Arabian Journal of Chemistry (2016) 9, 775–786

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Diverse biological effects of the essential oil from Iranian *Trachyspermum ammi*

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Received 23 April 2015; accepted 6 June 2015
Available online 20 June 2015

KEYWORDS

Trachyspermum ammi;
Ajwain oil;
Antimicrobial;
Antioxidant;
Cytotoxicity;
Immunostimulant

Abstract *Trachyspermum ammi* (Apiaceae) is a plant with a good reputation in the traditional Persian and Ayurvedic medicine. The hydrodistilled essential oil from the fruits of *T. ammi*, known as 'ajwain oil', is used in countries such as Iraq, Iran, Afghanistan, Pakistan, and India in the preparation of curry, to flavour several foods, as preservative, and in perfumery. At therapeutic level, ajwain oil is employed in the treatment of gastrointestinal ailments, lack of appetite and bronchial problems. In the present work, the essential oil of *T. ammi* growing in Iran was analysed by GC–FID and GC–MS showing thymol (67.4%), *p*-cymene (17.9%) and γ-terpinene (11.3%) as the major constituents. Afterwards, we investigated the biological effects displayed by ajwain oil, namely the antimicrobial and antioxidant activity, the cytotoxicity on tumour cells, and the induction of lymphocyte proliferation. In addition, the inhibition on nicotinate mononucleotide adenylyltransferase (NadD), which is a promising new target for developing novel antibiotics, was evaluated. The antimicrobial effects of ajwain oil, measured by the agar disc diffusion method, were relevant, with inhibition zones higher than those of reference antibiotics, especially on

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2015.06.002

Staphylococcus aureus and *Candida albicans* (34.7 and 54.3 mm, respectively). This effect was not due to the enzymatic inhibition on NadD. The ajwain oil exhibited a considerable dose-dependent inhibition on the ABTS radical cation, with an IC_{50} value of 22.4 μg/mL. MTT assay revealed that ajwain oil is particularly cytotoxic on colon carcinoma cells, with a IC_{50} value of 9.6 μg/mL. Finally, PBMC proliferation assay revealed some role for the ajwain oil within the network of interactions of the cells of the immune system.

1. Introduction

Herbal medicine represents one of the most important fields of traditional medicine all over the world. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way (Parekh and Chanda, 2007). Thousands of plant secondary metabolites have been identified and it is estimated that other thousands are yet to be discovered. Since secondary metabolites from natural sources have been elaborated within living systems, they are often perceived as showing more "drug – likeness and biological friendliness than totally synthetic molecules" (Koehn and Carter, 2005), making them good candidates for further drug development.

Trachyspermum ammi (L.) Sprague [syn. *Carum copticum* (L.) Benth. & Hook.f. ex C.B. Clarke], also known as ajwain (Bairwa et al., 2012), is a highly reputable plant as a source of constituents with promising bioactivity to be exploited at pharmaceutical level. *T. ammi* belongs to the Apiaceae family and is an annual herb up to 90 cm tall, native to arid and semi-arid regions of Egypt (Ashraf, 2002); it is also widely distributed and cultivated in Iraq, Iran, Afghanistan, Pakistan, and India. The plant is a highly valued medicinally seed spice. The fruits, having a bitter and pungent taste, are used to flavour and preserve foods, in perfumery for the manufacture of essential oil and in medicine (Pruthi, 1992). In the area of origin of the plant, the fruits are believed to exert aphrodisiac effects. They possess stimulant, antispasmodic and carminative properties, and are used traditionally in the treatment of flatulence, atonic dyspepsia, diarrhoea, abdominal tumours, abdominal pains, piles, bronchial problems, lack of appetite, asthma and amenorrhoea (Bairwa et al., 2012). The fruits contain 2–5% of a brown coloured essential oil, responsible for plant odour and taste, known as 'ajwain oil'. It is used in the treatment of gastrointestinal ailments, lack of appetite, and bronchial problems (Bairwa et al., 2012). The main component of this oil is thymol (35–60%), which is a strong germicide, antispasmodic and fungicide agent. The non-thymol fraction contains *p*-cymene, γ-terpinene, α-pinene, β-pinene, and other minor components (Zarshenas et al., 2014). However, sometimes γ-terpinene and *p*-cymene exceed the thymol content (Omer et al., 2014; Moein et al., 2015), and in other cases thymol and *p*-cymene are not among the predominant components (Singh et al., 2008).

The ajwain essential oil exhibited nematicidal (Park et al., 2007), scolicidal (Moazeni et al., 2012), antitermitic (Seo et al., 2009), antibacterial (Kumar et al., 2011; Paul et al., 2011; Moein et al., 2015), antifungal (Ashrafi Tamai et al., 2013; Moein et al., 2015; Kedia et al., 2015), and antioxidant (Chatterjee et al., 2013; Gandomi et al., 2014) effects. Interestingly, the oil showed appreciable spermicidal potential, which may be explored as an effective ingredient of male contraceptives (Paul and Kang, 2011, 2012). Ajwain oil also showed vapour toxicant and repellent effects against adults of *Anopheles stephensi* (Pandey et al., 2009), as well as larvicidal activity against *Aedes aegypti* (Seo et al., 2012), thus having promising applications in the management of malaria and yellow fever.

In the present work, we have evaluated the *in vitro* biological effects of ajwain oil, namely the antimicrobial and antioxidant activities, cytotoxicity on human tumour cells, and the induction of lymphocyte proliferation. These activities were evaluated by agar disc-diffusion, microdilution, DPPH, ABTS, FRAP, and MTT methods, and *in vitro* peripheral blood mononuclear cells (PBMC) proliferation assay, respectively. To complete the work we have evaluated the inhibitory effects of ajwain oil on nicotinate mononucleotide adenylyltransferase (NadD), which is a promising new target for developing novel antibiotics.

2. Materials and methods

2.1. Plant material

Fruits of *T. ammi* were collected at ripening from wild plants growing in Baluchistan (Iran) in the summer 2012. A voucher specimen was identified and deposited in the *Herbarium* of the University of Isfahan, Iran, under the code 125/21224/2012.

2.2. Hydrodistillation

The ripe fruits (78 g) were subjected to hydrodistillation in a Clevenger-type apparatus using 3 L of distilled water for 3 h until no more essential oil was obtained. The essential oil was stored in a sealed vial protected from light at – 20 °C before chemical analysis and biological assays. The oil yield (2.7%) was estimated on a dry weight basis.

