %), and light (12-h light/dark) in an accredited pathogen-free facility. All animals were provided ad libitum access to standard rodent chow and filtered water. All experiments were performed in accordance with guidelines for the care and use of laboratory animals as published by the US National Institute of Health. All experiments received the explicit approval of the Ethics Animal Committee in Tunisia.

Cell line and culture

B16F10 melanoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained at 37 °C in a humidified incubator with 5 % CO₂ at 37 °C. The cells were cultured in complete RPMI 1640, i.e., RPMI supplemented with 10 % (v/v) FBS, 2 mM glutamine, 1 % NEA (100×), and 1 % sodium pyruvate 100 mM.

Analysis of the essential oil (EO) product

The EO composition was investigated using GC and GC/MS. Analytical GC was done on an HP5890-series II gas chromatograph (Agilent Technologies, CA) equipped with flame ionization detectors (FID) using a fused silica capillary column, apolar HP-5 and polar HP Innowax (30 m×0.25 mm ID, film thickness of 0.25 mm). Oven temperature was held at 50 °C for 1 min then programmed to rise at a rate of 5 °C/min until 240 °C, and then held isothermal for 4 min. The carrier gas was N₂ at a 1.2-ml/min flow rate; injector temperature=250 °C; detector temperature=280 °C. The sample volume injected was always 0.1 ml of a 1 % solution (EO diluted in hexane). Constituent percentages were calculated by electronic integration of FID peak areas without use of response factor correction.

GC/MS was performed in a Hewlett Packard 5972 MSD System. An HP-5 MS capillary column (30 m×0.25 mm ID, film thickness of 0.25 mm) was directly coupled to the mass spectrometer. The carrier gas was He, with a 1.2-ml/min flow rate. Oven temperature was programmed (50 °C for 1 min, then 50–240 °C at 5 °C/min) and subsequently held isothermal for 4 min. Injector port temperature=250 °C, detector temperature=280 °C, split ratio=1:50. The sample volume injected was always 0.1 ml of a 1 % solution (EO diluted in hexane). Mass spectrometer parameters used were as follows: HP5972 recording at 70 eV; scan time=1.5 s; mass range=40–300 amu. ChemStation software was adopted to handle the mass spectra and chromatograms. Identification of compounds was based on spectra (compared with Wiley 275.L, 6th edition mass spectral library). Further confirmation was done using Retention Index data generated from a series of alkane retention indices (relatives to C₉–C₂₈ on the HP-5 and HP-20 M columns) [5].

Splenocyte proliferation and humoral responses

Spleen Balb/C lymphocytes were obtained as in Krifa et al. [6, 7]. Briefly, naïve mice were euthanized by cervical dislocation and each spleen isolated aseptically and minced with a sterile forceps. The splenocytes were then centrifuged (1500 rpm, 10 min), and any red blood cells present lysed by resuspending the pellet in lysing buffer (144 mM NH₄Cl, 1.7 mM Tris-base) and placing on ice for 10 min. Cells were then washed twice with PBS and then resuspended in complete RPMI.

EO effects on splenocyte proliferation were assayed using MTT [8]. Splenocyte suspension is a mixture of cells containing T lymphocytes, B lymphocytes, and monocytes among others, but at 24 h, only lymphocytes are going to proliferate. An aliquot of splenocyte suspension (5×10⁶ cells/ml; 100-μl aliquot/well) was preincubated in 96-well plate for 24 h in a humidified 5 % CO₂ atmosphere before lipopolysaccharide

[LPS, rough strain, *Escherichia coli* EH100, Sigma, Hamburg, Germany)] mitogen at 5 µg/ml was added alone or in combination with EO (at 0-, 1.25-, 2.5-, 5-, or 10-µg/ml final concentration in well) that freshly suspended in RPMI. The cells were then incubated at 37 °C for a further 48 h. Thereafter, the plates were centrifuged at 1500 rpm for 10 min, and the pellet in each well was then resuspended in 100 µl of MTT (1 mg/ml RPMI) solution. After incubation for 4 h at 37 °C, the plate was centrifuged, the MTT solution in each well removed, and 100 µl dimethyl sulfoxide (DMSO; 98 %) added. After a 15-min incubation at 37 °C, the absorbance (OD) of any formed formazan in each well was measured at 570 nm in a microplate reader (Thermo Scientific, Vantaa, Finland). The percentage proliferation (relative to that by cells that did not receive LPS) was calculated using proliferation (%) = $100 \times (\text{OD sample} - \text{OD control}) / \text{OD control}$ [9].

