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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Effect of citral and citral related compounds on viability of pancreatic and human B-lymphoma cell lines

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Abstract Citral, 3,7-dimethyl-2,6-octadienal, is a key component of the essential oils extracted from several lemon-scented herbal plants. It has been demonstrated to be an effective antifungal agent and in recent years, to have antiproliferative effect in certain tumor cell lines: thus citral could be a potential anticancer drug. In this study, we investigated the effect of citral on the proliferation of pancreatic cell tumor lines (MIA PaCa-2 cells) and human B-lymphoma (DeFew cells). To analyze which part of the molecule is pivotal for the activity, we also studied the effect of several citral related compounds, in particular focusing on some chemical aspects of the terpenic scaffold. The in vitro studies revealed the significant cytotoxic activity of some of the tested compounds.

Keywords Citral · Viability · Mia PaCa-2 cells · DeFew cells

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Introduction

Nowadays, one of the fastest growing areas of research is exploring natural plant products as an option to find new chemical entities as anticancer agents (Lee 1999). Citral **1** (3,7-dimethyl-2,6-octadienal), a key component of essential oils extracted among others, from lemongrass (*Cymbopogon citratus*), lemon balm (*Melissa officinalis*), and vervain (*Verbena officinalis*). Citral is a mixture of two isomeric acyclic monoterpene aldehydes used in foods and cosmetics (Kumar et al. 2000; Nakamura et al. 2003; Dudai et al. 2005): geranial (*trans* isomer) has a strong lemon odor, neral (*cis* isomer) has a lemon odor that is less intense and sweeter than geranial (Fig. 1).

The toxicity studies indicate that citral is devoid of major toxicity and carcinogenic potential in both mice and rats (Rabbani et al. 2005; Dieter et al. 1993); citral is also devoid of mutagenic effect on in vitro models (Vinitketkumnuen et al. 1994). Citral possesses many bioactivities such as antispasmodic (Ganjewala 2009; Devi et al. 2011), anti-inflammatory (Katsukawa et al. 2011), antipyretic, diuretic, and sedative. It also has strong antimicrobial properties (Onawunmi 1989) and pheromonal effects in insects (Kuwahara et al. 1983). Citral is also used in the synthesis

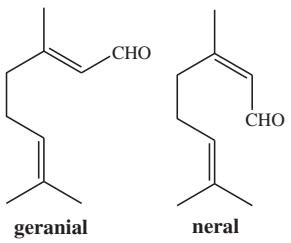

Fig. 1 Citral structure

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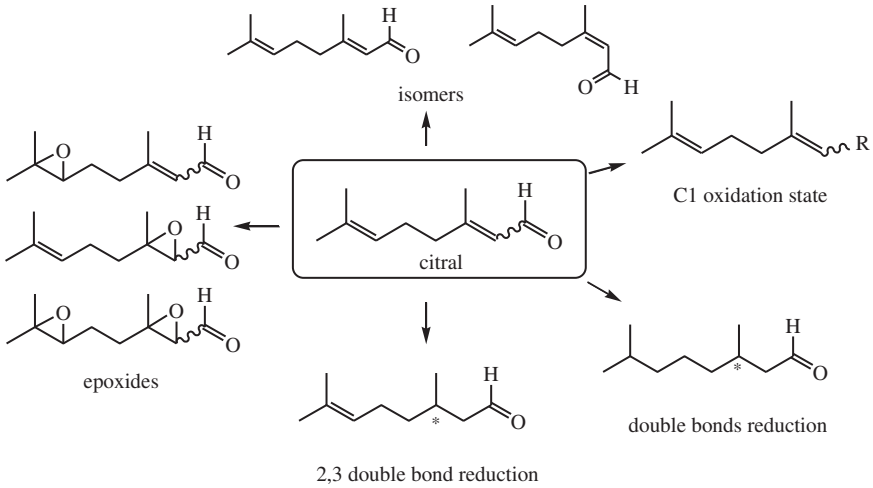


