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PII: S0378-8741(13)00487-X
DOI: http://dx.doi.org/10.1016/j.jep.2013.06.054
Reference: JEP8214

To appear in: *Journal of Ethnopharmacology*

Received date: 30 April 2013
Revised date: 26 June 2013
Accepted date: 27 June 2013

Cite this article as: Amel Bouzabata, Ophélie Bazzali, Célia Cabral, Maria José Gonçalves, Maria Teresa Cruz, Ange Bighelli, Carlos Cavaleiro, Joseph Casanova, Ligia Salgueiro, Félix Tomi, New compounds, chemical composition, antifungal activity and cytotoxicity of the essential oil from *Myrtus nivellei* Batt. & Trab., an endemic species of Central Sahara, *Journal of Ethnopharmacology*, http://dx.doi.org/10.1016/j.jep.2013.06.054

New compounds, chemical composition, antifungal activity and cytotoxicity of the essential oil from *Myrtus nivellei* Batt. & Trab., an endemic species of Central Sahara.

Amel Bouzabata[a], Ophélie Bazzali[a], Célia Cabral[b], Maria José Gonçalves[b], Maria Teresa Cruz[c], Ange Bighelli[a], Carlos Cavaleiro[b], Joseph Casanova[a], Ligia Salgueiro[b], Félix Tomi[a]*

[a]Université de Corse-CNRS, UMR 6134 SPE, Equipe Chimie et Biomasse, Route des Sanguinaires, 20000 Ajaccio, France.
[b]Centro de Estudos Farmacêuticos/Faculdade de Farmácia, Universidade de Coimbra, 3000-548 Coimbra, Portugal
[c]Centro de Neurociências e Biologia Celular e Faculdade de Farmácia, Universidade de Coimbra, 3000-295 Coimbra, Portugal

ABSTRACT

Ethnopharmacologic relevance: Myrtus nivellei Batt. & Trab. (Myrtaceae) known as Sahara myrtle is appreciated by the Touaregs as medicinal plant. Infusion of leaves is employed against diarrhea and blennorrhea. Crushed leaves added to oil or to butter ointment have been traditionally used for the treatment of dermatosis.
Aim of the study: Considering the traditional medicinal uses and the lack of scientific studies on their biological activities, the present study was designed to elucidate the chemical composition, the antifungal activity of its essential oils against fungi responsible for human infections, as well as, its cytotoxicity in the mammalian keratinocytes.
Materials and Methods: Chemical analysis of *M. nivellei* essential oil isolated by hydrodistillation of aerial parts (leaves and flowers), was carried out using a combination of chromatographic (CC, GC with retention indices) and spectroscopic techniques (MS, ^{13}C NMR, 2D NMR). The antifungal activity was evaluated by using broth macrodilution methods for yeasts and filamentous fungi. Cytotoxicity was tested in HaCaT keratinocytes through the MTT assay.
Results:–Ten samples coming from two localities of harvest were investigated. The chemical composition was largely dominated by 1,8-cineole (33.6-50.4%) and limonene (17.5-25.0%). The structure of two new compounds bearing the isoamylcyclopentane skeleton has been elucidated. The oil was more active against *Cryptococcus neoformans* with MIC of 0.16 µL/mL followed by dermatophytes, with MICs of 0.64 and 1.25 µL/mL. Furthermore, evaluation of cell viability showed no cytotoxicity in HaCaT keratinocytes at concentrations up to 1.25µL/mL.
Conclusions: The composition of *M. nivellei* oil differed from that of *M. communis*. The structure of two *di-nor*-sesquiterpenoids has been elucidated. It was possible to find appropriate doses of *M. nivellei* oil with both antifungal activity and very low detrimental effect on keratinocytes. These findings add significant information to the pharmacological activity of *M. nivellei* essential oils, specifically to its antifungal properties, thus justifying and reinforcing the use of this plant in traditional medicine.

Keywords: Myrtus nivellei, essential oil, chemical composition, 2D NMR, new terpenes, antifungal activity, cytotoxicity.

1. Introduction

In Algeria, the genus *Myrtus* L. (Myrtaceae) presently includes two species, *Myrtus communis* L. known as common myrtle and *Myrtus nivellei* Batt. & Trab. known as Sahara myrtle (Migliore et al., 2012). *M. nivellei* is an endemic species of Sahara, widespread in the Center and scarce in the North (Quézel, 1965). It has been considered two subspecies: subsp. *nivellei* in Algeria and subsp. *tibesticus* in Tchad (Tibesti) (Quézel, 1958).

M. nivellei grows in scattered populations, in rocky and sandy wades where subterranean water points exist, and generally at an altitude above 1400 m (Hammiche and Maiza, 2006). It is a shrub up to 2 m, with rough bark, leaves lanceolate, thick and linear (4- 5 cm), five white petals, indeterminate stamens and the fruits are black berries (Battandier and Trabut, 1911; Ozenda, 2004).

