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***Ziziphora tenuior* L. essential oil from Dana Biosphere Reserve (Southern Jordan);
chemical characterization and assessment of biological activities**

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Abstract

Ethnopharmacologic relevance:

Ziziphora tenuior L. (Lamiaceae) is a medicinal plant in Jordan, which is included in various antimicrobial, antiseptic, expectorant and wound healing preparations. It is used for the treatment

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of cough, stomach ache, dysentery, fever, uterus infection, gut inflammation and painful menstruation.

Aim of the study:

The aim of this study was to assess, for the first time, the chemical composition of the essential oil of *Z. tenuior* originated from southern Jordan and its antifungal effects against several yeasts. Concomitantly, the mechanisms behind the anti-fungal activity against *Candida albicans* were also disclosed. Since the *Z. tenuior* traditional uses are related with inflammatory-associated conditions, the putative anti-inflammatory activity of the oil was also unveiled. Importantly, the potential toxicity of pharmacologically active concentrations was screened in different types of mammalian cells.

Materials and methods:

Z. tenuior essential oil, isolated by hydrodistillation, was analyzed by gas chromatography, gas chromatography–mass spectrometry and ^{13}C nuclear magnetic resonance spectroscopy. Antifungal activity was evaluated against yeasts, dermatophytes and *Aspergillus* strains. Germ tube inhibition and biofilm formation assays were evaluated using *C. albicans*. Assessment of cell viability was made by the MTT assay using different types of mammalian cells, including hepatocytes, keratinocytes and macrophages. The *in vitro* anti-inflammatory potential of the oil was evaluated by measuring nitric oxide production using lipopolysaccharide-stimulated mouse macrophages.

Results:

Oxygen-containing monoterpenes are the main oil compounds: pulegone (46.8%), *p*-menth-3-en-8-ol (12.5%), isomenthone (6.6%) and 8-hydroxymenthone (6.2%). The highest antifungal activity was against *Cryptococcus neoformans*, with a MIC value of 0.16 $\mu\text{L}/\text{mL}$. The oil revealed an important inhibitory effect on germ tube formation with a filamentation inhibition rate higher than 80% at 0.16 $\mu\text{L}/\text{mL}$. The amount of the attached biomass was reduced. Importantly, concentrations devoid of toxicity on several mammalian cell types still displayed anti-inflammatory activity (0.16 and 0.32 $\mu\text{L}/\text{mL}$).

Conclusions:

These findings add significant information to the pharmacological activity of *Z. tenuior*, thus justifying and reinforcing the use of this plant in traditional medicine. Additionally, the

antifungal and anti-inflammatory potential of the oil at non-toxic concentrations, opens new avenues for its further exploitation, for instance in health-care product development.

Abbreviations:

GC, gas chromatography; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration; NO, nitric oxide; MLC, minimal lethal concentration; MS, mass spectrometry; NMR, nuclear magnetic resonance; TLR, toll-like receptor

Keywords: *Ziziphora tenuior*; essential oil; chemical composition; antifungal activity; anti-inflammatory activity; cytotoxicity

1. Introduction

Ziziphora tenuior L. (Arabic: *Zizfran*), is one of four species belonging to the genus *Ziziphora* (Lamiaceae). It is distributed in steppes, Al-Nagab desert, lower Jordan valley, Araba valley, Amman, Madaba and Edom (Zohary and Feinbrun, 1978; Al- Baba et al., 2015) and in the southern parts of Jordan mainly in Al-Shoubak, Tafila, Dana and Petra (Al-Rawashdeh, 2011; Oran 2013; Taifour and El-Oqlah, 2014). Additionally, this plant grows in Mediterranean and Iran-Turonian zones, including some parts of Iran, Turkey, Afghanistan, Iraq and Azerbaijan (Verdian-Rivi, 2008; Al-Rawashdeh, 2011; Darbandi et al., 2013; Taifour and El-Oqlah, 2014). *Z. tenuior* is also distributed in Russia, Turkmenistan, Afghanistan, Pakistan, Caucasus and Siberia (Rechinger, 1982).

Z. tenuior is an aromatic herbaceous plant used for many traditional preparations against various diseases. In Jordan, it is traditionally used as common teapot herb (Al-Rawashdeh, 2011; Al-Baba et al., 2015). Interestingly, in other Arabic regions, Turkey and Iran, *Z. tenuior* plays an important role in traditional medicine. In Iranian folk medicine, it is used to treat fever, dysentery and uterus infections (Talebi et al., 2012), gut inflammation and cough (Safa et al., 2012), bladder stone and painful menstruation (Naghbi et al., 2005). In the folk medicine of the Kalamoon Mountains area in Syria, it is used against cough, stomachache and dysentery (Dakah et al., 2015). In Turkish folk medicine, *Ziziphora* species have been used as infusion for various purposes such as antimicrobial, antiseptic, sedative and carminative effects as well as to treat

stomach ache and wound healing (Kilic and Bagci, 2013; Celik et al., 2016). Further emphasizing its ethnopharmacological relevance, the essential oils of *Ziziphora* species are used for treating edema, insomnia, lung abscess, tracheitis, hemorrhoids and hypertension (Senejoux et al., 2010).

Several antimicrobial properties have also been ascribed to the extract. For instance, the methanolic extract of the *Z. tenuior* exhibited significant antimicrobial effects against *Morganella morganii* and *Candida albicans* (Bazzaz et al., 2003).

Numerous other important immunological activities have been assigned to *Z. tenuior*. Azadmehr et al. (2014) showed that *Z. tenuior* ethanolic extract (80% ethanol) modulated the immune response by induction of CD40 expression in dendritic cells (DCs) and cytokine production, whereas it inhibited the T-cell stimulating activity of DCs at high concentrations. Furthermore, the water extract of *Z. tenuior* increased macrophage activity ($p < 0.05$) (Naeini et al., 2010) and significantly induced the generation of reactive oxygen species, revealing strong fungicidal activities in treated macrophages at concentrations of 10 and 20 mg/mL ($p < 0.05$) (Naeini et al., 2010).