Cytotoxicity against B16F10 melanoma cells

MTT was also used to assess the direct impact of the test concentrations of EO on B16F10 tumor cell viability. Cells were seeded into 96-well microtitre plates, and 24 h later, the test samples were added in serial dilutions before incubating the plates for an additional 48 h. Then, dedicated sets of cells were washed once with phosphate-buffered saline (PBS, pH 7.4) before receiving fresh medium containing 1 mg/ml MTT. After 1 h of incubation, the medium was discarded, and the formazan in the cells dissolved by adding 100 µl DMSO in ethanol (1:1). Negative control cells (without test extract treatments) were prepared in parallel. After incubation at 37 °C for 15 min, the OD in each well was measured at 570 nm in the Thermo plate reader. Cytotoxicity was expressed as IC₅₀, the concentration of EO that led to a reduction in absorbance of treated cells by 50 % with respect to that of the reference control (untreated cells).

Apoptotic effect in B16F10 cells

B16F10 cells were seeded into 6-well microtiter plates (at 5.10^5 cells/well; 2 ml) and incubated at 37 °C in the 5 % CO₂ incubator. After 24 h, serial dilutions of the test EO were added to dedicated wells. The plates were then incubated a further 48 h before the cells in each well were harvested, washed with PBS, resuspended in 1 ml PBS containing 10 µg acridine orange (AO) and 10 µg ethidium bromide, and incubated at room temperature for 15 min. Aliquots of these cells (10 µl) were then placed on glass slides, and triplicate samples of 100 cells each counted and scored for apoptotic chromatin condensation using a fluorescent microscope (Zeiss, Oberkochen, Germany). Stained nuclei with condensed chromatin (super-condensed chromatin at nuclear periphery) or nuclei fragmented into multiple smaller dense

bodies were considered as apoptotic. Nuclei with uncondensed and dispersed chromatin were considered not apoptotic.

Statistics

All data were expressed as mean (±SD) and compared using a Student's *t* test. Statistical significance was assigned at *p*-values <0.05. All data were analyzed using SPSS 11.0 software (SPSS Inc., Chicago, IL).

Results

Composition of *P. tortuosus* essential oil (EO)

The main EO constituents identified by GC-MS spectrometer analyses are summarized in Table 1 according to percentage composition. Seventeen compounds were identified as major constituents in *P. tortuosus* EO; sabinen (24.24 %) was the principal component followed by α-pinen (17.98 %), limonen (16.12 %), and terpinen-4-ol (7.21 %).

Splenocyte proliferation

Splenocytes from naïve mice were isolated and cultured with EO alone, or EO+LPS. In the absence of mitogen, EO was able to induce splenocyte proliferation in a biphasic dose-related manner (Fig. 1). EO at a concentration of 1.25 µg/ml only led to an increase in activity levels of 66 % above values for untreated unstimulated control cells, a dose of 2.5 µg/ml actually led splenocyte proliferation of 78.6 ± 4.2 %. In contrast, as the dose increased to ≥ 5 µg/ml, the trend was toward a lessening of extent of induction. In the LPS studies, higher cell proliferation occurred in a presence of the EO at a dose of 2.5 µg/ml compared to that by from control (stimulated) cell levels. Interestingly, at doses above that, there were decreases in proliferative capacity again.