M. nivellei, known under the names of "*Tafeldest"* or *"Tafaltasset"* in Tamahaq and "*Raihane Essahara El Wousta"* in Arabic, is appreciated by the Touaregs as medicinal plant, condiment and spice (flavoring of tea) (Maiza, 2008). According to the ethnobotanical research reported through interviews with nomad populations, leaves in infusion are employed by practitioners in internal use against intestinal diseases (diarrhea), fever and diabetes (Maiza, 2008, Hammiche and Maiza, 2006). In external use, the crushed leaves added to oil or butter ointment (poultice) are recommended in the treatment of dermatosis and for hair care (Hammiche and Maiza, 2006). In traditional medicine, the leaves in infusion or added to barley wafers, are also employed against the blennorrhea (Sahki and Boutamine-Sahki, 2004).

Very little is known about the phytochemistry and biological activities of *M. nivellei.* To our knowledge there is only one paper reporting the phenolic compounds and its antioxidant activity (Rached et al., 2010) and there is none about the composition of *M. nivellei* essential oil.

Therefore, in continuation of our ongoing work on the characterization of aromatic and medicinal plants from Algeria (Bekhechi et al., 2010; Bousmaha et al., 2006; Bouzabata et al., 2013; Mecherara-Idjeri et al., 2008), and considering the widely use of *M. nivellei* by the Touaregs in Algerian traditional medicine, the aims of the present work were to investigate the chemical composition of its essential oils, as well as to evaluate the antifungal potential and its topical safety on keratinocyte cells.

2. Materials and methods

2.1. Plant material and essential oil isolation

Aerial parts of *M. nivellei* were collected in July 2010 during the flowering stage from Central Sahara. Samples TAS1-TAS5 and TAM1-TAM5 were isolated from plants harvested near Djanet city (Tassili of N'Ajjers massif; altitude: 1710 m, latitude: 24°37, longitude: 9°35) and near Tamanrasset city (Hoggar massif; altitude: 1900 m, latitude: 22°50, longitude: 5°37), respectively (Fig. 1). A voucher specimen was deposited at the herbarium of the Museum d'Histoire Naturelle, Aix-Marseille University; reference PH-2011-17-1. The myrtle was identified as *M. nivellei* by Dr G. De Belaire, University of Badji-Mokhtar, Annaba (Algeria). Each sample of aerial parts (leaves and flowers, 100g) of *M. nivellei* was submitted to hydrodistillation using a Clevenger type apparatus during 3 hours. Essential oils were stored in the dark, at 4°C.

Figure 1: Locations of harvest of *Myrtus nivellei* in central Sahara (Algeria).

2.2. Fractionation of the essential oil

An aliquot of the sample TAM2 (1055 mg) was chromatographed on a silica gel column (63-200 µm, 20.5g) affording four fractions which were analyzed by GC(RI), GC-MS and ^{13}C NMR. Fractions F1 (159 mg) and F2 (61 mg) eluted with pentane contained terpene hydrocarbons, whereas oxygenated compounds were present in two fractions, F3 (501 mg, pentane/diethyl ether (P/DE) = 98/2) and F4 (334 mg; diethyl ether). The fraction F4 contained unidentified components A (26.8 %), and B (6.5 %). Part of F4 (250 mg) was fractionated once again (silica gel, 63-200 µm, 5 g) using a gradient of solvents, pentane/diethyl ether (96/4 to 0/100) as mobile phase, affording 28 fractions (F4.1-F4.28) which were analyzed by GC(RI) and ^{13}C NMR. Fraction F4.23 (5 mg, P/DE = 90/10) contained unidentified compound B (77% purity by GC). Unidentified compound A was present in fractions F4.10-F4.12 (19-20 mg each, P/DE = 95/5). The three fractions were mixed and the resulting mixture (60 mg) was chromatographed once again (silica gel, 35-70 µm, 5 g) affording 18 fractions. Fraction 16 (11 mg) eluted with P/DE = 95/5 contained unidentified compound A (purity: 84% by GC). Compounds A and B were accompanied by minor components that did not hinder structural elucidation by 2D NMR.

Compound A: ^{1}H and ^{13}C NMR data, see Table 1; EIMS 70 eV, m/z (relative intensity): 165 (92), 123 (100), 95 (35), 85 (17), 79 (10), 67 (11), 57 (27), 43 (64), 41 (30), 39 (11).

Compound B: ^{1}H and ^{13}C NMR data, see Table 2; EIMS 70 eV, m/z (relative intensity): 163 (77), 123 (40), 112 (7), 95 (20), 83 (100), 79 (6), 67 (11), 55 (35), 43 (41), 41 (21), 39 (16).

2.3. GC and GC-MS Analysis

GC analysis were carried out using a Perkin-Elmer Autosystem apparatus equipped with two Flame Ionization Detectors (FID), and fused capillary columns (50 m x 0.22 mm i.d., film thickness 0.25 µm), BP-1 (polydimethylsiloxane) and BP-20 (polyethyleneglycol). The oven temperature was programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min); injector temperature: 250°C (injection mode: split 1/60); detector temperature: 250°C; carrier gas: helium (0.8 mL/min). The relative proportions of the essential oil constituents were expressed as percentage obtained by peak area normalization, without using correcting factors. Retention indices (RI) were determined relative to the retention times of a series of n-alkanes with linear interpolation ("Target Compounds" software from Perkin Elmer).