2.3. GC–FID and GC–MS analyses

For GC separations, an Agilent 4890D instrument coupled to an ionization flame detector (FID) was used. Volatile components were separated on a HP-5 capillary column (5% phenylmethylpolysiloxane, 30 m, 0.32 mm i.d.; 0.25 mm film thickness; J and W Scientific, Folsom, CA, USA), with the following temperature programme: 5 min at 60 °C, subsequently 4 °C/min up to 220 °C, then 11 °C/min up to 280 °C, held for 15 min, for a total run of 65 min. Injector and transfer line

temperatures were 280 °C; He was used as the carrier gas, at a flow rate of 1.8 mL/min; split ratio, 1:34. Oil samples were diluted to 1:100 in *n*-hexane and injected at a volume of 1 μL. For each sample, the analysis was repeated three times and the mean value was reported. Data were collected by using HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01). Quantification of essential oil components was achieved by FID peak-area internal normalization without using correction factors. GC–MS analysis was performed on an Agilent 6890 N gas chromatograph coupled to a 5973 N mass spectrometer using a HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 mm film thickness; J and W Scientific) capillary column. The temperature programme was the same as described above. Injector and transfer line temperatures were 280 °C; He was used as the carrier gas, at a flow rate of 1 mL/min; split ratio, 1:50; acquisition mass range, 29–400 *m*/*z*. Mass spectra were acquired in electron-impact mode with an ionization voltage of 70 eV. The oil sample was diluted to 1:100 in *n*-hexane, and the volume injected was 2 μL. A mixture of aliphatic hydrocarbons (C8–C30; Sigma, Milan, Italy) in *n*-hexane was directly injected into the GC–MS under the above temperature programme, in order to calculate the temperature programmed RIs of peaks in the chromatograms. Data were analysed by using MSD ChemStation software (Agilent, Version G1701DA D.01.00). Major oil constituents were identified by co-injection with authentic standards. Otherwise, the peak assignment was carried out according to the recommendations of the International Organization of the Flavor Industry (http://www.iofi.org/), i.e. by the interactive combination of chromatographic linear retention indices that were consistent with those reported in the literature (Adams, 2007; NIST 08, 2008; FFNSC 2, 2012) for apolar stationary phases, and MS data consisting in computer matching with the WILEY275, NIST 08, ADAMS, FFNSC 2 and home-made (based on the analyses of reference oils and commercially available standards) libraries.

2.4. Antimicrobial activity

Ajwain essential oil was tested against a panel of microorganisms including *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, MD, USA), *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 24433. Bacterial strains were cultured overnight at 37 °C in blood agar plates (Oxoid, Basingstoke, UK). *C. albicans* was grown in Sabouraud Dextrose Agar Oxoid. Tests were performed following the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009). For paper disc diffusion method, a suspension of the tested microorganism ($1–2 \times 10^8$ cells per mL in saline-10^6 per mL for *Candida*) was spread on the solid media plates using a sterile cotton swab. Sterile paper discs (6 mm in diameter) were placed on the surface of inoculated plates and spotted with 10 μL of the essential oil. The plates were incubated 24 h at 35 ± 1 °C (48 h for *C. albicans*). The diameters of zone inhibition (including the 6 mm disc) were measured with a calliper. A reading of more than 6 mm indicated growth inhibition. No zone inhibition was observed using DMSO alone.

For microdilution method, a stock solution of essential oil was prepared in DMSO (oil to DMSO ratio 1:3). Then, two-fold serial dilutions of each compound were prepared in 96-well plates, starting from 10 mg/mL, in Cation Adjusted Mueller Hinton Broth (RPMI1640 for *C. albicans*). An equal volume of the microbial inoculum (10^6 cfu/mL), obtained by direct colony suspension of an overnight culture, was added to each well of the microtiter plate containing 0.1 mL of the serially diluted test molecule. After incubation for 18–24 h at 35 °C (24–48 h in the case of *Candida*), in normal atmosphere, Minimum Inhibitory Concentrations (MICs) were defined as the lowest concentration of compound able to inhibit the growth of the microorganisms. All tests were done in duplicate. Ciprofloxacin (5 μg disc) and Nystatin (100 Units disc) (both from Oxoid) were used as reference antimicrobials against bacteria and fungi, respectively. Each test was done in triplicate.

2.5. Enzyme inhibition assay

Recombinant NadD enzyme from *S. aureus* subsp. *aureus* N315 was a generous gift from Dr. Andrei Osterman. *Mus musculus* NMNAT isoform 1 (NMNAT1) was obtained as previously described (Orsomando et al., 2012). NadD in bacteria is responsible for the penultimate step of NAD synthesis, and it catalyses the conversion of NaMN and ATP into nicotinate adenine dinucleotide (NaAD) and inorganic pyrophosphate (PPi). NMNAT1 in mammals catalyses the same reaction but can also use nicotinamide mononucleotide (NMN) as an alternative substrate to yield nicotinamide adenine dinucleotide (NAD). A phosphate detection assay method based on Malachite Green reagent (Biomol Green, Enzo Life Sciences) was adapted to measure the activity of both enzymes (Sorci et al., 2009). Briefly, the common by-product of each catalysed reaction, inorganic pyrophosphate (PPi), was hydrolysed by inorganic pyrophosphatase and the resulting orthophosphate was detected by the malachite green reagent. The reaction mixtures contained, in a final volume of 0.1 ml, 1.2 nM *S. aureus* NadD (or an equal amount of murine NMNAT1), 100 mM buffer (Sigma), pH 7.5, 0.1 mM ATP, 10 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 0.2 U yeast inorganic pyrophosphatase, and 0.05 mM NaMN (for NadD) or 0.1 mM NMN (for NMNAT1). Ajwain oil was added to the reaction mixtures at a final concentration of 0.1 mg/ml. After preincubation of each enzyme with the oil for 5 min at 37 °C, the reaction was started by adding the corresponding mononucleotide substrate. The reactions were finally quenched with two volumes of Malachite Green reagent and, after 20–30 min incubation to allow for complex/colour formation, their absorbance at 620 nm was measured.