Several biological activities have also been attributed to *Z. tenuior*. For instance, the essential oil of *Z. tenuior* originated from Turkey showed antimicrobial activity against some Gram-positive and Gram-negative bacteria (Sarac and Ugur, 2009). Additionally, Celik et al. (2016) evaluated the anti-biofilm and antimicrobial properties of *Z. tenuior* L. essential oil against multidrug-resistant *Acinetobacter baumannii*, where minimal inhibition concentration (MIC) values of 0.6-1.25 $\mu\text{L/mL}$ and minimal bactericidal concentration values of 2.5-5.0 $\mu\text{L/mL}$ were found. Furthermore, minimal biofilm inhibition concentration values of 0.3-1.25 $\mu\text{L/mL}$ and minimal biofilm eradication concentration values of 5-10 $\mu\text{L/mL}$ were observed.

Concerning the chemical composition of the essential oil, pulegone, isomenthone, thymol, menthone and piperitone were the major constituents in the oil of *Z. tenuior* originated from Turkey (Sezik et al., 1991), while pulegone (30.00%) and 1,8-cineole (9.65%) were identified as major components in *Z. tenuior* originated from Adiyaman/Turkey (Kilic and Bagci, 2013). Pulegone (71.2 to 85.3%), limonene (0.51 to 7.8%), thymol (1.0 to 4.3%), and menthone (0.1 to 3.7%) were the major components in the oils from various natural habitats in the alpine regions, Kerman province, and Iran (Pirbalouti et al., 2013). Essential oil of *Z. tenuior* collected from the

northern part of Shiraz, Iran contained *p*-mentha-3-en-8-ol (54.0%) and pulegone (38.5%) but not piperitenone (Darbandi et al., 2013) compared to the oil of other *Ziziphora* species from Turkey. These data indicate that the yield and composition of essential oil of *Z. tenuior* may be affected by genetic and ecological factors, including plant genetics, environmental conditions, and geographic origin such as climate precipitation, altitude, temperature, plant competition and nitrogen concentration in the soil (Pirbalouti et al., 2013; Maral et al., 2015).

We previously evaluated the content of heavy metals (Fe, Mn, Zn) in aerial parts of *Z. tenuior* originated from Shoubak (Ofir and Abu-Darwish, 2012). Apart from this study, there is currently no other report available concerning the chemical composition as well as the antifungal and anti-inflammatory properties of *Z. tenuior* originated from Jordan. Taking into account that *Z. tenuior* is one of the medicinal plants of Jordan, which are counted as important elements of folk medicine for treating certain health disorders, it is of utmost importance to validate its traditional use by modern pharmacological research. In order to fill this gap, the aim of this study was to assess, for the first time, the chemical composition of the essential oil of *Z. tenuior* originated from southern Jordan and its antifungal effect against several yeasts, dermatophytes and *Aspergillus* strains. Concomitantly, the mechanisms behind the anti-fungal activity against *Candida albicans* were also disclosed with a special focus on the inhibition of germ tube formation and on preformed biofilms. Additionally, and since most chronic illnesses, including cancer, diabetes, and cardiovascular disease, are mediated through chronic inflammation, the anti-inflammatory activity of the oil was also unveiled. Importantly, the potential toxicity of bioactive concentrations was screened in different types of mammalian cells, including hepatocytes, keratinocytes and macrophages.

2. Materials and methods

2.1. Isolation of essential oil

Aerial parts of *Ziziphora tenuior* were collected in various parts of the Dana Biosphere Reserve (DBR) near Tafilah in southern Jordan, latitude (30° 40' 33N), longitude (35° 36' 39E), altitude (1543 m), during spring in 2015, in order to obtain a collective sample representative of the region. In Jordan, it is one of the endangered medicinal plants that is included into the red list (Taifour and El-Oqlah, 2014). The investigations of the present paper have been done with

official permission of the Royal Botanical Garden, Jordan with letter from July 50th 2015. The plant sample was identified by Mr. Hatem Taifour, head botanist at Royal Botanic Garden, Jordan. A voucher specimen was deposited in the Herbarium of Shoubak University College, Al-Balqa Applied University, Jordan under the registration number 00014- Zt-DAsh-2015. The collected plant material was dried at room temperature and crushed into small pieces with a hammer mill.

Essential oil was isolated by hydro-distillation for 3 h using a Clevenger-type apparatus (Normax, Lisboa, Portugal), according to the procedure described in the European Pharmacopoeia (Council of Europe, 1997). The oil obtained after extraction was dried over anhydrous sodium sulphate and kept under 4°C until analyzed and tested.

2.2 Chemical composition

2.2.1 Gas chromatography (GC) analysis

Analyses were performed on a Clarus 500 Perkin Elmer (Courtaboeuf, France) chromatograph equipped with flame ionization detectors FID and two fused-silica capillary columns (50 m × 0.22 mm, film thickness 0.25 µm), BP-1 (polydimethyl siloxane) and BP-20 (polyethylene glycol). The oven temperature was programmed from 60-220°C at 2°C/min and then held isothermal at 220°C for 20 min; injector temperature: 250°C; detector temperature: 250°C; carrier gas: helium (1.0 mL/min); split: 1/60; injection volume, 0.5 µL. 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).

2.2.2 Gas chromatography-mass spectrometry (GC-MS) analysis

The essential oils were analyzed with a Perkin-Elmer (Courtaboeuf, France) TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem equipped with two fused silica capillary columns (50 m × 0.22 mm × 0.25 µm film thickness), BP-1 (polydimethyl siloxane) and BP-20 (polyethylene glycol). Carrier gas was helium at 0.8 mL/min, split 1/75. Further conditions were: injection volume, 0.5 µL; injector temperature, 250°C; oven temperature programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min); ion source temperature, 250°C; energy ionization, 70 eV. The electron ionization mass spectra were acquired over a mass range of 40-400 Da.

2.2.3 ^{13}C NMR analysis

All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 (Wissembourg, France) (400.132 MHz for ^1H and 100.623 MHz for ^{13}C) equipped with a 5 mm probe, in deuterated chloroform (CDCl_3), with all shifts referred to internal tetramethylsilane (TMS). ^{13}C NMR spectra were recorded with the following parameters: pulse width, 4 μs (flip angle 45°); acquisition time, 2.7 s for 128 K data table with a spectral width of 24 000 Hz (240 ppm); digital resolution, 0.183 Hz/pt. The number of accumulated scans was 3000 (around 50 mg of the oil sample in 0.5 mL of CDCl_3).