GC-MS analysis: EOs were analyzed with a Perkin-Elmer TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem XL, equipped with a fused-silica capillary column (60 m x 0.22 mm i.d., film thickness 0.25 µm), Rtx-1 (polydimethylsiloxane). Carrier gas, helium at 1 mL/min; split, 1/80; injection volume, 0.2 µL; injector temperature, 250°C; oven temperature programmed from 60°C to 230°C at 2°C/min and then held isothermal (45 min); ion source temperature, 150°C; energy ionization, 70 eV; electron ionization mass spectra were acquired over the mass range 35-350 Da.

GC-TOF-MS analyses were performed on an Agilent 6890 gas chromatograph coupled to a time of Flight (ToF) mass spectrometer GCT Premier from Waters equipped with a column (30 m x 0.25 mm i.d., film thickness 0.25µm), DB-5MS UI. Injection volume: 1µL mode splitless; oven temperature programmed from 60 °C (1 min) to 320 °C (25 °C/min), 320°C during 10 min. Transfer line temperature at 250°C and source temperature at 200°C. The mass spectrometer was operated in the electron impact mode (energy ionization, 70 eV). Multichannel plate voltage was set at 2600V, acquisition rate at 10 spectra/sec (*i.e.* 5 spectra/sec with "Dynamic Range Enhancement" mode on) and pusher interval at 40µs. Acquisition was performed in the full scan mode with a scan range of m/z 50-550. Calibration was done using the calibration wizard, with heptacosane as the reference. The mass resolution was around 5000 FMWH for m/z 218.9856. During acquisitions, an internal standard, pentafluorobromo benzene, was introduced continuously into the EI source, from a reference reservoir at 50°C and through a reference inlet at 120°C. The mass m/z 181.0077 was used as lock mass. Data were processed with MassLynx 4.1.

2.4. ^{13}C NMR Analysis

All NMR spectra were recorded on a Bruker AVANCE 400 Fourier Transform spectrometer, equipped with a 5 mm probe, in deuterated chloroform, with all shifts referred to internal tetramethylsilane (TMS). ^{13}C NMR spectra of mixtures (EO or fractions of chromatography) were recorded with the following parameters: pulse width = 4 µs (flip angle 45°); acquisition time = 2.7 s for 128K data table with a spectral width of 25 000 Hz (250 ppm); CPD mode decoupling; digital resolution = 0.183 Hz/pt. The number of accumulated scans was 3000 (around 40 mg of the sample in 0.5 mL of $CDCl_3$). Standard pulse sequences from Bruker library were used for two dimensional spectra. Gradient enhanced sequences were used for the heteronuclear two-dimensional experiments.

2.5. Identification of components

Identification of the individual components was based: i) on comparison of their GC retention indices (RI) on apolar and polar columns, with those of authentic compounds ii) on computer matching with commercial mass spectral libraries (National Institute of Standards and Technology, 1996; König et al., 2001; Adams, 2007), iii) on comparison of the signals in the ^{13}C NMR spectra of the mixtures with those of reference spectra compiled in the laboratory spectral library, with the help of a laboratory-made software (Bighelli and Casanova, 2009; Rezzi et al., 2002; Tomi and Casanova, 2006).

2.6. Antifungal activity evaluation

2.6.1. Fungal strains

The antifungal activity of the oil was evaluated against yeasts and filamentous fungi strains (dermatophytes):
- Yeasts: three ATCC (American Type Culture Collection) type strains (*Candida albicans* ATCC 10231, *C. parapsilosis* ATCC 90018, *C. tropicalis* ATCC 13803); one CECT (Colección Española de Cultivos Tipo) type strain (*Cryptococcus neoformans* CECT 1078); and two clinical strains isolated from recurrent cases of vulvovaginal candidiasis (*Candida guillermondii* MAT23 and *C. krusei* H9).
- Dermatophytes: three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9*, Trichophyton mentagrophytes* FF7, *Microsporum canis* FF1); and four CECT (Colección Española de Cultivos Tipo) type strains (*Trichophyton rubrum* CECT 2794*, M. gypseum* CECT 2908, *T. mentagrophytes* var. *interdigitale* CECT 2958, *T. verrucosum* CECT 2992).
- *Candida parapsilosis* ATCC 90018 was used as control.

The fungal isolates were identified by standard microbiology methods and stored on Sabouraud broth with glycerol at –70ºC. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud agar to ensure optimal growth characteristics and purity.