2.6. Antioxidant activity

Free radical scavenging activity (DPPH assay) of ajwain essential oil was assessed on a microplate analytical assay according to the procedures described by Srinivasan et al. (2007), while the total radical scavenging capacity of the same products was measured by the ABTS assay as modified by Re et al. (1999), for application to a 96-well microplate assay. Determination of antioxidant activity by FRAP assay was carried out according to the procedure described by Müller et al.

(2011), by monitoring the reduction of Fe^{3+}-tripyridyl triazine (TPTZ) to blue-colored Fe^{2+}-TPTZ. The ability of ajwain oil to scavenge the different radicals in all assays was compared to Trolox used as standard and expressed as tocopherol-equivalent antioxidant capacity mmol TE/g of product. Each experiment was repeated at least three times.

2.7. MTT assay

A375 (human malignant melanoma cells) (ATCC-CRL-1619, American Type Culture Collection, Rockville, MD, USA) and MDA-MB 231 cells (human breast adenocarcinoma cells) ATCC-HTB-26) were cultured in Dulbecco's modified Eagle's medium with 2 mM L-glutamine (PAA), 100 IU/mL penicillin, 100 mg/mL streptomycin and supplemented with 10% heat-inactivated foetal bovine serum (HI-FBS) (PAA). HCT116 cells (human colon carcinoma cells) (ATCC-CCL-247) were cultured in RPMI1640 medium (PAA) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin and supplemented with 10% HI-FBS. Cells were cultured in a humidified atmosphere at 37 °C in the presence of 5% CO_2. The MTT assay was used as a relative measure of cell viability. In brief, cells were seeded at the density of 2×10^4 cells/mL. After 24 h, samples were exposed to different concentrations of ajwain essential oil, standard compounds, and reconstituted oil (0.78–200 μg/mL). Cells were incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37 °C. At the end of incubation, the cytotoxic activity was evaluated by the MTT assay (Quassinti et al., 2013). Cisplatin (Sigma) was used as the positive control. Experiments were conducted in triplicate. Cytotoxicity is expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}). The IC_{50} values were determined with GraphPad Prism 4 computer program (GraphPad Software, San Diego, CA, USA).

2.8. PBMC proliferation assay

The proliferation assay was performed on pig blood lymphocytes isolated from fresh heparinized blood samples (PB, 20 mL/pig). Pigs (Danish pigs, weighing 65–70 kg) were conventionally reared in a hilly area of Umbria (Rustici Farm, Parco del Subasio, Assisi, Italy). In laboratory, the PB samples were mixed with an equal volume of sterile NaCl 0.9%. The mixture was laid on the top of 15 mL of Lympholyte® (Cedarlane®) in two 50 ml sterile centrifuge tubes for each sample and centrifuged (400*g* for 20 min at room temperature) to obtain the PBMC by density gradient. The PBMC layer was then transferred to sterile tubes and washed twice with Hank's balanced salt solution (HBSS; Gibco®, Life Technologies Italia) without Ca^{2+} and Mg^{2+}. Then the cells were suspended in complete RPMI-1640 medium (Euroclone®) that contained 10% heat-inactivated pig serum, L-Glutamine (2 mM; Euroclone®), penicillin (100 U/mL; Biochrom^{AG}, Berlin), and streptomycin (100 μg/mL; Biochrom^{AG}, Berlin). The number of live lymphocytes was determined using a counting chamber and a trypan blue dye exclusion procedure. Cells were suspended in medium and left overnight in the fridge (4 °C). For the assessment of cell proliferation by flow cytometry, the day after a number of PBMC was prestained with carboxyfluorescein diacetate succinimidyl ester (CFSE) cell tracer (BioLegend, San Diego, CA) and cultured for five days at 37 °C in 5% CO_2. Briefly, PBMC (15×10^6 cells in 2 mL of pre-warmed PBS) were incubated with 2 μL/mL CFSE at 37 °C for 10 min in the dark. Subsequently, 44 mL of cold medium was added and the cells were washed twice. The final concentration of live cells was adjusted to 2×10^6/mL in complete medium and 100 μL of suspension/well (2×10^5 live cells) was dispensed in flat bottom 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ). Proliferation was stimulated by adding 1 μg/mL of pokeweed mitogen (PWM, which stimulates the B lymphocyte only in the presence of T cells; Sigma–Aldrich) or 1.2 μg/mL of phytohemagglutinin (PHA, a polyclonal T-cell activator; Biochrom^{AG}, Berlin), as positive controls, or by adding different dilution of ajwain oil (5 μg/mL; 2.5 μg/mL; 2 μg/mL) alone or together with the same mitogens. Each culture condition was repeated in triplicate. The essential oil was initially diluted to 1:100 (v/v) in ethanol, and further dilutions were made in HBSS. The tested essential oil concentrations were all lower than the IC_{50} values of cytotoxicity. Finally, medium volume was adjusted to 200 μl/well. A negative control was represented by PBMC cultured without any mitogen, so that the base proliferation could be estimated (Liu et al., 1996). With each cell division, the intensity of CFSE staining will be halved and lymphocyte proliferation was calculated as frequency of $CFSE^{low}$ cells within gated cell population compared to intensity of parent population. Flow cytometry analyses were performed on a standard FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA) operated by the CELLQuest™ software. Within a tight lymphocyte gate, 10,000 cells were acquired and the data were saved in the list mode. Cells from each culture condition were pooled and analysed together. The residual cells after FACS analysis, stimulated with 2.5 μL of ajwain oil, were stained using a PE labelled mouse anti-pig CD8a mAb (76-2-11 clone, BD Pharmingen) and an APC labelled mouse anti-human $CD79a^+$ mAb (clone HM47, BD Pharmingen), a cross-reactive antibody against pig B^+-cells antigen, for intracellular staining (Lee et al., 2008). Briefly, each tube was incubated at 4 °C for 20 min in the dark with 5 μl of PE mouse anti-pig $CD8^+$; after incubation, cells were twice washed in PBS (1 mL), by centrifuging the cells at 400*g* for 5 min at room temperature and carefully eliminating the supernatant. After a 4% paraformaldehyde fixation, the same samples were finally permeabilized with 0.1% saponin blocking buffer to 'intracellularly' label the $CD79^+$ cells (APC pig B-lymphocytes) according to a protocol recommended by the producers, and data were again acquired by flow cytometer. This procedure has permitted a simultaneously three colour analysis on the same sample: FITC-CFSE, PE -$CD8^+$ and APC-$CD79a^+$.