Fig. 2 Chemical modifications carried out on the terpenic scaffold

of vitamin A and to mask the smell of smoke (Lewinsohn et al. 1998). Citral is a potent inducer of glutathione-*s*-transferase class of enzymes, involved into protection of healthy hepatocytes against apoptosis during chemotherapy of liver cancers (Dudai et al. 2005). A good superoxide scavenging activity ($EC_{50} = 19$ µg/ml) was reported in Swiss albino mice in citral treated groups, suggesting that the antioxidant action could be responsible for the anti-clastogenic effect of citral against nickel chloride (Rabbani et al. 2006). Citral also disrupts animal microtubules and inhibits polymerization of microtubules in vitro (Chaimovitsh et al. 2012) and at a concentration comparable to that found in a cup of tea brewed with 1 g of lemongrass, citral was found to induce apoptosis in cancer cells. Apoptosis was accompanied by DNA fragmentation and caspase-3 catalytic induction (Rabbani et al. 2005). Citral treatment also caused inhibition of breast cancer MCF-7 cell growth (IC_{50} 48 h: 180 µM) with a cycle arrest in G2/M phase and apoptosis induction suggesting a potential chemo-preventive effect (Chaouki et al. 2009). Pro-apoptotic activity of citral in patients with chronic lymphocytic leukemia as well as its effect on the acute promyelocytic leukemia cell line NB4 were also reported (De Martino et al. 2009; Hailong et al. 2013). Citral is also capable to induce cancer cell death by decreasing cell proliferation, altering mitochondrial membrane potential, and initiating apoptosis in HeLa and ME-180 cell lines (Ghosh 2013). Recently it has been demonstrated that citral potentiated cytotoxicity of doxorubicin by increasing apoptotic effects in human lymphoma Ramos cells (Dangkong and Limpanasithikul 2015).

Taking into account these published reports, the aim of the present study was to evaluate the effect of citral **1** on viability of malignant cells such as pancreatic cell tumor lines (MIA PaCa-2 cells) and human B-lymphoma cell lines (DeFew cells). To analyze which part of the molecule is pivotal for the activity, we also studied the effect of several citral related compounds, in particular focusing on four chemical aspects of the terpenic scaffold (Fig. 2):

a) geometry of double bond in 2,3 position (as already mentioned citral is a mixture of two isomeric acyclic monoterpene aldehydes which are double bond isomers)
b) oxidation state of carbon in C_1 position
c) oxidation of double bonds into epoxides
d) removal of double bonds

Our initial experiments focused on the effect of citral **1** on cell growth and proliferative potential of MIA PaCa-2 cells and DeFew cell lines at a concentration range between 12.5 and 400 µM in DMSO (Dimethyl sulphoxide). Cancer cells were exposed to the compound 1 day after seeding and their viability was followed for 3 days using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) cell proliferation assay and the results are presented as percentage of viability. Figure 3 shows the effect of citral on the viability of MIA PaCa-2 cells and DeFew cell lines estimated by MTT assay, in particular the exposure to citral at a range between 50 and 400 µM strongly inhibited the growth of the cell line by 10–90 % indicating that the compounds affect cell viability in a dose-dependent manner. IC_{50} values were calculated to be 238 µM on MIA PaCa-2 cells and 300 µM on DeFew cells. DMSO concentration did not affected cell viability.