2.6.2. Antifungal activity methods

Broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 (CLSI, 2008a) and M38-A2 (CLSI, 2008b) for yeasts and filamentous fungi, respectively, were used to determine MICs and MLCs of the essential oils. The serial doubling dilution of the essential oil was prepared in dimethyl sulfoxide (DMSO), with concentrations ranging from 0.08 to 5 µL/mL. Final concentration of DMSO never exceeded 2%. Recent cultures of each strain were used to prepare the cell suspension adjusted to 1-2x10^3 cells per mL for yeasts, and 1-2x10^4 cells per mL for filamentous fungi. The concentration of cells was confirmed by viable count on Sabouraud agar. The test tubes were incubated aerobically at 35ºC for 48h / 72h (*Candida* spp./ *Cryptococcus neoformans*) and at 30ºC for 7 days (dermatophytes) and MICs were determined. To evaluate MLCs, aliquots (20 µL) of broth were taken from each negative tube after MIC reading, and cultured in Sabouraud dextrose agar plates. Plates were then incubated at 35ºC for 48h (*Candida* spp.) and 72h for *Cryptococcus neoformans,* and 30ºC for 7 days (dermatophytes). In addition, two reference antifungal compounds, amphotericin B (Fluka) and fluconazole (Pfizer), were used to control the sensitivity of the tested microorganisms. All tests were performed in RPMI medium. For each strain tested, both growth conditions and sterility of the medium were checked in two control tubes. The innocuity of the DMSO was also checked at the highest tested concentration. All experiments were performed in duplicate and repeated three times,

yielding essentially the same results (a range of values is presented when different results were obtained).

2.7. Evaluation of cytotoxicity

2.7.1. Cell culture and materials

The fetal calf serum was from Biochrom KG (Berlin, Germany) and trypsin from Gibco (Paisley, UK). The proteases inhibitor cocktail was from Roche (Carnaxide, Portugal). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and all the other reagents were from Sigma Chemical Co. The human keratinocyte cell line HaCaT, obtained from DKFZ (Heidelberg), was kindly supplied by Dr. Eugénia Carvalho (Centro de Neurociências e Biologia Celular, Universidade de Coimbra, Coimbra, Portugal). Keratinocytes were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 4 mM glutamine, 10% heat inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Along the experiments, cells were monitored by microscope observation in order to detect any morphological change.

2.7.2. MTT assay for cell viability

Assessment of cell viability was made through a colorimetric assay, using MTT (Mosmann, 1983). In this method, the optical density of the solution containing the formazan produced by metabolically active cells is measured spectrophotometrically. The HaCaT cells (0.1×10^6 cells/well, cultured in 48-well microplates) were incubated in a final volume of 600 µL, allowed to stabilize for 12h, and then incubated for 24h with varying concentrations of the oil. After adding 60 µL of MTT solution (5 mg/mL in PBS) to each well, the cells were further incubated at 37ºC for 15 min, in a humidified atmosphere of 95% air/5% CO_2. Supernatants were then discarded and 300 µL of acidified isopropanol (0.04N HCl in isopropanol) were added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an automatic plate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

2.8. Data analysis

All the experiments were performed in duplicate. The MTT results are presented as mean ± standard error of the mean (S.E.M.) of the indicated number of experiments, and the means were statistically compared using the one-way ANOVA test, with a Dunnett's post-test. The differences between the means were considered significant for values of $p < 0.05$.

3. Results and Discussion

Leaves and flowers of *M. nivellei* were collected on ten individual shrubs in two localities of Algerian Central Sahara (Djanet, Tassili and Tamanrasset, Hoggar) (Fig. 1). Essential oil was isolated using a Clevenger-type apparatus. Yields ranged from 0.5 to 0.9% for Hoggar samples and from 1.4 to 2.0% for Tassili samples. All the samples were analyzed by GC(RI) and ^{13}C NMR without isolation of individual components, following a computerized method developed in our laboratory (Bighelli and Casanova, 2009; Rezzi et al., 2002; Tomi and

Casanova, 2006). One sample was also analyzed by GC-MS. It resulted from the analyses that two components remained unidentified. Therefore, the following items will be successively reported i) structural elucidation of compounds A and B; ii) detailed analysis of a selected sample; iii) comparison of the ten samples composition; iv) antifungal activity of the essential oil, v) skin cytotoxicity.

3.1. Structure elucidation of new compounds

A sample, chosen among those exhibiting the higher yields of essential oil as well as the higher contents of unidentified compounds A and B was repeatedly chromatographed over silica gel column in order to purify these compounds. Compounds A and B were found into two different fractions of CC and their structural elucidation was achieved using a full set of two dimensional NMR experiments.

3.1.1. Compound A

According to GC-TOF-MS, compound A had an exact mass of *m/z* =268.1684 corresponding to the formula $C_{15}H_{24}O_4$ (calc =268.1668), in agreement with ^{1}H NMR, ^{13}C NMR and DEPT spectra (Table 1, Fig. 2). The molecule bears four insaturation centers, three of them being identified by the chemical shift values of selected carbons: a keto and an ester functions (215.46 ppm and 170.73 ppm, respectively), a trisubstituted double bond (131.34 ppm and 130.04 ppm), the fourth insaturation belonged to a ring. The deshielded values of aliphatic carbons (81.15 ppm and 77.26 ppm) confirmed the acetate function (methyl group at 20.94 ppm and 2.17 ppm) and suggested the occurrence of another oxygenated function (alcohol). The 3-methylbutoxy substructure [-CO-CH_2-$CH(CH_3)_2$], frequently found in natural compounds, was evidenced by characteristic proton and carbon chemical shifts and confirmed by adequate correlation plots in the HMBC spectrum (Table 1). If we take care of three remaining methyl groups, the molecule contains a cyclopentene moiety in its structure. The various oxygenated functions (acetate, hydroxyle and keto fragment) as well as the three methyl groups were located on the cyclopentene moiety using long range proton-carbon connectivities on the HMBC spectrum. Therefore, compound A was identified as 1-hydroxy-1-(3-methylbutoxy)-2-acetoxy-3,5,5-trimethyl-3-cyclopentene. Unfortunately, neither the chemical shift values of protons and carbons, nor through space proton-proton correlation plots in the NOESY spectrum (none was observed) allowed the determination of the relative stereochemistry of the oxygenated groups present in the molecule.