3. Results and discussion

3.1. Essential oil composition

Hydrodistillation of ajwain fruits gave 2.7% of a brownish essential oil whose composition is reported in Table 1. Nineteen volatile constituents were identified, accounting for 99.8% of the total composition. The essential oil was mainly composed of aromatic compounds (86.2%) and monoterpene hydrocarbons (13.1%). Among the former, the phenol thymol (67.4%) was by far the predominant component, followed by

Table 1 Composition of the essential oil from seeds of *Trachyspermum ammi*.

N.	Component[a]	RI exp.[b]	RI ADAMS[c]	%[d]	ID[e]
1	α-Thujene	916	924	0.2	RI,MS
2	α-Pinene	922	932	0.1	Std
3	Sabinene	960	969	tr[f]	RI,MS
4	β-Pinene	962	974	0.7	Std
5	Myrcene	983	988	0.2	Std
6	α-Phellandrene	998	1002	Tr	Std
7	δ-3-Carene	1003	1008	Tr	Std
8	α-Terpinene	1010	1014	0.3	RI,MS
9	*p*-Cymene	1017	1020	17.9	Std
10	β-Phellandrene	1020	1025	0.3	RI,MS
11	1,8-Cineole	1023	1026	Tr	Std
12	γ-Terpinene	1051	1054	11.3	Std
13	*cis*-Sabinene hydrate	1060	1065	Tr	RI,MS
14	Terpinolene	1080	1086	Tr	Std
15	*trans*-Sabinene hydrate	1091	1098	0.1	RI,MS
16	Terpinen-4-ol	1168	1174	0.2	Std
17	α-Terpineol	1184	1186	0.1	Std
18	Thymol	1294	1289	67.4	Std
19	Carvacrol	1299	1298	0.9	Std
	Total identified (%)			99.7	
	Grouped components (%)				
	Aromatic compounds			86.2	
	Monoterpene hydrocarbons			13.1	
	Oxygenated monoterpenes			0.4	

[a] Compounds are listed in order of their elution from a HP-5MS column.
[b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8–C_{30} alkanes.
[c] Linear retention index taken from Adams (2007).
[d] Percentage values are means of three determinations at GC–FID, with a RSD% in all cases below 10%.
[e] Identification methods: std, based on comparison with authentic compounds; MS, based on comparison with WILEY, ADAMS and NIST 08 MS databases; RI, based on comparison of LRI with those reported in ADAMS, FFNSC 2 and NIST 08.
[f] tr, % below 0.1%.

p-cymene (17.9%), while carvacrol was present in scant amount (0.9%). γ-Terpinene (11.3%) was the most representative compound among monoterpene hydrocarbons. Finally, none of the other constituents occurring in the oil exceeded 0.7%. The essential oil composition herein detected is fully consistent with the standard reported in the literature for ajwain oil (Zarshenas et al., 2014). However, different chemical profiles for ajwain oil were recently reported from Iran (Moein et al., 2015) and India (Kedia et al., 2015). In these works, the authors found γ-terpinene (48%) and *p*-cymene (76.3%) more abundant than thymol (17.4% and 13.3%, respectively). We assume that these quantitative differences may be related to the geographic origin, genetic variability and harvesting time of the samples.

3.2. Antimicrobial activity

Results for antimicrobial activity are shown in Table 2. As evaluated by the disc diffusion test, ajwain essential oil inhibited Gram-positive bacteria quite effectively, with *S. aureus*

Table 2 Antimicrobial activity of ajwain oil by the diffusion disc (IZD) and the microdilution (MIC) methods. Each value is the average of three determinations. Where appropriate, standard deviation is indicated (±SD).

Compound		*S. aureus*	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
		ATCC 29213	ATCC 29212	ATCC 25922	ATCC 27853	ATCC 24433
Essential oil						
	IZD (mm)	34.7 ± 2.3	27.7 ± 1.5	29.3 ± 1.1	8.5 ± 0.5	54.3 ± 1.1
	MIC (μg/ml)	500	1000	500	10,000	500
Std. antibiotic						
Ciprofloxacin	IZD (mm)	23 ± 1	n.r.[a]	29 ± 1	30 ± 2	n.a.[b]
	MIC (μg/ml)	0.25	0.5–1	0.016	0.5	n.a.
Nystatin	IZD (mm)	n.a.	n.a.	n.a.	n.a.	15–16
	MIC (μg/ml)	n.a.	n.a.	n.a.	n.a.	4.0

[a] n.r.: Not recommended.
[b] n.a.: No activity (no IZD).

Table 3 *In-vitro* radical-scavenging activities of ajwain oil.

	DPPH		ABTS radical cation		FRAP
	TEAC[a] μmol TE/g	IC_{50}[b] μg/mL	TEAC[a] μmol TE/g	IC_{50}[b] μg/mL	TEAC μmol TE/g
Ajwain oil	72.6 ± 13.9	239.3 ± 33.3	266.7 ± 32.3	22.4 ± 2.4	90.6 ± 0.3
Positive control					
Trolox		4.26 ± 0.4		1.49 ± 0.8	

[a] TEAC = Trolox equivalent (TE) antioxidant concentration.
[b] IC_{50} = the concentration of compound that affords a 50% reduction in the assay.

Table 4 *In vitro* cytotoxic activity of essential oil from *Trachyspermum ammi*.

	Cell line (IC_{50} μg/ml)[a]		
	A375[b]	MDA-MB 231[c]	HCT116[d]
Ajwain oil	16.93	66.52	9.6
95% C.I.[e]	13.20–21.71	60.81–72.76	8.15–11.29
Reconstituted oil (thymol + *p*-cymene + γ-terpinene)	21.07	65.33	30.58
95% C.I.[e]	17.83–24.89	59.13–72.50	25.43–36.77
Thymol	9.12	15.75	13.62
95% C.I.[e]	8.38–9.92	14.52–17.09	13.15–14.10
p-Cymene	13.00	31.40	19.32
95% C.I.[e]	10.23–16.52	28.17–34.99	15.20–24.56
γ-Terpinene	22.48	59.59	13.35
95% C.I.[e]	19.61–25.76	49.39–71.90	10.23–17.41
Positive control			
Cisplatin	0.49	2.54	2.12
95% C.I.[e]	0.34–0.56	2.02–3.04	1.92–2.57