Cytotoxic effect of EO

Exposure of B16F10 cells to EO inhibited cell proliferation in a dose- and time-related manner (Fig. 2). IC₅₀ values were determined, and inhibition percentages were calculated. Inhibition of proliferation of the tumor cells by EO at 400 µg/ml reached a maximum of 91.3 ± 3.6 % after 48 h of incubation. From the data, an IC₅₀ value of ≈ 80 µg EO/ml after 48 h was calculated.

EO induces apoptosis on B16F10 cells

To examine whether the studied EO induces apoptosis in B16F10 cells, we assessed cell morphology using the AO/ethidium bromide double-staining and fluorescence microscopy.

Table 1 Composition of the essential oil (% w/w) of *Pituranthos tortuosus*

No.	%	Compounds
1	17.98	α -pinene
2	24.24	Sabinene
3	2.26	L-Phellandrene
4	2.31	Tricyclene
5	0.44	Alpha-Terpipene
6	16.12	Limonene
7	0.59	beta-Phellandrene
8	2.14	Trans-.Beta.-Ocimene
9	3.05	Gamma.-Terpinene
10	0.50	1,3,7-Octatriene
11	4.83	Para Cymene
12	3.21	Alpha.-Terpinolene
13	0.09	3-Nonen-5-yne
14	1.46	Allo-Ocimene
15	0.10	1-(2-Propenyl)Tricyclo[4.1.0.0(2,7)]Heptane
16	0.07	Benzene, Pentyl- (CAS)
17	0.09	P-Mentha-1,5,8-Triene
18	0.15	Benzene, 1-Methyl-4-(1-Methylethen Yl)- (CAS)
19	0.09	Trans-Limonene Oxide
20	1.32	Cis-Sabinene Hydrate
21	0.20	Decanal
22	0.07	Ethanone
23	0.48	Cis-Sabinene Hydrate
24	0.08	1-Tetradecanol (Cas)
25	0.70	2-Cyclohexen-1-ol
26	0.40	Bornyl Acetate
27	7.21	Terpinen-4-ol
28	0.44	1-Terpineol
29	0.06	4-Methyl-7-Methylethyl-3,8-Dioxatr Cyclo[5.1.0(2-4)]octane
30	0.07	Alpha.-Amorphene
31	0.13	Alpha.-Humulene
32	0.21	2-Propylcyclopentanone
33	0.18	Sabinyl Acetate
34	0.12	Naphthalene
35	0.71	Alpha.-Terpineol
36	0.11	Germacrene D
37	0.15	Beta.-Selinene
38	0.16	Alpha.-Muuroolene
39	0.11	Carvone
40	0.23	Piperitol Isomer II (Trans?)
41	0.11	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl- (CAS)
42	0.42	Delta.-Cadinene (Armoise-Maroc)
43	0.48	1-Decanol
44	0.06	Benzaldehyde
45	0.08	Cadina-1,4-Diene
46	0.09	2-Caren-10-Al

Table 1 (continued)

No.	%	Compounds
47	0.15	1-(2-Propenyl)Tricyclo[4.1.0.0(2,7 23478115798-16-8 49)]Heptane
48	0.11	2,4-Nonadienal (Cas)
49	0.23	1-Pentene, 3-Methyl- (CAS)
50	0.24	Para-Cymen-8-ol
51	0.07	Caryophyllene Oxide
52	0.06	Citronellyl Propionate
53	0.23	Benzene
54	0.09	Myrtenol
55	0.08	Alpha.-Cubebene
56	0.14	Naphthalene
57	0.10	3-Pentenal, 4-Methyl- (CAS)
58	0.24	2-Methyl-4-Methylenetetrahydropyra
59	0.15	T-Muurolol
60	0.09	Benzene
61	0.08	Alpha.-Copaene
62	0.21	Carvacrol
63	0.47	Beta.-Eudesmol
64	0.07	Myristcin
65	0.07	Limonene Glycol
66	0.91	1,2-Methylenedioxy-5,6-Dimethoxy-4-Allylbenzene
67	0.92	Butylidene Phthalide
68	0.93	Butylidene Dihydro-Phthalide
69	0.23	Butylidene Phthalide
70	0.07	1-(1-Cyanoethyl)3-Ethyl-1,2,5,6-Tetrahydropyridine