Materials and methods

Chemistry

Citral, citronellol and citronellal were purchased from Sigma Aldrich (Germany). Geraniol, nerol and geranic acid were purchased from Alfa Aesar (Germany). Citral isomers

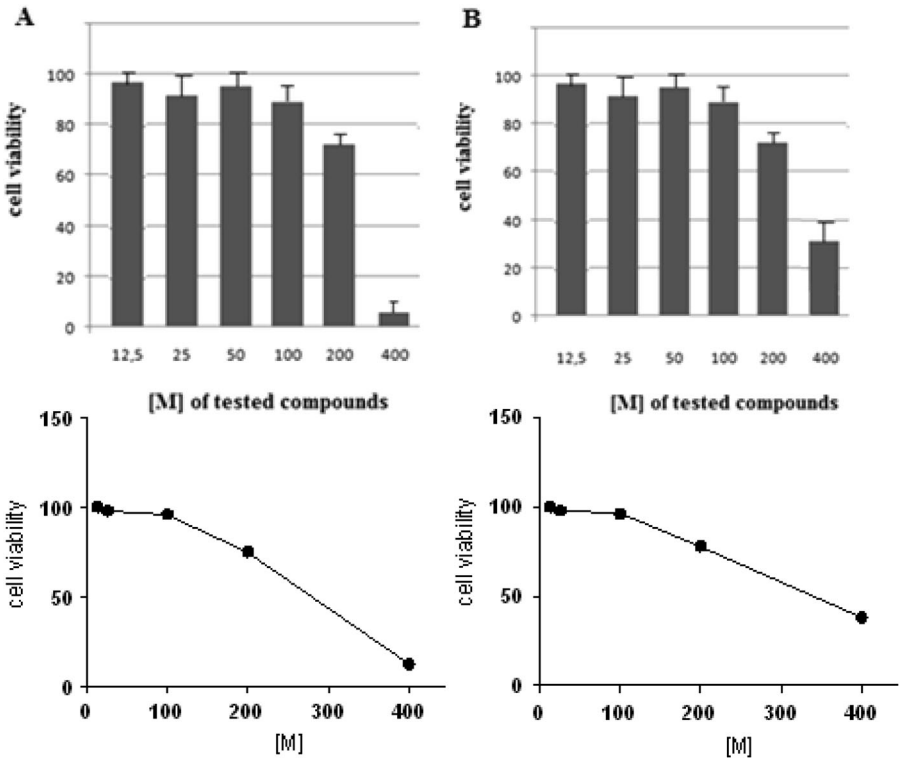
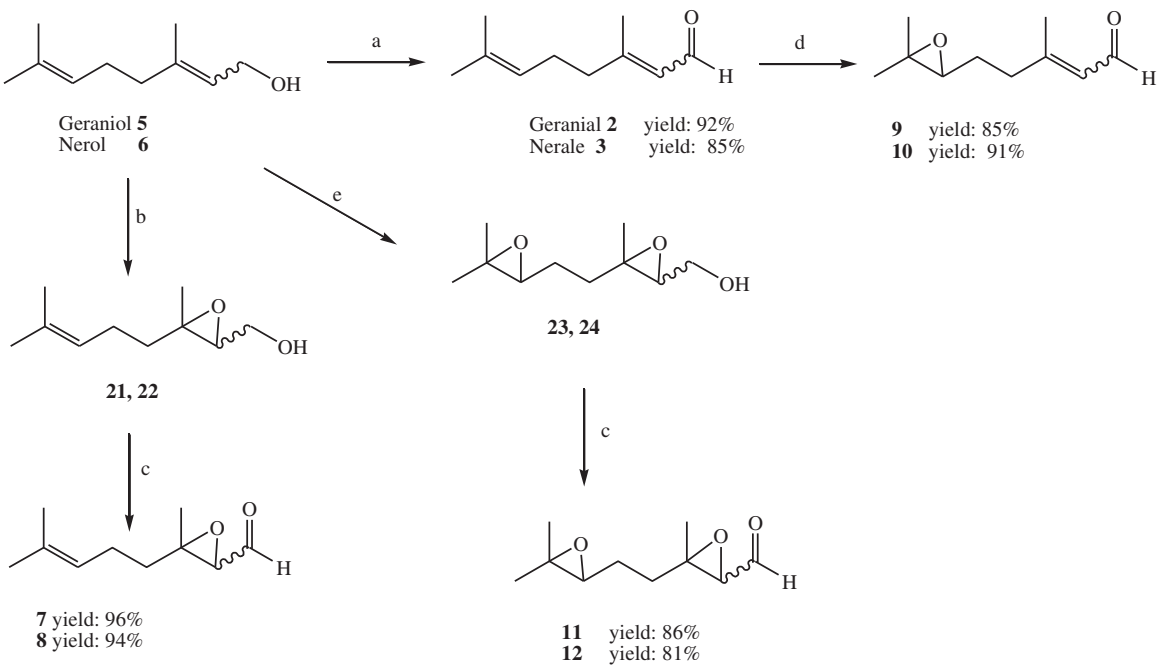
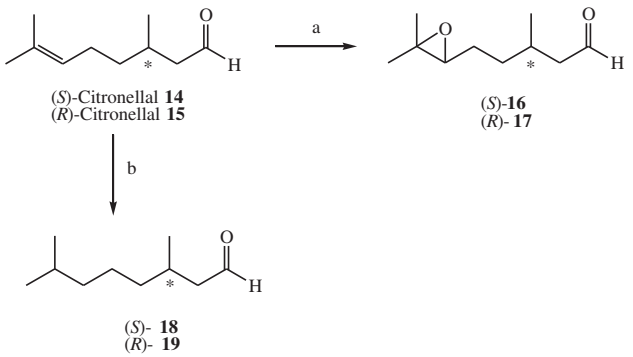