[a] IC_{50} = the concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation).
[b] Human malignant melanoma cell line.
[c] Human breast adenocarcinoma cell line.
[d] Human colon carcinoma cell line.
[e] Confidence interval.

being more susceptible than *E. faecalis*. This result was also confirmed by microdilution method. Accordingly, MIC of the essential oil toward *E. faecalis* was double than the one obtained for the staphylococcal species. Among Gram-negative species only *E. coli* was inhibited to the same extent as the Gram-positives. *P. aeruginosa* was almost insensitive to the oil as confirmed by the MIC determination, which is actually very high (10,000 μg/mL). On one hand, the obtained results were in good agreement with previously reported data (Moein et al., 2015). On the other hand, Paul and coll. found comparable MIC values in the range of 150–500 μg/mL toward *S. aureus* and *E. coli* (Paul et al., 2011), but a remarkable MIC of 12.5 μg/mL toward *P. aeruginosa*, which is extremely different from our value (MIC = 10,000 μg/mL). The difference may be accounted for by the different strain used (KCTC 2004 *vs* ATCC 27853) or by the different experimental condition used to perform the test (LB medium *vs* MH medium). According to the composition of the oil, thymol was the main component and it might fully explain the inhibitory activity observed (Burt, 2004; Rota et al., 2008). Of note, the ajwain oil antibacterial activity was comparable to that of the reference antibiotic ciprofloxacin, in the disc diffusion test. In the microdilution test ciprofloxacin was found to act at lower concentrations (Table 2). A fair explanation of this result could be the solubility of the essential oil in the water-based medium used for the microdilution test assay. The limitation is most probably circumvented in the disc diffusion test, where the growth of bacteria occurs on the surface of the semi-solid medium. The same observation has been made for the fungus *C. candida*. Ajwain oil had a MIC of 500 μg/mL, which was 125 fold higher than the reference antimicotic molecule nystatin. The MIC value against the *Candida* species is in full agreement with Moein and coll., which tested a fraction of *T. ammi* essential oil having a composition similar to ours (Moein et al., 2015). Conversely, inhibition zone diameters from the disc diffusion experiments revealed a very strong inhibitory effect, generating a consistent halo of up to 55 mm.

3.3. Enzyme inhibition assay

Prompted by the herein observed bacterial growth suppressive activity of ajwain oil, we tested whether the antibacterial property could be attributed, at least partially, to the inhibition of the bacterial metabolic enzyme nicotinate mononucleotide

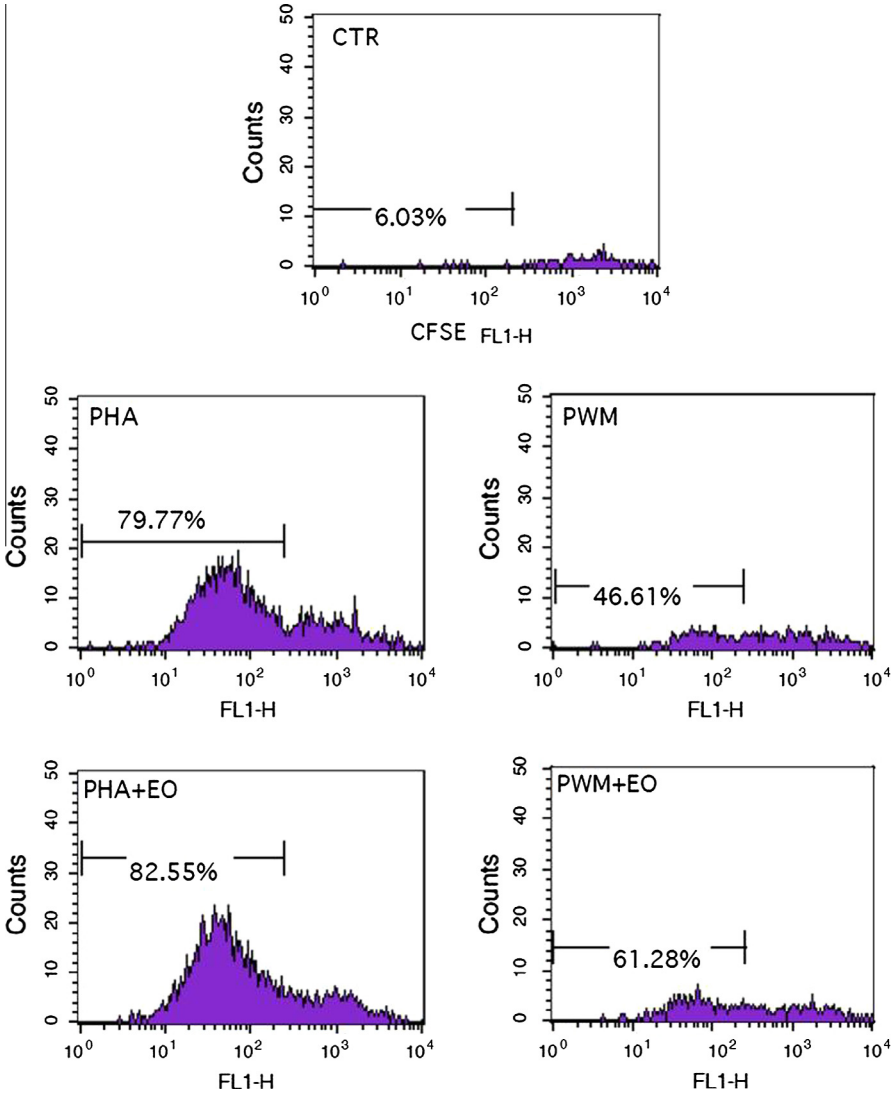

Figure 1 Peripheral blood mononuclear cells (PBMC) proliferation monitored by CFSE labelling in response to *in vitro* stimulation with PHA or PWM. An example of CFSE pattern in the PBMC in a pig whose CFSE-labelled cells were cultured *in vitro* in the presence of PHA, PWM or in media only (CTR). After 5 days, the PBMC were analysed by flow cytometry for CFSE intensity.