Evaluation of nuclear morphology indicated that the percentage of apoptotic cells increased from 6.0 ± 2.5 % (control) to $70.5 \pm$

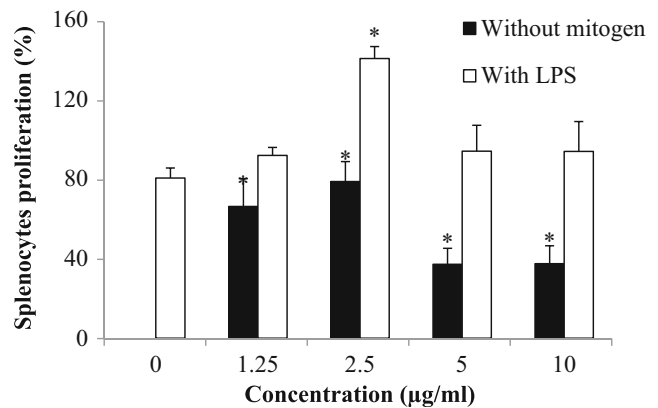


Fig. 1 In vitro effects of EO from *P. tortuosus* on splenocyte proliferation responses. Cells from naïve mice were incubated for 48 h with increasing concentrations of EO without mitogen or with LPS (5 µg/ml) in the absence/presence of EO. Control cells were incubated with RPMI 1640 only. Cell proliferation was assessed using an MTT test. Data shown are mean (\pm SE) values of three independent measures in separate experiments. *^aValue significantly different compared with that of negative (RPMI) control ($p < 0.05$). *^bValue significantly different compared with that of mitogen (LPS)-treated cells ($p < 0.05$)

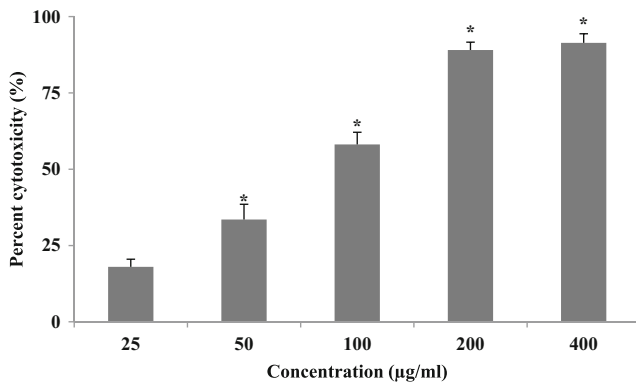


Fig. 2 Effects of EO on B16F10 cell proliferation. B16F10 cells were treated with different concentrations of EO for 48 h. Cell proliferation rate was then assessed using MTT. Values shown are mean (\pm SE) of three independent experiments. *Value significantly different vs corresponding untreated group ($p < 0.05$)

0.7 % and 94.5 ± 2.1 % in EO-treated cells after 48 h of exposure at concentrations of 160 and 320 $\mu\text{g/ml}$, respectively (Fig. 3).

Discussion

A large number of plants used in traditional medicines have been shown to possess immunomodulating and/or antitumor activities [10]. The aims of this study were to evaluate any anticancer effect of EO against B16F10 melanoma cancer cells in vitro and to clarify any underlying mechanisms. The results showed that, in vitro, EO inhibited cell proliferation in a concentration- and time-related fashion. To evaluate if the extract decreased cell