Fig. 3 **a**. Cell viability of MIA PaCa 2-cells treated with citral. **b**. Cell viability of DeFew cells treated with citral. MIA PaCa-2 and DeFew cells were treated with citral (12.5., 25, 50, 100, 200, 400 µM) for 72 h. Their viability was evaluated using the MTT assay as described in the materials and methods Section. The optical densities (OD 620) of citral-treated cells were compared with the OD of the control wells (untreated cells). Each percent value is the mean ± SD of three independent experiments



Scheme 1 Reagents and conditions: **a** MnO_2, benzene, r.t; **b** *tert*-butyl hydroperoxide (TBHP), vanadyl acetyl acetonate, CH_2Cl_2, r.t; **c** Py-SO_3, CH_2Cl_2, DMSO, Et_3N, r.t; **d** MCPBA 77% (1.1 equiv.), CH_2Cl_2, 0 °C; **e** MCPBA 77% (2.2 equiv), CH_2Cl_2, 0 °C

2 and **3** were prepared according to the Scheme 1, by oxidation of commercially available geraniol and nerol using manganese dioxide in benzene (Surendra and Corey 2008). The 2,3 Citral epoxides **7,8** were synthesized by oxidation of 2,3 double bond in the presence of *tert*-butyl hydroperoxide and vanadylacetyl acetonate and following oxidation of the alcohols **21, 22** to aldehydes with pyridinium SO_3 complex (Rose et al. 2006). The 6,7 Citral epoxides **8, 9** were synthesized by oxidation of 6,7 double bond in the presence of 3-chloroperoxybenzoic acid (77%)



Scheme 2 Reagents and conditions: **a** MCPBA 77%, CH_2Cl_2, 0 °C.; **b** H_2, 5% Pd/C, ethyl acetate, r.t

in dichloromethane (Elgendy and Khayyat 2008; Yarovaya et al. 2002). Epoxides were recovered in very good yields. Diepoxides **11, 12** were prepared treating geraniol and nerol with an excess of 3-chloroperoxybenzoic acid (MCPBA77%) in dichloromethane affording the corresponding alcohols diepoxides which were converted into aldehydes with pyridinium SO_3 complex.