2.2.4 Identification of Individual Components

Identification of the individual components was based: (i) on comparison of their GC retention indices (RIs) on apolar and polar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation ('Target Compounds' software of Perkin-Elmer), with those of authentic compounds, or literature data, (ii) on a computer search using digital libraries of mass spectral data (National Institute of Standards and Technology, 1999; König et al., 2001) and/or literature data (Joulain and König, 1998; Adams, 2007) and (iii) by ^{13}C NMR spectroscopy, following a computerized method developed in our laboratory (Tomi et al., 1995; Tomi and Casanova, 2006; Bighelli and Casanova, 2010), using a tailor-made software, by comparison of the chemical shift values of the signals in the essential oil spectrum with those of reference compounds compiled in a laboratory-built library. This method allowed the identification of individual components of the essential oil at contents as low as 0.3-0.4%. A few compounds have been identified by comparison with literature data.

2.3 Antifungal activity

2.3.1. Fungal strains

The following clinical isolates and collection strains of *Candida*, *Cryptococcus neoformans*, *Aspergillus*, and dermatophyte species were used: two *Candida* clinical strains (*Candida guilliermondii* MAT23 and *C. krusei* H9), isolated from recurrent cases of vulvovaginal candidiasis, and three ATCC type strains (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803, and *C. parapsilosis* ATCC 90018); *Cryptococcus neoformans* type strain from the Colección

Espanõla de Cultivos Tipo (*C. neoformans* CECT 1078), one *Aspergillus* clinical strain isolated from bronchial secretions (*A. flavus* F44), two ATCC *Aspergillus* type strains (*Aspergillus niger* ATCC 16404 and *Aspergillus fumigatus* ATCC 46645), and three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9, *Microsporum canis* FF1, and *Trichophyton mentagrophytes* FF7), as well as four dermatophyte type strains (*M. gypseum* CECT 2908, *T. mentagrophytes* var. *interdigitale* CECT 2958, *T. rubrum* CECT 2794, and *T. verrucosum* CECT 2992). The clinical isolates were identified by standard microbiological methods and stored on Sabouraud dextrose broth (Becton-Dickinson) with 20% glycerol at -80°C . Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud dextrose agar (SDA; Becton-Dickinson) to ensure optimal growth and purity. The fungal isolates were identified by standard microbiological methods and stored on Sabouraud broth with glycerol at -70°C .

2.3.2. 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 MIC and minimally lethal concentration (MLC) 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 10 $\mu\text{L}/\text{mL}$. The final concentration of DMSO never exceeded 2%. Recent cultures of each strain were used to prepare the cell suspensions and adjusted to $1-2 \times 10^3$ cells per mL for yeasts, and $1-2 \times 10^4$ cells per mL for filamentous fungi. The concentration of the cells was confirmed by viable count on Sabouraud agar. The test tubes were incubated aerobically at 35°C for 48 h or 72 h (*Candida* spp./*Cryptococcus neoformans*/*Aspergillus*) and at 30°C for 7 days (dermatophytes) and MICs were determined. To evaluate MLCs, aliquots (20 μL) of broth were taken from each negative tube after MIC measurement and cultured in Sabouraud dextrose agar plates. Plates were then incubated at 35°C for 48 h (*Candida* spp./*Aspergillus* spp.), 72 h for *Cryptococcus neoformans*, or 30°C for 7 days (dermatophytes). In addition, two reference antifungal compounds, amphotericin B (Fluka) and fluconazole (Pfizer), were used to monitor 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 controlled in two control tubes. The

innocuity of DMSO was also proven at the highest tested concentration. All experiments were performed in triplicate and repeated, if the results differed.

2.3.3 Germ tube inhibition assay

In order to determine the effect of the volatile oil on the yeast-mycelium transition, cell suspensions from overnight SDA of *C. albicans* strains ATCC 10231 cultures were prepared in NYP medium (N-acetylglucosamine [Sigma; 10-3mol/L], Yeast Nitrogen Base [Difco; 3.35 g/L], proline [Fluka; 10-3mol/L], NaCl [4.5 g/L], and pH 6.7±0.1; (Marichal et al., 1986). The suspensions were adjusted to obtain a density of yeast cell suspensions at $1.0\pm 0.2 \times 10^6$ CFU/mL and distributed into glass test tubes in a volume of 990 μ L. Each dilution of essential oil was added into the cell suspension tubes, in 10 μ L volumes, to obtain appropriate sub-inhibitory concentration. After incubation at 37°C without agitation for 3 h, the treated and untreated yeast cells were counted for germ tube formation under light microscope and the percentage of germinating cells was calculated. The formation of germ tube was considered positive, if the germinating tube was at least as long as the diameter of the blastospore. DMSO at a maximal concentration of 1% (v/v) was used as control.

2.3.4 Biofilms formation

C. albicans biofilms were formed as described by Taweechaisupapong et al. (2010) with slight modifications. Briefly, yeast cells were cultured in Yeast Peptona Dextrose broth (YPD) overnight at 37°C, washed with PBS and re-suspended in RPMI-1640 (1×10^6 cells/ml). Biofilms were formed on commercially available pre-sterilized polystyrene flat-bottom 96-well microtiter plates by pipetting standardized cell suspensions (100 μ L of 10^6 cells/mL) into selected wells of the microtiter plate and further incubated at 37°C for 24 h. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by thorough washing of the biofilms for three times in sterile PBS. Residual PBS was removed by blotting with paper towels before addition of the essential oil. The oil was then added to the biofilms in serially double-diluted concentrations in DMSO (0.32 to 2.5 μ L/mL) and incubated for 24 h at 37°C. A series of essential oil-free wells were also included: DMSO at a maximal concentration of 1% (v/v) (control) and biofilm-free wells. The effect of the oil on pre-biofilm growth was determined as

reported by Raut et al. (2014) with the XTT/Menadione metabolic assay as well as the crystal violet assay.

2.3.5 Biofilms quantification

2.3.5.1 XTT assay

Biofilm growth was quantified using the XTT metabolic assay. The XTT solution was prepared by mixing 1 mg/mL XTT salt (SIGMA) in PBS and stored at -70°C . Prior to use, menadione (Sigma) solution was prepared in acetone and added to XTT to a final concentration of $4\ \mu\text{M}$. The wells containing biofilms were washed with PBS to remove non-adhered cells and incubated with $100\ \mu\text{L}$ of XTT-menadione solution in the dark, at 37°C for 2 h. The color formation by the water-soluble formazan product was measured at 450 nm using a microplate reader (Thermo Scientific™ Multiskan™ FC) and indicated the metabolic activity relatively to biofilm growth.