adenylyltransferase (NadD, arising from the *nadD* gene). NadD represents a novel, actively pursued antibacterial target (Sorci et al., 2009, 2013; Petrelli et al., 2011; Rodionova et al., 2015), for which no natural products inhibitors have been reported yet. The choice to test NadD target was dictated by the observed resistance of *P. aeruginosa*, in our hands, toward *T. ammi* essential oil as opposed to the significant susceptibility of *S. aureus*, *E. faecalis* and *E. coli*. Indeed, a comparative genome analysis indicates that *P. aeruginosa* is one of the few bacteria, and the only major bacterial pathogen, that carries two copies of the *nadD* gene (loci PA4006 and PA4917) (Sorci L, unpublished observation) in its genome. These two paralogs are quite divergent, bearing just 31% of sequence identity, and thus potentially increasing or granting full resistance to an antibacterial substance that targets the NadD enzyme. Nevertheless, our assay did not reveal any significant inhibition of bacterial NadD at 0.1 mg/mL concentration of *T. ammi* oil. Similarly, the eukaryotic functional counterpart of bacterial NadD, NMNAT1, was not affected by the ajwain oil in our assay conditions (data not showed). Such absence of inhibition on NAD synthesis suggests that the antimicrobial activity of ajwain oil is likely to be attributed solely to the recognized alteration of the cell membrane permeability, by action of its major component thymol (Burt, 2004).

3.4. Antioxidant activity

We assayed the ajwain oil, with thymol as the major constituent (67.4%), for which a significant radical scavenging activity and a major contribution to the antioxidant potential have been reported (Alma et al., 2003; Öztürk, 2012; Quiroga et al., 2015).

To provide better insights into the antioxidant potential of the ajwain oil we used alternative free radical generating systems to assess its free radical scavenging and reducing properties.

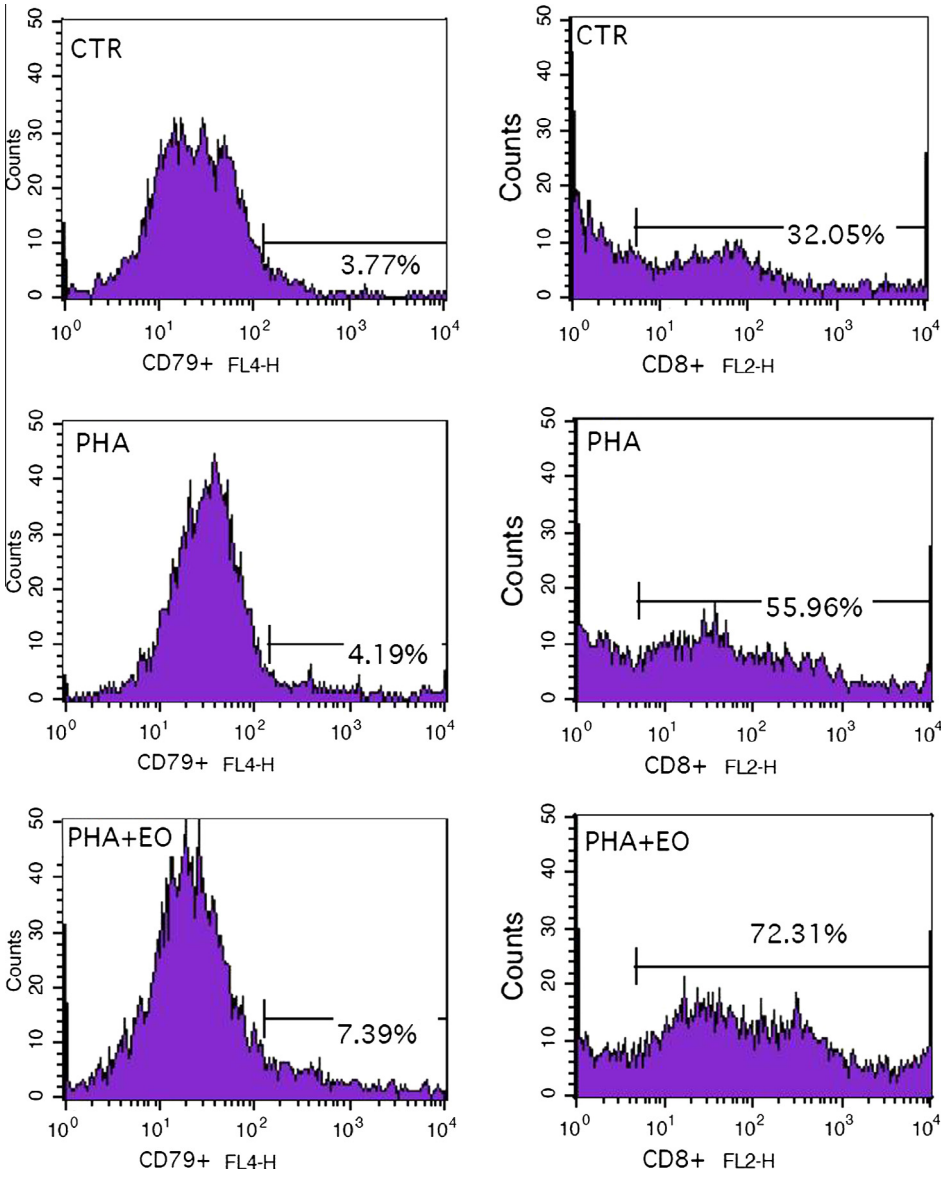

Figure 2 T and B sub-population proliferation monitored by CFSE labelling in response to *in vitro* stimulation with PHA. PBMC proliferation in a pig whose CFSE-labelled cells were cultured *in vitro* in the presence of PHA or in media only (CTR). After 5 days, cells were stained with anti-CD8a and anti-CD79a monoclonal antibody and analysed by flow cytometry. T and B cell subsets were identified on the basis of their $CD8^+$ and $CD79^+$ expression.

As reported in Table 3, the essential oil showed a good antioxidant power as evidenced from the high value of scavenging activity towards the ABTS radical cation (TEAC = 266.7 ± 32.3 µmol TE/g, IC_{50} = 22.4 µg/mL), while a lower activity is measured for the DPPH radical (TEAC = 72.6 ± 13.9 µmol TE/g, IC_{50} = 239.3 µg/mL). Moreover, the antioxidant compounds occurring in the oil seem to act as good reducing agents (FRAP activity, TEAC = 90.6 ± 0.3 9 µmol TE/g). Thus, our overall results obtained with different assays indicate good antioxidant properties of the ajwain oil. Minor differences in the observed scavenging activities may be attributed to the intrinsic mechanisms of the radical antioxidant reactions in the different assays, or to factors such as stereo-selectivity of the radicals and the solubility of antioxidant components. Our data are in accordance with those previously reported for the ajwain oil (Nickavar et al., 2014; Singh et al. 2004; Goswami and Chatterjee, 2014), where a noticeable free radical scavenging and antioxidant activity is shown, and confirm that *T. ammi* is a natural source of important antioxidant substances to be used as efficient antioxidant agents comparable to commercially used antioxidants.