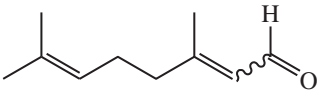
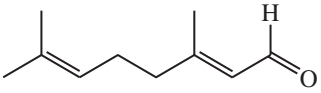
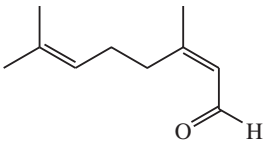
Citronellal derivatives were prepared as reported in Scheme 2. (*S*)-citronellal **14** and (*R*)-citronellal **15** were treated with 3-chloroperoxybenzoic acid (77%) in dichloromethane affording compounds **16** and **17**. Compound **18** and **19** were obtained after reduction of both double bonds of citronellal using 5% Pd/C in ethyl acetate under hydrogen atmosphere (Mikami et al. 2011).

Cytotoxic activity evaluation

To analyze which part of the citral molecule is pivotal for the activity, we also considered some chemical structural modifications of the terpenic scaffold, focused on the effect of several citral related compounds on cell growth and proliferative potential of same cells. IC_{50} values of tested compounds are reported in Tables 1–4. As already mentioned, citral is a mixture of two isomeric acyclic monoterpene aldehydes that are double bond isomers, but few studies have directly compared their antitumor properties (Zeng et al. 2015). Thus first of all, we considered if the geometry of double bond in 2,3 position could affect the citral antiproliferactive effect. As reported in Table 1 no substantial difference was observed for the *trans/cis* isomers **2, 3** in comparison with citral mixture on MIA PaCa-2 cells proliferation and DeFew cells proliferation.

Considering C-1 oxidation state, both reduction of aldehyde to alcohol or oxidation to carboxylic acid did not seem to affect cytotoxic activity of citral on MIA PaCa-2 cells lines, except in the case of nerol **5** which showed higher IC_{50} than citral (Table 2). IC_{50} value of geraniol was found to be in according to that reported by Burke, who

Table 1 IC_{50} values of citral and cis/trans isomers

compounds	IC_{50} μM MIA PaCa 2-cell	IC_{50} μM DeFew cells
 citral **1**	238	300
 geranial **2**	270	320
 neral **3**	280	250

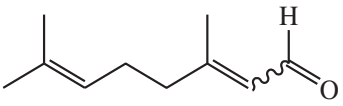
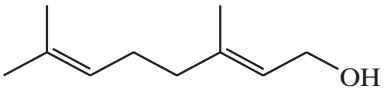
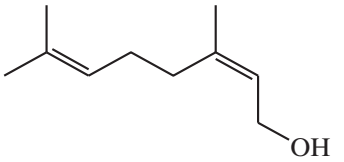
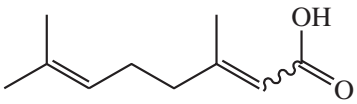
investigated the anticancer activity of geraniol finding a significant (60–90%) inhibition of the anchorage-independent growth of human MIA PaCa-2 cells (Burke et al. 1997). When we consider the effect on DeFew cells survival, oxidation of citral aldehyde to geranic acid **6** acid strongly affect viability, confirming the results reported by Dudai for similar leukemia lines (Dudai et al. 2005).

Next, we focused on double bonds oxidation considering selective 2,3 epoxidation, 6,7 epoxidation and diepoxidation as reported in Table 3. Epoxidation of double bond in position 2,3 increased three fold times cytotoxic activity in both malignant tested cell lines, compared with citral. Compound 7 was already reported as active to inhibit cell growth in various human cancer cell lines in culture (Rose et al. 2006). In particular, on DeFew cells, epoxide **7** was very active if compared with epoxide **8** suggesting the importance of trans epoxide geometry for the reduction of cell viability in human B-lymphoma cells. When we considered epoxidation of double bond in position 6,7 (compounds **9** and **10**) and diepoxidation (compounds **11** and **12**), a complete loss of cytotoxic effect was observed, highlighting the necessary presence of double bond in 6,7 position for optimal activity.