2.3.5.2 Cristal violet assay

Biofilm cells were fixed with 99% methyl alcohol for 15 min. Supernatants were removed, and wells were air-dried. One hundred microliters of 0.02% crystal violet were added to each well and allowed to stand for 15 min for staining of the biofilm. Excessive crystal violet was removed by washing the wells 2–3 times with sterile distilled water. Absorbed stain was released by addition of $150\ \mu\text{L}$ of 33% of acetic acid and transferred to the new wells. Absorbance measurement was read at 620 nm using a microplate reader. The percentage of biofilm mass was calculated by comparing the absorbance measurement of treatment with that of the control biofilm.

2.4 Measurement of anti-inflammatory activity

2.4.1 Cell culture and materials

The macrophage cell line (Raw 264.7), obtained from the American Type Culture Collection (TIB-71) was kindly supplied by Dr. Otilia Vieira (Center for Neuroscience and Cell Biology, University of Coimbra, Portugal). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) non-inactivated fetal bovine serum, 3.02 g/L sodium

bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 in a humidified atmosphere of 95% air and 5% CO₂.

2.4.2 Nitric oxide (NO) measurement

The anti-inflammatory potential of the essential oil of *Z. tenuior* was evaluated in the macrophage cell line (RAW 264.7). The cells (0.6×10^6 cells/well) were cultured in 48-well microplates, in a final volume of 600 µL and allowed to stabilize for 12 h. Following this period, cells were either maintained in culture medium (control) or pre-incubated with different concentrations of the essential oil or its main compounds for 1 h, and later activated with LPS (1 µg/mL) for 24 h. The production of NO was measured by the accumulation of nitrites in the culture supernatants, by the colorimetric reaction with the Griess reagent (Cruz et al., 2001; Green et al., 1982). Briefly, 170 µL of medium were diluted with equal volumes of the Griess reagent [0.1 % (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 1% (w/v) sulphanilamide containing 5% (w/v) H₃PO₄ (v/v)] and maintained during 30 min, in the dark. The absorbance at 550 nm was measured in an automated plate reader. The results were expressed as the percentage of nitrite production by cells cultured with LPS.

2.5 In vitro cytotoxicity evaluation

2.5.1 Cell culture and materials

The human keratinocytes cell line (HaCaT), obtained from the German Cancer Research Center (DKFZ, Heidelberg), was kindly supplied by Dr. Eugenia Carvalho (Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal). Keratinocytes were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% (v/v) inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/ml penicillin, at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

The human hepatocyte cell line (HepG2) was purchased from ATCC (number: 77400) and kindly provided by Dr. Conceição Pedroso Lima. Hepatocytes were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% (v/v) inactivated fetal bovine serum, with 1.5 g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin and kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

The method for culturing the cell line of macrophages (RAW 264.7) is described above.

2.5.2 MTT assay for cell viability

Cell viability was assessed for the essential oil of *Z. tenuior* in different mammalian cell types, namely macrophages (Raw 264.7), keratinocytes (HaCaT) and hepatocytes (HepG2).

Evaluation of cell viability was performed by a colorimetric assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction colorimetric assay, as previously reported (Mosmann, 1983). Macrophages, keratinocytes and hepatocytes were cultured at densities of 0.6×10^6 ; 0.2×10^6 ; 0.2×10^6 cells/well, respectively, in 48-well microplates in a final volume of 600 μL for 12 h and were further treated with different concentrations of the oil (0.16 to 1.25 $\mu\text{L}/\text{mL}$).

After 24 h, 60 μL of a MTT solution (5 mg/mL in PBS) were added per well to keratinocytes and hepatocytes and 43 μL of MTT solution per well to macrophages. Cells were further incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 30 min (keratinocytes) / or 1 h (hepatocytes and macrophages). After this time period of incubation with MTT, the supernatants were discarded and 300 μL of acidic isopropanol (0.04 N HCl in isopropanol) were added to each well. Quantification of formazan crystals was performed using an ELISA microplate reader at 570 nm with a reference wavelength of 620 nm. The results were expressed as the percentage of MTT reduction relatively to control cells.

2.6 Data analysis

All the experiments were performed in duplicate, being the results expressed as mean \pm SEM of three independent experiments. Statistical analyses were performed using one-way ANOVA with a Dunnett's multiple comparison test. The statistical tests were applied using GraphPad Prism, version 6.00 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Essential oil composition

The essential oil of Jordanian *Ziziphora tenuior* was obtained with a yield of 0.72%, which is comparable to previously published yields obtained from this species growing in other countries. The oil yield from the aerial parts of *Z. tenuior* from alpine regions, Kerman province, Iran,

ranged from 0.61 to 0.75 mL/100 g dry plants (Pirbalouti et al., 2013), and a range of 0.2% to 2.3% oils was measured in flowering branches of *Z. tenuior* grown in Isfahan, Iran (Batooli et al., 2012). This variation in essential oil yield is related to many different factors. For instance, environmental conditions, geographic origin and climate can affect the yield and the content of *Z. tenuior* essential oil (Pirbalouti et al., 2013). The levels of altitudes also influenced the contents of essential oils of *Ziziphora*. The highest essential oil yield was obtained from plants growing at 908 m altitude above sea level in comparison with those growing at 1286 m (Maral et al., 2015).

The essential oil was analyzed by GC(RI), GC-MS and ^{13}C NMR. In total, 36 compounds were identified accounting for 91.0% of the entire composition (Table 1).