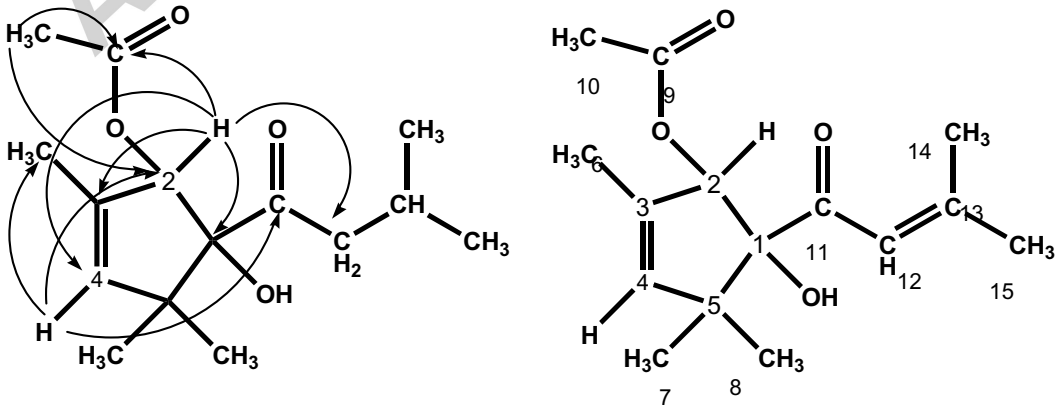

Figure 2. Structures of compounds A and B and illustrative long-range proton-carbon connetivities observed in the HMBC spectrum of A.

Table 1 : NMR data of compound A.

C	δ(^{13}C)	DEPT	δ(^{1}H)	Proton	Multiplicity (*J*)	COSY ^{1}H-^{1}H	HMBC (H→C)
C1	81.15	C					
C2	77.26	CH	5.62		m	4, 6	1,3,4,9,12
C3	131.34	C					
C4	130.07	CH	5.36		m	2, 6	2,5,6,11
C5	44.32	C					
C6	18.59	CH_3	1.68		t (1.3)	2,4	2,3,4,8
C7	27.17	CH_3	1.26		s		4,5,8,11
C8	26.72	CH_3	1.24		s		1,4,5,7,11
C9	170.43	C					
C10	20.94	CH_3	2.17		s		2, 9
C11	215.46	C					
C12	39.33	CH_2	1.87	a	dd (14.7, 8.6)	13	1,2,11,13,14
			1.78	b	dd (14.7, 4.0)	13	1,2,11,13,14
C13	24.26	CH	1.5		m	12, 14, 15	
C14	24.03	CH_3	0.97		d (6.7)	13	12,13
C15	24.45	CH_3	0.83		d (6.7)	13	12,13

δ: ppm; J: Hz; *d: doublet, t: triplet, m: multiplet, dd: doublet of doublet.

3.1.2. Compound B

The exact mass of compound B was measured as *m/z* =266.1552 by GC-TOF-MS corresponding to the formula $C_{15}H_{22}O_4$ (calc = 266.1512) in agreement with ^{1}H NMR, ^{13}C NMR and DEPT spectra (Table 2, Fig. 2). The formula of compound B differs from that of compound A by the loss of two hydrogen atoms. Examination of ^{1}H and ^{13}C NMR spectra demonstrated that B contains a supplementary double bond on the one hand and that the chemical shifts of most carbons are close to those of compound A, suggesting that both compounds exhibited the same framework. Moreover, the acetyl function was present and in contrast, the 3-methylbutoxy substructure was no more found. Indeed, the side chain contained the isopentenyl moiety evidenced by proton and carbon chemical shifts as well as by the shielding of the carbonyl carbon (211.80 ppm in B instead of 215.46 ppm in A). The isopentenyloxy substructure was confirmed by correlation plots in the HMBC spectrum between vinylic hydrogen H12 on the one hand and methyl hydrogens H13 and H14 with carbons C11-C15. The location of the side chain on the cyclopentene moiety was ascertained by the observation of connectivity between H12 and C1. As this has been done in compound A, various long range hydrogen-carbon correlation plots allowed the location of the three methyls, the acetyl function on the cyclopentene substructure. Therefore, compound A was identified as 1-hydroxy-1-(3-methyl-2-butenoxy)-2-acetoxy-3,5,5-trimethyl-3-cyclopentene. Here again, the relative stereochemistry of the oxygenated groups present in the molecule, could not be assigned (no through space proton-proton correlation plots was observed in the NOESY spectrum).