Other natural monoterpenes structurally related to citral are citronellal and citronellol, also present in the essential oils extracted from several lemon-scented herbal plants. It has been demonstrated that a number of monoterpenes have antitumoral activity that can prevent the formation or progress of cancer and cause tumor regression (Zhaung et al. 2009).

Citronellal (in which 2,3 double bond is missing) showed antiproliferactive effect similar to citral, on MIA PaCa-2

Table 2 IC_{50} values of citral and related C_1 oxidation state compounds

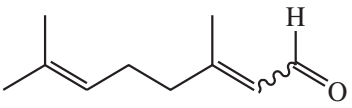
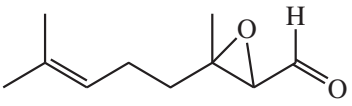
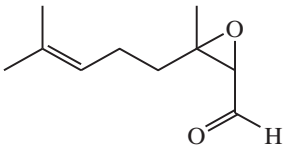
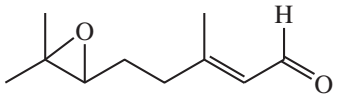
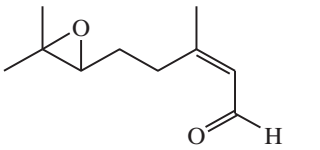
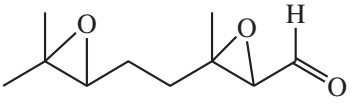
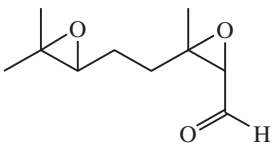
compounds	IC_{50} μM MIA PaCa 2-cells	IC_{50} μM DeFew cells
 citral **1**	238	300
 geraniol **4**	257	280
 nerol **5**	392	323
 geranic acid **6**	263	580

cells, when we consider the racemic mixture **13** (Table 4), suggesting that removal of 2,3 double bond in not crucial for activity.

When testing the two enantiomers, a crucial role of stereochemistry was observed since *R*-citronellal **15** was found to be two fold times more potent than racemic citronellal and six fold times than *S*-citronellal **14**. Proliferation measurement showed a different trend on DeFew cells, because in this case *S*-citronellal was four fold times more potent than *R*-citronellal and two fold times than racemic mixture. Epoxidation of 6,7 double bond (compounds **16** and **17**) or removal of 6,7 double bond (compounds **18** and **19**) led to a complete loss of cytotoxic effect, highlighting also in this case the necessary presence of double bond in 6,7 position for optimal activity. Citronellol showed a good cell viability reduction effect only on human B-lymphoma, in particular *S*-citronellol **21** was almost seven fold times more active than *R*-citronellol and two fold times than citronellal. Also Zhuang reported that citronellol has anticancer and anti-inflammatory properties (Zhuang et al. 2009). No effect was observed on MIA PaCa-2 cell lines.

Taking into account these data which revealed a significant cytotoxic activity of some of the tested compounds (citral **1**, epoxides **7–8**, citronellal enantiomers **14–15,** and

Table 3 IC_{50} values of citral and related epoxides

compound	IC_{50} μM MIA PaCa 2-cells	IC_{50} μM DeFew cells
 citral **1**	238	300
 **7**	88.7	45
 **8**	94	89
 **9**	–	–
 **10**	–	–
 **11**	–	–
 **12**	–	–

S-citronellol **21**), in vitro studies were also carried out on HUVEC (Human Umbilical Vein Endothelial cells) to evaluate eventual toxicity (Table 5). As already reported in literature, citral was found to be devoid of toxicity as well as the other natural monoterpenes. Indeed synthetic epoxides **7–8** affect the survival of HUVEC cells with calculated IC_{50} values as reported in Table 5, however very high value when compared to IC50 values on the tumor cell lines.