Table 1. Composition of *Ziziphora tenuior* essential oil from Jordan

	Components	RI _a	RI _p ^c	%	Identification
1	3-methylcyclohexanone	920	1340	0.3	RI, MS
2	α -pinene	930	1016	0.2	RI, MS
3	camphene	943	1065	tr	RI, MS
4	oct-1-en-3-ol	960	1456	tr	RI, MS
5	sabinene	964	1122,	0.1	RI, MS
6	β -pinene	969	1111	0.3	RI, MS
7	octanal	983	1282	tr	RI, MS
8	<i>p</i> -cymene	1011	1270	0.1	RI, MS
9	limonene*	1020	1201	3.2	RI, MS, ^{13}C NMR
10	1,8-cineole*	1020	1210	1.2	RI, MS, ^{13}C NMR
11	<i>p</i> -mentha-3,8-diene	1056	1264	0.2	RI, MS
12	α -thujone	1085	1422	0.3	RI, MS
12	β -thujone	1096	1441	0.3	RI, MS
14	<i>trans</i> - <i>p</i> -mentha-2,8-dien-1-ol	1102	1625	0.1	RI, MS
15	<i>cis</i> - <i>p</i> -mentha-2,8-dien-1-ol	1115	1657	0.1	RI, MS
16	camphor	1120	1516	0.7	RI, MS, ^{13}C NMR
17	<i>p</i> -menth-3-en-8-ol	1132	1605	12.5	RI, MS, ^{13}C NMR
18	isomenthone	1141	1491	6.6	RI, MS, ^{13}C NMR
19	<i>cis</i> -isopulegone	1148	1572,	0.4	RI, MS
20	<i>trans</i> -isopulegone	1149	1584	0.4	RI, MS
21	terpinen-4-ol	1160	1599	0.2	RI, MS
22	isomenthol	1171	1663	4.7	RI, MS, ^{13}C NMR
23	<i>trans</i> -carveol	1197	1831	0.3	RI, MS
24	pulegone	1214	1645	46.8	RI, MS, ^{13}C NMR

25	8-hydroxymenthone	1223	1758	6.2	RI, ¹³ C NMR
26	carvone	1225	1732	0.2	RI, MS
27	<i>trans</i> -pulegone oxide	1228	1813	0.9	RI, MS
28	8-hydroxyisomenthone	1255	1818	0.9	RI, ¹³ C NMR
29	<i>cis</i> -pulegone oxide	1265	1909	0.6	RI, MS
30	isomenthyl acetate	1278	1559	1.8	RI, MS, ¹³ C NMR
21	piperitenone	1307	1917	0.6	RI, MS
32	mintlactone	1448	2316	0.4	RI, MS
33	spathulenol	1567	2115	0.2	RI, MS
34	caryophyllene oxide	1578	1975	0.1	RI, MS
35	viridiflorol	1591	2076	tr	RI, MS
36	manool	2035	2644	0.1	RI, MS
Total				91.0	

Order of elution and percentages of individual components are given on apolar column (BP-1) except for the compounds with an asterisk * (polar column BP-20). RI_a, RI_p: retention indices calculated on apolar and polar columns respectively. tr = traces, <0.05%.

The oil was dominated by C-3 oxygenated *p*-menthane derivatives: pulegone (46.8%) was by far the most abundant component, followed by *p*-menth-3-en-8-ol (12.5%), isomenthone (6.6%), 8-hydroxymenthone (6.2%) isomenthol (4.7%) and isomenthyl acetate (1.8%). Other constituents present at appreciable contents were limonene (3.2%) and 1,8-cineole (1.2%). Only three oxygenated sesquiterpenes have been identified as minor components: caryophyllene oxide, spathulenol, viridiflorol and the diterpene manool.–The use of NMR spectroscopy assuredly allowed the identification of several isomers bearing the menthane skeleton, which exhibited similar mass spectra.

3.2. Antifungal activity

The antifungal activity of the essential oil against human and animal pathogens is presented in Table 2. The highest antifungal activity was observed against *Cryptococcus neoformans* with a MIC value of 0.16 μ L/mL. Regarding filamentous fungi, the essential oil has similar activity against dermatophytes and *Aspergillus* strains with MICs values of 0.64 to 1.25 μ L/mL and showed fungicidal effects against *Epithermophyton flocosum* with MIC and MLC values of 0.64 μ L/mL.

Table 2. Antifungal activity (MIC and MLC) of *Ziziphora tenuior* for yeasts, dermatophytes and *Aspergillus* strains.

Strains	<i>Ziziphora tenuior</i>		Fluconazole		Amphotericin B	
	MIC ^(a)	MLC ^(a)	MIC ^(b)	MLC ^(b)	MIC ^(b)	MLC ^(b)
<i>Candida albicans</i> ATCC 10231	1.25	1.25	1	>128	N.T	N.T
<i>Candida tropicalis</i> ATCC 13803	1.25	1.25	4	>128	N.T	N.T
<i>Candida krusei</i> H9	1.25	1.25	64	64-128	N.T	N.T
<i>Candida guilliermondii</i> MAT23	1.25	1.25	8	8	N.T	N.T
<i>Candida parapsilosis</i> ATCC 90018	1.25	2.5	<1	<1	N.T	N.T
<i>Cryptococcus neoformans</i> CECT 1078	0.16	0.64	16	128	N.T	N.T
<i>Trichophyton mentagrophytes</i> FF7	1.25	1.25	16-32	32-64	N.T	N.T
<i>T. mentagrophytes</i> var. <i>interdigitale</i> CECT 2958	1.254	2.5	128	≥128	N.T	N.T
<i>T. rubrum</i> CECT 2794	0.64	1.25	16	64	N.T	N.T
<i>T. verrucosum</i> CECT 2992	0.64	1.25	128	>128	N.T	N.T
<i>Microsporium canis</i> FF1	1.25-0.64	1.25	128	128	N.T	N.T
<i>M. gypseum</i> CECT 2905	1.25	1.25	128	>128	N.T	N.T
<i>Epidermophyton floccosum</i> FF9	0.64	0.64	16	16	N.T	N.T
<i>Aspergillus niger</i> ATCC16404	0.64	5	N.T	N.T	1-2	4
<i>A. fumigatus</i> ATCC 46645	0.64	2.5	N.T	N.T	2	4
<i>A. flavus</i> F44	1.25	2.5	N.T	N.T	2	8

^a MIC and MLC were determined by a macrodilution method and expressed in $\mu\text{L}/\text{mL}$ (v/v).

^b MIC and MLC were determined by a macrodilution method and expressed in $\mu\text{g}/\text{mL}$ (w/v).

N.T not tested.

Antimicrobial activity of *Ziziphora tenuior* essential oil was previously reported against strains of Gram-negative and Gram-positive bacteria, *C. albicans* and *Aspergillus* (Mahboubi et al., 2012). The authors reported that the essential oil is active against *A. flavus* and *A. niger* as well as against *C. albicans*. In our study, we evaluated the effect on other *Candida* species, *C. neoformans* and dermatophytes.