Table 2: NMR data of compound B.

C	δ(^{13}C)	DEPT	δ(^{1}H)	Multiplicity (*J*)	COSY ^{1}H-^{1}H	HMBC (H→C)
C1	79.48	C				
C2	77.90	CH	5.55	m	4,6	1,3,4,9
C3	130.87	C				
C4	130.54	CH	5.33	m	2,6	2,5,6,11
C5	45.02	C				
C6	18.64	CH_3	1.72	t (1.3)		
C7	27.92	CH_3	1.21	s		4,5,8,11
C8	25.99	CH_3	1.16	s		4,5,7,11
C9	170.73	C				
C10	20.99	CH_3	2.17	s		9
C11	211.80	C				
C12	120.41	CH	5.55	m	14,15	1,11,14,15
C13	141.98	C				
C14	18.56	CH_3	1.57	d (1.3)	12	11,12,13,15
C15	26.89	CH_3	1.74	d (1.3)	12	11,12,13,14

δ: ppm; J: Hz; *d: doublet, t: triplet, m: multiplet,

3.2. Chemical composition of M. nivellei essential oil

The chemical composition of the *M. nivellei* leaf oil sample is reported in Table 3 resulting from the GC(RI) and GC-MS analysis of the EO, as well as ^{13}C NMR analysis of the EO and fractions of chromatography and structure elucidation of new compounds A and B. 1,8-Cineole (37.5%) and limonene (25.0%) were by far the major components of that monoterpene-rich oil sample. Indeed, various oxygenated monoterpenes were present at appreciable contents: geranyl acetate, 5.1%; α-terpineol, 5.0%; linalyl acetate, 4.2%, α-terpinyl acetate, 3.8% and linalool, 1.7%. The two *di-nor*-sesquiterpene new compounds A and B accounted for 4.3% and 0.9%, respectively of the whole composition.

Table 3: Components of the essential oil isolated from aerial parts of *Myrtus nivellei.*

	Components	***RI*^{a}**	***RI*^{p}**	%	Identification
1	α-Thujene	923	1013	tr	RI, MS, *^{13}C NMR*
2	α-Pinene	930	996	3.2	RI, MS, ^{13}C NMR
3	β-Pinene	971	1114	0.2	RI, MS, *^{13}C NMR*
4	Myrcene	981	1163	0.2	RI, MS
5	α-Phellandrene	997	1168	0.1	RI, MS, *^{13}C NMR*
6	δ-3-Carene	1005	1151	0.1	RI, MS, *^{13}C NMR*
7	α-Terpinene	1009	1183	0.1	RI, MS, *^{13}C NMR*
8	p-Cymene	1011	1273	0.6	RI, MS, ^{13}C NMR
9	1,8-Cineole*	1020	1213	37.5	RI, MS, ^{13}C NMR
10	Limonene*	1020	1204	25.0	RI, MS, ^{13}C NMR
11	*(Z)-β*-Ocimene	1025	1235	0.1	RI, *^{13}C NMR*
12	*(E)-β*-Ocimene	1036	1252	0.2	RI, MS, *^{13}C NMR*
13	γ-Terpinene	1048	1248	0.6	RI, MS, ^{13}C NMR

14	Terpinolene	1079	1285	0.2	RI, MS, *^{13}C NMR*
15	Linalool	1082	1544	1.7	RI, MS, ^{13}C NMR
16	Terpinen-4-ol	1161	1600	0.5	RI, MS, ^{13}C NMR
17	α-Terpineol	1172	1693	5.0	RI, MS, ^{13}C NMR
18	Geraniol	1233	1829	0.1	RI, *^{13}C NMR*
19	Linalyl acetate	1239	1556	4.2	RI, MS, ^{13}C NMR
20	α-Terpinyl acetate	1332	1694	3.8	RI, MS, ^{13}C NMR
21	Neryl acetate	1341	1725	0.4	RI, MS, ^{13}C NMR
22	Geranyl acetate	1359	1755	5.1	RI, MS, ^{13}C NMR
23	*(E)-β*-Caryophyllene	1417	1596	0.2	RI, MS, *^{13}C NMR*
24	α-Humulene	1451	1665	0.4	RI, ^{13}C NMR
25	Compound A	1582	2179	4.3	MS, NMR†
26	Compound B	1594	2270	0.9	MS, NMR†
	Total			94.7	

Order of elution and percentages of individual components are given on apolar column (BP-1), those with an asterisk (*) excepted, percentages on polar column (BP-20. Limonene and cineole co-eluted on the BP1 column (GC) while they were separated on the Rtx1 column (GC-MS). RI^{a}, RI^{p}: retention indices measured on apolar and polar columns, respectively. tr <0,05%, $^{\#}$RI not available. † Identification by NMR: ^{1}H and ^{13}C NMR, 2D NMR.