Table 4 IC_{50} values of citral , citronellal , citronellol and related compounds

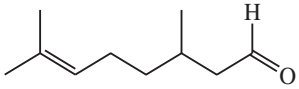
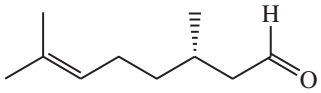
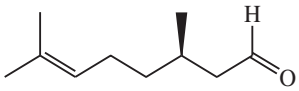
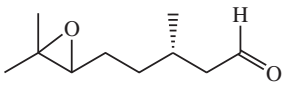
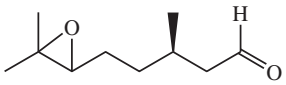
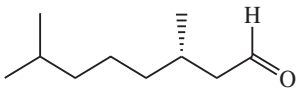
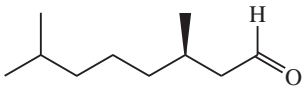
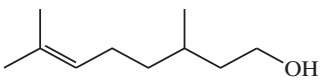
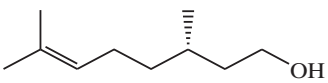
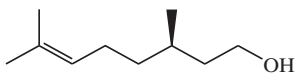
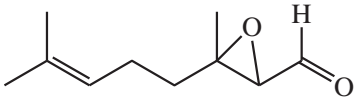
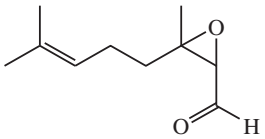
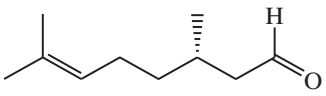
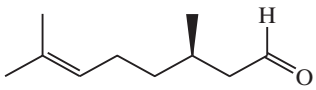
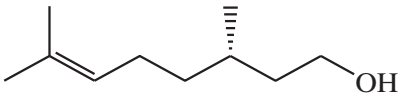
compounds	IC_{50} μM MIA PaCa 2-cells	IC_{50} μM DeFew cells
Citral **1**	238	300
 (*RS*)-Citronellal **13**	231	61
 (*S*)-Citronellal **14**	668	31
 (*R*)-Citronellal **15**	128	129
 (*S*)- **16**	–	–
 (*R*)- **17**	–	–
 (*S*)- **18**	–	–
 (*R*)- **19**	–	–
 (*RS*)-Citronellol **20**	567	252
 (*S*)-Citronellol **21**	463	95
 (*R*)-Citronellol **22**	–	679

Table 5 IC_{50} values of most promising compounds on HUVEC cells proliferation

compounds	IC_{50} μM HUVEC cells
Citral **1**	–
 **7**	200
 **8**	162
 (*S*)-Citronellal **14**	–
 (*R*)-Citronellal **15**	–
 (*S*)-Citronellol **21**	–

Conclusions

Results presented in the current study showed a remarkable effect of citral, citronellal, and citronellol on viability of pancreatic cell tumor lines (MIA PaCa-2 cells) and human B-lymphoma lines (DeFew cells), such that they should be considered good candidates for chemoprevention and chemotherapy. In addition a careful structure-activity relationship study carried out on the terpenic scaffold to analyze which part of the citral molecule is pivotal for the cell viability reduction, highlighted substantial no role of 2,3 double bond and crucial importance of 6,7 double bond. In addition some citral related compounds like 2,3 epoxides, which are very easy to synthesize, strongly reduced cell viability in both malignant cell lines. Thus, current studies are in course to identify and to understand well the mechanisms (apoptosis or cell cycle arrest or both of these phenomena) involved in the effect induced by the most potent compounds.

Experimental Section

All reactions were performed using commercially available compounds without further purification. Column chromatographic purification of products was carried out using silica gel 60 (70–230 mesh, Merck). The NMR spectra were recorded on Bruker DRX 400, 300, 250 spectrometers (400, 300, 250 MHz, ^{1}H). Spectra were referenced to residual $CHCl_3$ (7.26 ppm, ^{1}H, 77.23 ppm, ^{13}C). Coupling constants *J* are reported in Hz. Yields are given for isolated products showing one spot on a TLC plate and no impurities detectable in the NMR spectrum. Mass spectral analyses were carried out using an electrospray spectrometer Waters 4 micro quadrupole or Agilent GC-MSD 5975C with triple axis detector. Elemental analyses were performed with FLASHEA 1112 series-Thermo Scientific for CHNS-O apparatus.