Although some studies on the antifungal activity of *Ziziphora tenuior* oil were previously carried out, the mechanism of action underlying this effect remains unknown. Therefore, in the present study, we attempted to elucidate possible modes of action on *C. albicans*. Two assays were selected, namely the inhibition of germ tube formation and the disruption of preformed biofilms. The essential oil revealed an important inhibitory effect on germ tube formation in *C. albicans* tested strain at sub-inhibitory concentrations (Figure 1). At a concentration of 1/16 MIC (0.08 $\mu\text{L}/\text{mL}$), the inhibition of filamentation was more than 80% in comparison to untreated control

cells. The yeast-mycelium transition in *C. albicans* has been described as important virulence factor in this species, and it seems that filamentation inhibition *per se* is sufficient to treat disseminated candidiasis (Saville et al., 2006).

The striking difference between MICs and filamentation-inhibiting concentrations suggest that different mechanisms of action may be responsible for these two biological effects.

Figures 2 and 3 represent the effect of the essential oil on preformed *C. albicans* biofilms. The XTT assay evaluates cell viability by analyzing the formation of a water soluble crystal formed after mitochondrial metabolization, whereas the crystal violet method quantifies the biomass of the biofilm by its staining with the dye. The oil decreased the metabolic activity compared to control at MIC and 2×MIC values (Fig.2),

Comparable results were also observed for biofilm biomass reduction demonstrating that 2.5 $\mu\text{L}/\text{mL}$ (2×MIC) and 1.25 $\mu\text{L}/\text{mL}$ (MIC) of the oil were able to interfere with pre-formed biofilms by reducing the amount of the attached biomass.

3.3 Anti-inflammatory activity

To explore whether *Ziziphora tenuior* oil modulates NO production, we used an *in vitro* inflammation model consisting of macrophages stimulated with the Toll-like receptor (TLR) 4 agonist, LPS. Indeed, upon TLR activation, macrophages produced large NO amounts. This result is comparable to the situation in several inflammatory-related diseases, namely atherosclerosis, obesity, diabetes and neurodegenerative diseases. For this reason, NO represents a well-established marker of inflammation, and inhibition of its production serves as useful strategy to screen compounds with anti-inflammatory potential (Weinberg et al., 2007). The effect of the oil on NO production was analyzed by measuring the nitrites accumulation in the culture medium of LPS-stimulated macrophages. As shown in Figure 4, NO production in macrophages decreased in the presence of the four tested concentrations of the essential oil: 18.57 ± 0.55 (1.25 $\mu\text{L}/\text{mL}$), 36.45 ± 0.27 (0.64 $\mu\text{L}/\text{mL}$), 48.74 ± 1.07 (0.32 $\mu\text{L}/\text{mL}$) and 78.51 ± 0.29 (0.16 $\mu\text{L}/\text{mL}$). It is important to emphasize that the essential oil inhibited NO production evoked by LPS at concentrations, which revealed only very low toxicity (0.64 $\mu\text{L}/\text{mL}$) to macrophages (Figure 5).

3.4 Evaluation of cell viability

In a drug discovery perspective, it is important to assess the toxicological profile of compounds at concentrations presenting relevant bioactivity. Therefore, we further tracked the safety of *Z. tenuior* essential oil in three mammalian cell types, namely macrophages, keratinocytes and hepatocytes, using the MTT assay. Figures 5, 6 and 7 demonstrate that the essential oil of *Z. tenuior* showed very low cytotoxicity towards macrophages and hepatocytes at concentrations up to 0.64 $\mu\text{L}/\text{mL}$, and keratinocytes at concentrations up to 0.32 $\mu\text{L}/\text{mL}$. Therefore, we conclude that *Z. tenuior* essential oil may represent a safe herbal drug with low toxicity at the doses indicated.

4. Conclusions

There is currently no literature available concerning the chemical composition as well as the antifungal and anti-inflammatory properties of *Ziziphora tenuior* L. essential oil from Dana Biosphere Reserve (Southern Jordan), a medicinal plant used in Jordan for treating several disorders. Therefore, we performed a detailed phytochemical investigation on *Z. tenuior* essential oil and the associated pharmacological properties, especially its mechanism of action and toxicology, in order to validate its ethnomedicinal use.

The chemical analysis revealed that *Z. tenuior* essential oil was dominated by C-3 oxygenated *p*-menthane derivatives: pulegone (46.8%), *p*-menth-3-en-8-ol (12.5%), isomenthone (6.6%), 8-hydroxymenthone (6.2%) isomenthol (4.7%) and isomenthyl acetate (1.8%). Interesting results were obtained concerning the antifungal activity against *Cryptococcus neoformans*, dermatophytes and *Aspergillus* strains. Remarkably, *Z. tenuior* essential oil significantly inhibited germ tube formation and disrupted preformed biofilms of *C. albicans*, two important virulence factors in this species. Hence, the oil represents a good candidate for the treatment of candidiasis. Importantly, concentrations devoid of toxicity in several mammalian cell types also displayed anti-inflammatory activity.

These findings add significant information to the pharmacological activity of *Z. tenuior*, thus justifying and reinforcing the use of this species in traditional medicine and opening new avenues for their further exploitation, for instance in health-care product development.

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Figure 1. Influence of sub-inhibitory concentrations of *Z. tenuior* essential oil on germ tube formation of *Candida albicans* ATCC 10231. Ctrl (control); tested concentrations of the essential oil (0.08-1.25 $\mu\text{L}/\text{mL}$). Results are shown as mean \pm SEM of three independent determinations carried out in duplicate (**** $p < 0.0001$, compared to control).

Figure 2. Biofilm viability after treatment with *Z. tenuior* essential oil using the XTT viability assay. Ctrl (control); tested concentrations of the essential oil (0.32-2.5 $\mu\text{L}/\text{mL}$). Results are shown as mean \pm SEM of three independent determinations carried out in duplicate (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, compared to control).