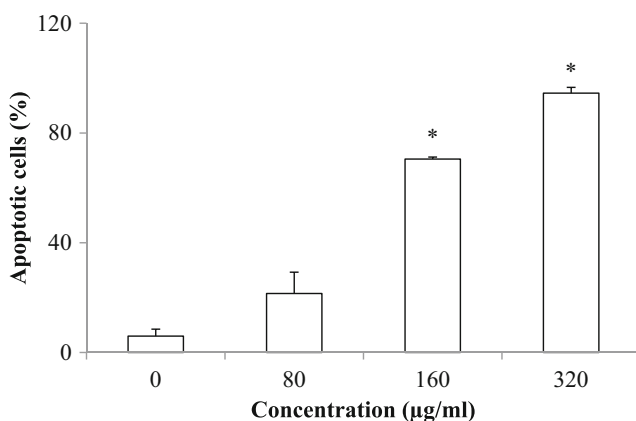


Fig. 3 Effects of EO on apoptosis in B16F10 cells. Cells were stained with AO/EB after treatment with different concentrations of EO for 48 h, and the presence of apoptotic cells then evaluated by fluorescence microscopy. Values shown are mean (\pm SE). The histogram is representative of two independent experiments (each performed in duplicate). *Value significantly different vs corresponding untreated group ($p < 0.05$)

viability via apoptosis, morphological changes characteristic of apoptotic cells (condensed chromatin) were analyzed and quantified. Evaluation of nuclear morphology indicated that the percentage of apoptotic cells increased among cells treated with EO. These results were in agreement with our previous studies that demonstrated how the EO of *P. tortuosus* induced cytotoxicity against several human cancer cells such as HepG2, HCT116, K562 [3, 4].

As with many cancers, development of melanoma is associated with immune suppression [11]. The capacity to elicit effective T and B cell immunity, including anti-tumor cell activity, is ultimately related to stimulation of lymphocyte proliferation and the upregulation of the activity of both NK and CTL cells [12, 13]. Factors released by activated NK and CTL cells, in turn, promote proliferation and differentiation of immune cells and can activate macrophages to phagocytize and then kill target (tumor) cells [14]. Thus, an efficient feedback mechanism is triggered in a host to help bring about removal of transformed cells.

In our study, we showed that lymphocyte subpopulations were differentially activated by EO (at varying levels) and that proliferation decreased with increasing concentrations. EO effects on LPS-stimulated cell proliferation were also analyzed. It is generally known that LPS stimulates B cells to proliferate [15, 16]. At 2.5 $\mu\text{g/ml}$, EO upregulated LPS-induced cell proliferation, suggesting to us potential synergistic effects of EO with this mitogen. The EO itself may also contain some factors that are acting as mitogens. Overall, this data indicates that EO might act as modulator of B cell function and, consequently, of humoral immune responses in situ.

Taken together, the findings here suggested to us that EO could potentially increase splenocyte proliferation in situ and that this, in turn, would lead to increased cytotoxic activity among splenic NK/CTL cells—resulting in reductions in tumor cell survival (and progression) in transformed cell-injected hosts. These results were in agreement with our previous studies that demonstrated how some natural products imparted antitumor effects via immune system activation. For example, we recently demonstrated that an aqueous extract of *Limoniastrum guyonianum* gall induced antitumor effects in melanoma-injected mice [17].

Based on the findings here and by other investigators, it may be assumed that much of the anticancer activity of EO could likely be ascribed to several compounds in the EO. GC-MS analysis revealed a presence of sabinen, α -pinene, limonene, and terpinen-4-ol. With regard to the former, terpinen-4-ol present in the EO is considered to be a very active anticancer agent. It was reported that terpinen-4-ol exhibited antimelanoma activity in vitro as well as in vivo [18]. It has also been reported that the D-

limonene significantly inhibited the growth and metastasis of gastric cancers *in vivo* [19].

Conclusions

In conclusion, our data presented that the tested EO of *P. tortuosus* is chemically characterized by the presence of sabinene, terpinen-4-ol, as major constituents, and possesses *in vitro* antimelanoma activities. In cell-based assay, it was able to stimulate the immune system and to induce apoptosis in B16F10 cells. Nevertheless, further investigations, such as studying effects against melanoma, *in vivo*, are still necessary to confirm our belief about utility.

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Conflict of interest The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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