3.5. Cytotoxicity on tumour cells

In the literature few data are available about anticancer activity of the essential oil from *T. ammi*. To investigate the cytotoxicity of ajwain oil, we evaluated the antiproliferative

effects of pure essential oil, standard compounds, and reconstituted oil (with available standards of the three main components) on human tumour cell lines by the MTT assay. Three human cell lines, a malignant melanoma cell line (A375), a breast adenocarcinoma cell line (MDA-MB 231), and a colon carcinoma cell line (HCT116), were treated with different concentrations of essential oil for 72 h. As shown in Table 4, the essential oil was active against all of them, and induced a concentration-dependent inhibitory effect in the dilutions range 0.78–200 μg/mL. The highest activity was observed on HCT116 cell line, with IC_{50} value of 9.6 μg/mL, while the lowest activity was obtained on MDA-MB 231, with IC_{50} value of 66.52 μg/mL. Such a cytotoxic activity of ajwain oil could also be attributed to its main components. Indeed, in our experiments, thymol resulted to be cytotoxic on all three cell lines, with IC_{50} values ranging from 9.19 μg/mL (60.7 μM) to 15.75 μg/mL (104.8 μM) for A375 and MDA-MB 231, respectively, while *p*-cymene and γ-terpinene exerted generally lower cytotoxic activity. These data are in good agreement with the reported antiproliferative activity of thymol against human acute promyelotic leukaemia cell line HL-60 (Deb et al., 2011), human glioblastoma cell line (Hsu et al., 2011), and human lung cancer cell line H1299 (Ozkan and Erdogan, 2012). Bourgou et al. (2010) studied the cytotoxic activity of γ-terpinene against human lung carcinoma A-549 and colon adenocarcinoma DLD-1 cells and achieved $IC_{50} \geqslant 100$ μM (13.62 μg/mL) on both cell lines. Also γ-terpinene showed some cytotoxic properties against Jurkat cell line (Döll-Boscardin et al., 2012). Finally, the above listed compounds with demonstrated antiproliferative activity were appropriately combined to reconstitute an 'artificial' mixture of essential oil, using commercially available compounds. Hence, thymol, *p*-cymene, and γ-terpinene were mixed at the same percentage reported in Table 1 and the solution obtained was tested on the above cell lines. The cytotoxic activities resulted in all cases slightly lower than those of the original essential oil (Table 4). This result highlights that some other minor compounds occurring in the essential oil might contribute to potentiate its cytotoxic activity in a synergistic way with respect to thymol, *p*-cymene, and γ-terpinene.

Finally, the inhibition of ajwain oil on colon carcinoma cells could partially support the traditional use of fruits in the treatment of abdominal tumours (Bairwa et al., 2012)

3.6. PBMC proliferation assay

The three different concentrations of ajwain essential oil tested were not able to stimulate a lymphocyte proliferation *in vitro* when added alone to the culture plates. However, when the PBMC were activated by mitogens such as PHA and PWM and cultured together with the ajwain essential oil, the latter seemed to generate a dose-dependent synergistic effect. Indeed, a slight increase of the response to the proliferation assay was observed for cells stimulated with PHA at all the three concentrations tested (mean increase ± standard deviation: 7.03% ± 1.9; percentage of increase: +11.1%. Fig. 1), whereas, the combination of ajwain oil with PWM seemed to induce an higher response to the proliferation assay, proportional to the oil concentration (mean increase ± standard deviation: 17.03% ± 7.7; percentage of increase: +75.3% Fig. 1). Furthermore, the level of proliferation (determined as the percentage of $CFSE^{low}$) in some lymphocyte subsets was evaluated for cells stimulated with PHA, PWM and 2.5 μg/mL of ajwain essential oil. These cells, when stimulated by PHA with/without ajwain oil revealed an increase of both the percentages of APC-$CD79a^+$ cells and PE-$CD8a^+$ cells (Fig. 2), while, those cells stimulated by PWM with/without ajwain oil, revealed a substantial increase of the percentage of $CD8^+$ cells, besides a maintained percentage of $CD79a^+$ cells (Fig. 3) in the presence of the ajwain oil. Unfortunately, it was not possible to investigate the percentage of $CD4^+$ cells because the FITC channel in the cytometer was already occupied by CFSE.

Several T cell subsets in pig have been identified on the basis of CD4 and CD8 cell surface expression, and are generally defined as: naive/nonactivated $CD4^+CD8^-$ T cells, cytotoxic $CD4^-CD8^+$ T cells (CTL) and memory/activated $CD4^+CD8^+$ T cells (T_{helper}) (Lefevre et al., 2012). However, T-helper cells express CD4 as well as other activation-related markers, including CD8alpha, MHC class II and CD45RC (Gerner et al., 2009).

The pig B cells could be identified by the anti-human CD79a mAb that recognizes the pig CD79a on the cell surface (Lee et al., 2008). Besides these lymphocyte subsets, pigs possess a high proportion of circulating TCR-γδ T cells (Takamatsu et al., 2006) that recognize different types of antigen in alternative ways to αβ T cells, and thus appear to play a complementary role in the immune response. The latter subset can express CD8a and MHC class II, two molecules which in swine seem to be correlated with an activation status of T cells. Functional properties of these cells seem to include cytolytic activity as well as antigen presentation; however, both aspects require further investigation (Gerner et al., 2009; Takamatsu et al., 2006).

In this study the ajwain oil, utilized at a concentration much lower than the cytotoxic one (2.5 μg/mL) and combined with a specific mitogen for T cells (PHA), seemed to be effective in the induction of an higher proliferation of PBMC *vs* PHA alone, increasing both the proportion of $CD8a^+$ and $CD79a^+$ cells *in vitro*. On the other hand, the combination of ajwain oil with a specific mitogen for B cells (PWM), induced a higher proliferation response by PBMC, together with an increase of $CD8a^+$ and a maintained proportion of $CD79a^+$ and *in vitro*, with respect to the cells stimulated only by PWM.