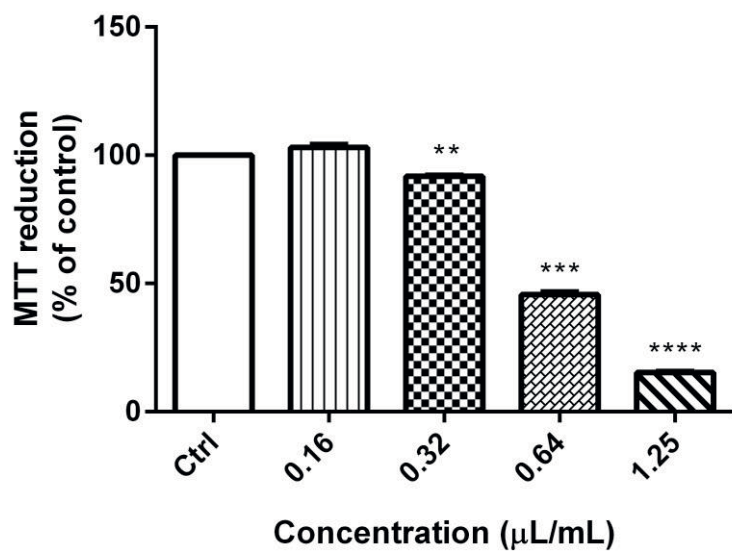
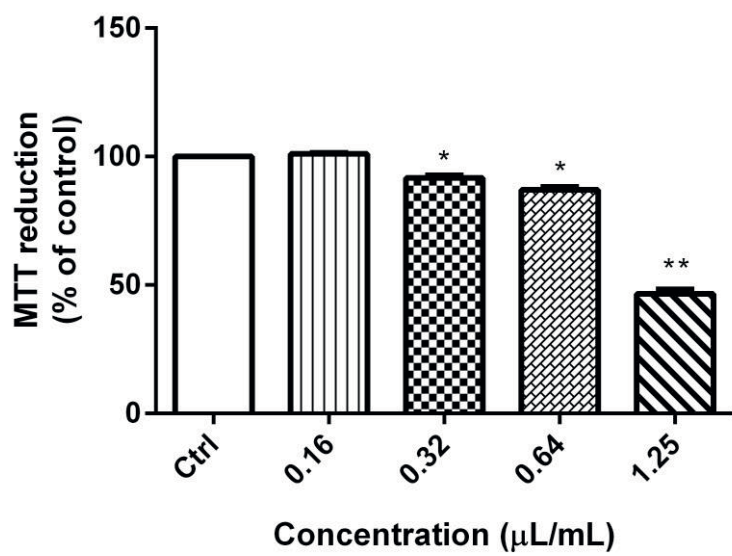
Figure 3. Biofilm biomass after treatment with *Z. tenuior* essential oil, using the crystal violet assay. Ctrl (control); tested concentrations of the essential oil (0.32-2.5 μ L/mL). Results are shown as mean \pm SEM of at least three independent determinations carried out in duplicate (** p <0.001, **** p <0.0001, compared to control).

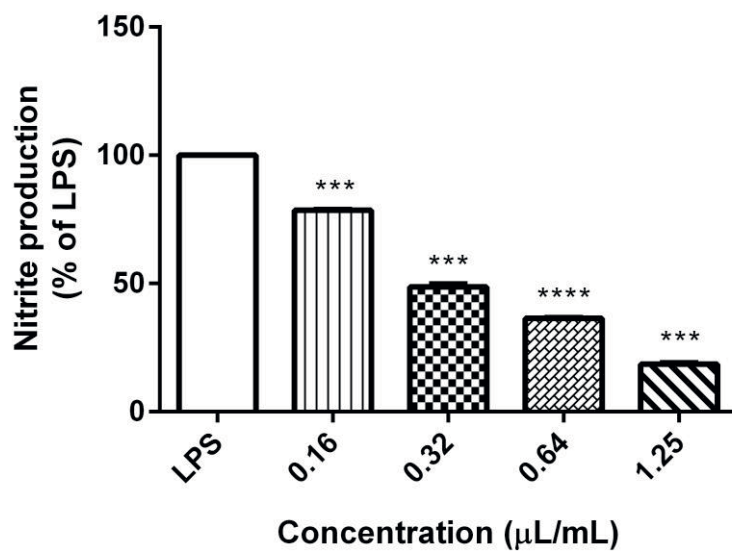
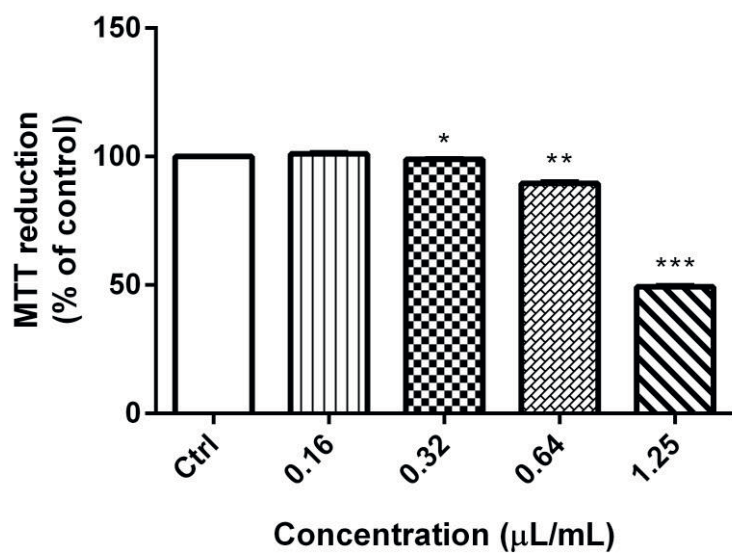
Figure 4. Effect of essential oil of *Z. tenuior* on NO production in macrophages (Raw 264.7). LPS (control); tested concentrations of the essential oil (0.16-1.25 μ L/mL). Results are expressed as a percentage of the nitrite production by cells cultured in the presence of LPS. Each value represents the mean \pm SEM of three independent experiments performed in duplicate (** p <0.001, **** p <0.0001, compared to LPS).