In order to check the homogeneity of the composition of *M. nivellei* essential oil or to evidence an eventual chemical variability, ten oil samples isolated from plants growing wild in Sahara have been investigated. The stations of harvest are located in Tassili des N'Ajjers mountains, (samples TAS-1 to TAS-5) and in Hoggar mountains (near Tamanrasset, samples TAM-1 to TAM-5). Both stations exhibit a meso-Mediterranean climate. The yields of EO isolated from *M. nivellei* growing in the Tassili des N'Ajjers station (TAS samples, 1.4 - 2.0 %, w/w) were twice higher than those of EO isolated from plants growing in Hoggar station (TAM samples, 0.5 -0.9 %, w/w). Analysis of the essential oils by GC(FID) and ^{13}C NMR allowed the identification of twenty four constituents representing from 77.3 to 92.6% of the whole composition (all the unidentified compounds were minor components) (Table 4).

Table 4: Composition of *M. nivellei* essential oils from two stations in Central Algerian Sahara .

Samples	**TAS 1-5**				**TAM 1-5**			
Components	Min	Max	M.	SD.	Min	Max	M.	SD.
α-Pinene	2.9	3.3	3.1	0.2	3.6	5.8	5.2	1.0
p-Cymene	0.6	0.7	0.6	0.0	0.9	1.0	0.9	0.0
Limonene*	17.5	25.0	20.5	3.7	18.8	20.6	19.9	1.4
1.8-Cineole*	36.6	50.4	43.9	6.3	33.6	40.7	39.0	3.7
γ-Terpinene	0.0	0.6	0.4	0.0	0.0	0.2	0.1	0.0
Linalool	0.7	1.7	1.1	0.5	0.6	1.0	0.8	0.1
Terpinen-4-ol	0.5	0.6	0.5	0.1	0.3	0.3	0.3	0.0
a-Terpineol	3.5	5.2	4.4	0.8	4.7	5.5	5.0	0.3
Linalyl acetate	1.7	4.2	2.8	1.3	1.5	2.1	1.6	0.3
α-Terpinyl acetate	3.6	7.8	5.7	2.0	3.6	4.7	4.0	0.4
Geranyl acetate	5.0	5.3	5.1	0.1	3.2	4.1	3.6	0.4
(E)-β-Caryophyllene	0.2	0.2	0.2	0.0	0.4	0.6	0.5	0.1
α-Humulene	0.3	0.4	0.4	0.0	0.8	1.3	1.0	0.2
Compound A	2.4	4.5	3.4	0.9	4.8	9.8	6.7	2.0
Compound B	0.4	0.9	0.6	0.3	1.0	2.1	1.5	0.4

Order of elution and percentages are given on apolar column (BP-1), except for compounds with an asterisk (*), percentage on BP-20; tr, traces, TAS1-5: Tassili des N'Ajjers samples, TAM1-5: Tamanrasset samples

The ten oil samples exhibited similar chemical composition dominated by 1,8-cineole (up to 50.4%) and limonene (up to 25.0%). It could be noticed that the content of cineole is slightly higher in the samples from Tassili (TAS) than those from Hoggar (TAM). Conversely, the contents of the new compounds A and B are higher in Hoggar samples (means values = 6.7 and 1.5%) than in Tassili samples (mean values = 3.4 and 0.6%).

The composition of *M. nivellei* essential oil differed from that of *M. communis* growing wild in North Algeria, characterized by a high amount of α-pinene (Bouzabata et al., 2010, 2013). In fact, it differed from the various compositions of EOs isolated from *M. communis* growing wild all around the Mediterranean basin and in the islands (Bazzali et al., 2012). Compounds A and B have been found in all the investigated samples of *M. nivellei* EO. In contrast, they were never identified in *M. communis* EOs (Bazzali et al., 2012; Bouzabata et al., 2013). Both compounds may be considered as markers of *M. nivellei.*

3.3. Antifungal activity

The antifungal activity of *M. nivellei* essential oil presented in Table 5 showed a variability of inhibition among all the fungal strains tested. Our results showed that *Myrtus nivellei* essential oil had effective antifungal activity against *Cryptococcus neoformans* with MIC and MLC values of 0.16 µL/mL and 0.32 µL/mL, respectively. *Cryptococcus neoformans* is the major causative agent of fungal meningoencephalitis. It is the best known form of this mycosis in both immunocompromised and immunocompetent individuals. Also against dermatophytes, the oil demonstrated antifungal potential, particularly for *Microsporum canis, Trichophyton rubrum* and *Epidermophyton floccosum* with MIC and MLC of 0.64 µL/mL (Table 5). For most of the fungi tested MIC and MLC are similar revealing fungicidal activity of the oil. These results support the use of this species in traditional medicine for the treatment of dermatophytosis.

Table 5**:** Antifungal activity (MIC and MLC) of *Myrtus nivellei* essential oil.