In the first case, the increased response to the proliferation assay by PBMC, supported by an increase of $CD8^+$ subset, could be attributed to an increase of proliferation of both the $CD4^-CD8^+$ (CTL) and the $CD4^+CD8^+$ subsets that, although not investigated in this study, similarly respond to PHA mitogen induction. While, the higher percentage of $CD79a^+$ could represent a direct effect of ajwain essential oil, in the case of stimulation with PWM and ajwain oil, besides the increase of proliferation by PBMC, the higher proportion of $CD8a^+$ could be expression of the activation of γδ T cells or derived from the proliferation of the $CD4^+CD8^+$ T and $CD4^-CD8^+$ T subsets. Indeed, as mentioned above, the γδ T cells can express CD8a when activated and autologous B cells can stimulate γδ T cells after a period of mitogen-activation (Häcker et al., 1995), whereas a possible increase of the $CD4^+CD8^+$ T (activated T_{helper}) and $CD4^-CD8^+$ T (CTL) subsets could be a direct effect of ajwain essential oil. Thus, these data suggest some role for the ajwain essential oil within the network of interactions of the cells of the

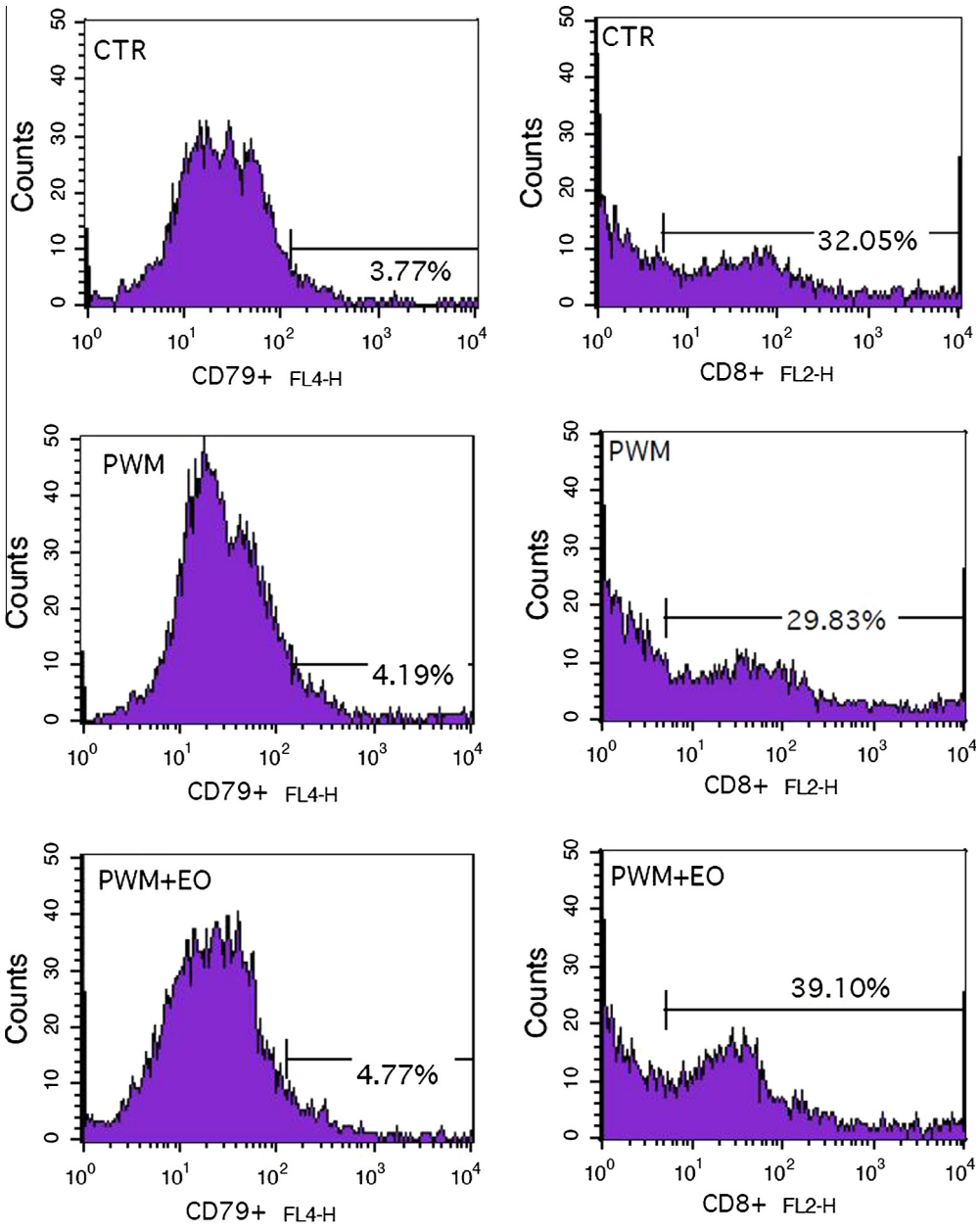

Figure 3 T and B sub-population proliferation monitored by CFSE labelling in response to *in vitro* stimulation with PHA. PBMC proliferation in a pig whose CFSE-labelled cells were cultured *in vitro* in the presence of PWM or in media only (CTR). After 5 days, cells were stained with anti-CD8a and anti-CD79a monoclonal antibody and analysed by flow cytometry. T and B cell subsets were identified on the basis of their $CD8^+$ and $CD79^+$ expression.

immune system. Future studies on its possible effect on the $CD4^+$ T cells, γδ T cells are recommended.

4. Conclusions

T. ammi represents a medicinal plant with a consolidated profile in the oriental traditional medicine. Most of its original uses are expected to be exported also to the Western medicine. Ajwain oil, which is characterized by thymol as the major bioactive constituent, was proven to exert important biological effects such as antimicrobial and antioxidant, confirming what previously reported in the literature and supporting its potential application as a food preservative with efficacy comparable to that of commercially used substances. For the first time we have reported the cytotoxic activity of ajwain oil on colon carcinoma cells together with its interaction with immune system cells, which could open new interesting applications for health. However, further studies are recommended.

Ethical conduct of research

For the blood sampling, the animal care procedures were in accordance with the recommendations of the Directive 2010/63/EU of the European Parliament and of the Council of the European Union for the protection of animals used for scientific purposes.

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