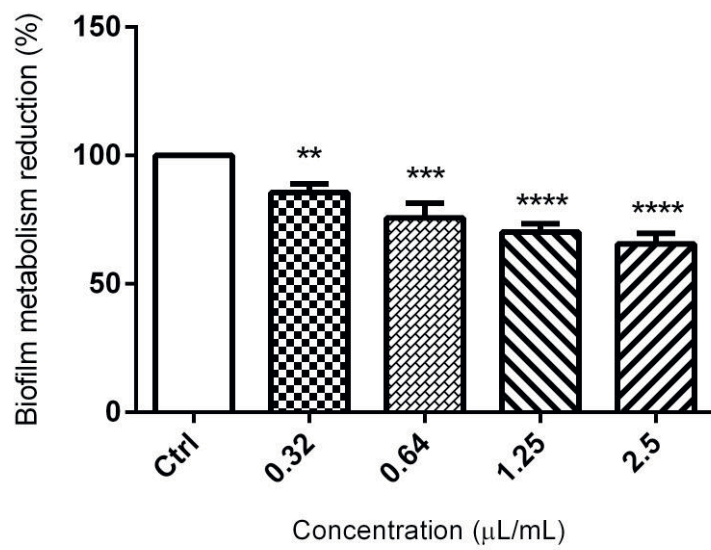
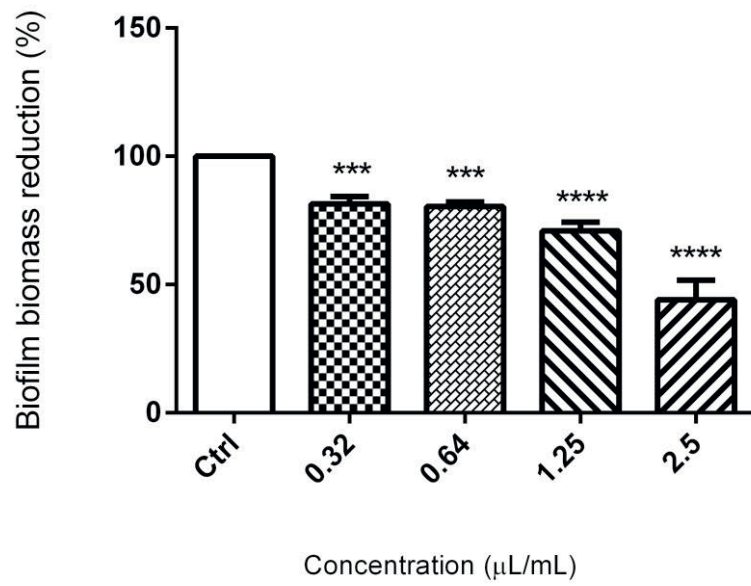
Figure 5. Effect of essential oil of *Z. tenuior* on macrophages (Raw 264.7) viability by the MTT assay. Ctrl (control); tested concentrations of the essential oil (0.16-1.25 μ L/mL). Results are expressed as a percentage of MTT reduction by control cells. Each value represents the mean \pm SEM of three independent experiments performed in duplicate (* p <0.05, ** p <0.01, *** p <0.001, compared to control).

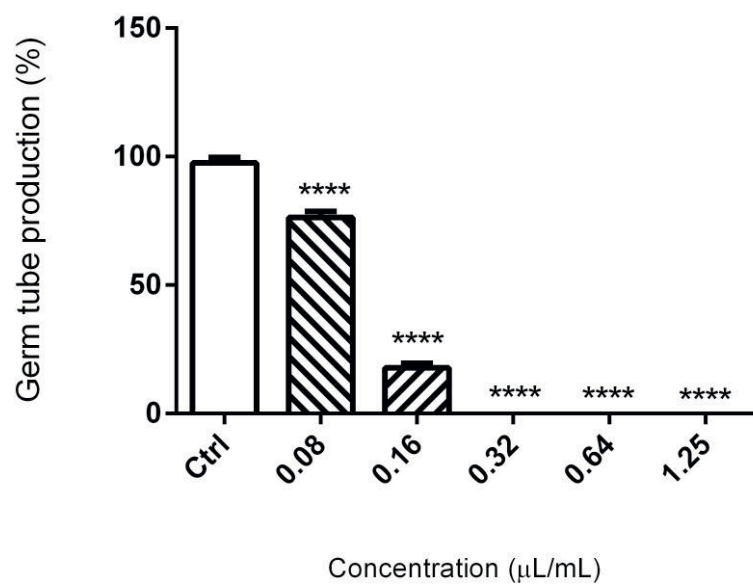
Figure 6. Effect of essential oil of *Z. tenuior* on keratinocytes (HaCaT) viability by the MTT assay. Ctrl (control); tested concentrations of the essential oil (0.16-1.25 μ L/mL). Results are expressed as a percentage of MTT reduction by control cells. Each value represents the mean \pm SEM of three independent experiments performed in duplicate (** p <0.01, *** p <0.001, **** p <0.0001, compared to control).

Figure 7. Effect of essential oil of *Z. tenuior* on hepatocytes (HepG2) viability by the MTT assay. Ctrl (control); tested concentrations of the essential oil (0.16-1.25 μ L/mL). Results are expressed as a percentage of MTT reduction by control cells. Each value represents the mean \pm SEM of three independent experiments performed in duplicate (* p <0.05, ** p <0.01, compared to control).





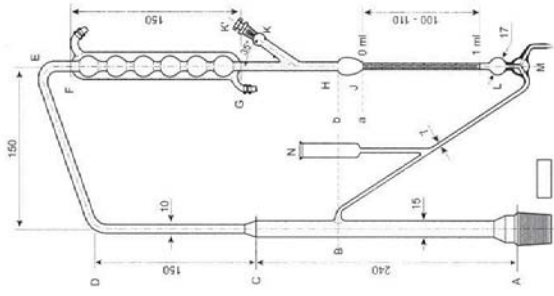




Accepted manuscript



Ziziphora tenuior L. in a Jordan traditional market



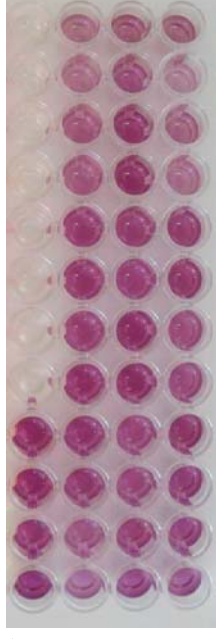
Essential oil characterized by GC, GC/MS, ^{13}C NMR



Antifungal activity (MIC, MLC, germ tube inhibition, biofilm formation)



Anti-inflammatory activity (NO production)



Cell viability (MTT assay)