Strains	*M. nivellei* oil		Fluconazole	
	MIC[a]	MLC[a]	MIC[b]	MLC[b]
Candida albicans ATCC 10231	1.25-2.5	1.25-2.5	1	>128
Candida tropicalis ATCC 13803	2.5	2.5	4	>128
Candida krusei H9	2.5	2.5	64	64-128
Candida guillermondii MAT23	1.25-2.5	1.25-2.5	8	8
Candida parapsilosis ATCC 90018	2.5	2.5	<1	<1
Cryptococcus neoformans CECT 1078	0.16	0.32	16	128
T. mentagrophytes FF7	1.25	1.25	16-32	32-64
Microsporum canis FF1	0.64	0.64	128	128
Trichophyton rubrum CECT 2794	0.64	0.64	16	64
M. gypseum CECT 2908	1.25	1.25	128	>128
Epidermophyton floccosum FF9	0.64	0.64	16	16
T. mentagrophytes var. *interdigitale* CECT 2958	1.25	2.5	128	≥128
T. verrucosum CECT 2992	1.25	2.5	>128	>128

Results were obtained from 3 independent experiments performed in duplicate.
[a]MIC and MLC were determined by a macrodilution method and expressed in µL/mL (V/V).

[b]MIC and MLC were determined by a macrodilution method and expressed in µg/mL (W/V).

3.4. Cytotoxicity evaluation

As shown in Figure 3, only for 2.5 µL/mL of essential oil the value of cell viability in human keratinocyte is significantly different from the control (32.42 ± 1.62). Cell viability evaluation demonstrated that the oil in concentrations until 1.25 µL/mL are safe for topical use supported by the absence of cytotoxicity in HaCaT keratinocytes.

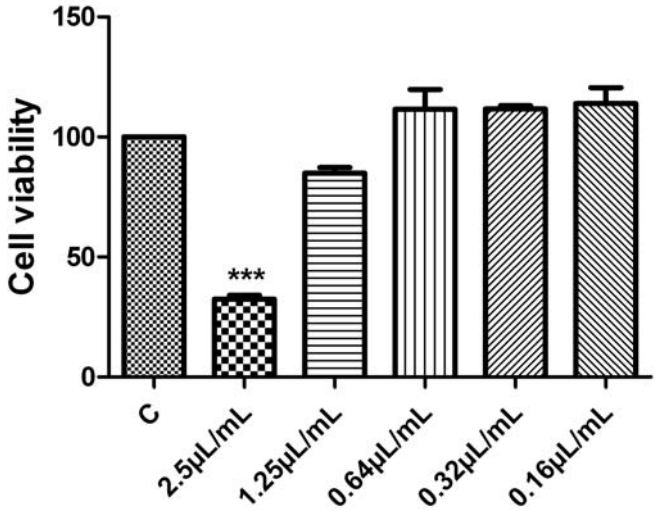

Figure 3. Effect of *M. nivellei* essential oil on keratinocytes viability (MTT assay). HaCaT cells were exposed to different concentrations of the essential oil (0.16-2.5 µL/mL) for 24h. Results are expressed as a percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three experiments, performed in duplicate (***$p < 0.001$, compared with control).

4. Conclusions

This paper reported for the first time the chemical composition and the antifungal activity of the essential oil isolated from *M. nivellei*, an endemic species to the Central Saharan mountains. 1,8-Cineole and limonene were the major components. Two new natural components, which possess a cyclopentene framework, were identified by structural analysis from 1D and 2D NMR. It could be assumed that these components were probably markers of this species.

Skin cytotoxicity evaluation for doses with antifungal activity revealed that *M. nivellei* oil in concentrations up to 1.25 µL/mL is safe for topical applications against *Cryptococcus neoformans* and all dermatophytes tested. These results demonstrate the efficacy and safety of *M. nivellei* and support its use by the Touaregs in Saharan traditional medicine for the treatment of dermatophytosis.

Acknowledgements – A. Bouzabata wishes to thank the cooperation program between France and Algeria (PROFAS «B» program) for a research grant and PEst-OE/SAU/UI0177/2011 (Center for Pharmaceutical Studies, Portugal). We also thank Dr G. De Belaire (Badji-Mokhtar University, Annaba, Algeria) for identification of the plant, Dr J.

Migliore, (IMBE, Aix-Marseille University, France) who deposited the voucher specimen, and Dr H. Casabianca (SCA-CNRS, Solaize, France) for exact mass measurement.

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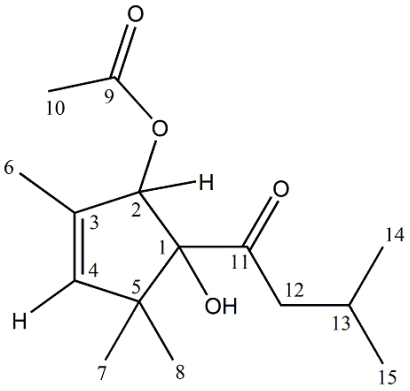

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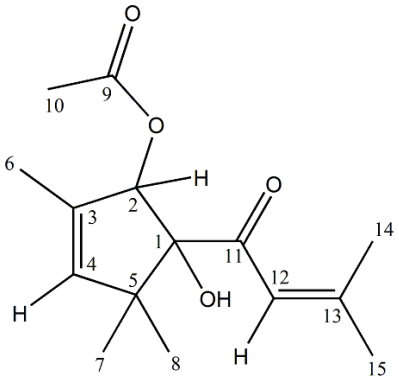

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