General procedure for the synthesis of *trans/cis* isomers 2 and 3

To a solution of geraniol or nerol 300 mg (1.94 mmol) in benzene (6 ml), manganese dioxide (4 equiv.) was added and the mixture was allowed to stir for 6 h. Filtration on celite and evaporation of the solvent gave a pale oil which was purified by chromatography (Petroleum ether/ diethyl ether 9:1).

(*E*)-3,7-dimethylocta-2,6-dienale (Geranial) 2

Pale oil. Yield: 92%. ESI (m/z): 153 $[M + H]^+$. Anal. calcd for $C_{10}H_{16}O$: C 78.90, H 10.59. Found: C 78.80, H 10.69. Spectroscopic data are in agreement with literature (Elgendy and Khayyat 2008; Yarovaya et al. 2002)

(*Z*)-3,7-dimethylocta-2,6-dienale (Neral) 3

Pale oil. Yield: 85%. ESI (m/z): 153 $[M + H]^+$. Anal. calcd for $C_{10}H_{16}O$: C 78.90, H 10.59. Found: C 78.83, H 10.63. Spectroscopic data are in agreement with literature (Elgendy and Khayyat 2008; Yarovaya et al. 2002)

General procedure for the synthesis of 2,3 epoxides 7, 8

To a solution of geraniol or nerol (300 mg, 1.94 mmol) in CH_2Cl_2 (10 ml) keeping the mixture at 0 °C, TBHP (5.5M in decano, 3.5 mmol, 635 µL) and vanadylacetylacetonate (10.3 mg, 3.5 mmol) were added and the mithure was stirred till starting material disappeared. Purification by chromatography (Petroleum ether/ diethyl ether 8:2), gave pure products which were solubilised (50 mg, 0.294 mmol) in CH_2Cl_2 (1 ml) and under nitrogen atmosphere Et_3N (1.47 mmol, 204 µl) was added. A solution of Py-SO_3 (140 mg, 0,882 mmol) in DMSO (22 mmol, 460 µl) was added droppwise and the reaction mixture was allowed to stir overnight. Purification by chromatography (Petroleum ether/ethyl acetate 9:1) gave a yellow oil.

(*E*)- 3-methyl-3-(4-methylpent-3-enyl)oxiran-2-carbaldehyde 7

Yellow oil. Yield: 96%. ESI (m/z): 191 $[M^+ + 23]$. Anal. calcd for $C_{10}H_{16}O_2$: C 71.39, H 9.59. Found: C 71.19, H 9.49. Spectroscopic data are in agreement with literature (Elgendy and Khayyat 2008).

(*Z*)- 3-methyl-3-(4-methylpent-3-enyl)oxiran-2-carbaldehyde 8

Yellow oil. Yield: 94%. ESI (m/z): 191 $[M^+ + 23]$. Anal. calcd for $C_{10}H_{16}O_2$: C 71.39, H 9.59. Found C 71.19, H 9.49. Spectroscopic data are in agreement with literature (Leal et al. 1898).

General procedure for the synthesis of 6,7 epoxides 9,10

To a solution of geraniol or nerol (50 mg, 0.328 mmol) in CH_2Cl_2 (2 ml) , MCPBA 77% (92 mg, 1.76 mmol), was added keeping the mixture at 0 °C for 1 h. The organic layer was washed three times with $NaHCO_3$ and brine; purification by chromatography (Petroleum ether/diethyl ether 7:2) gave a yellow oil.

(*E*)-3-methyl-5-(3,3-dimethyloxiran-2-yl)pent-2-enal 9

Yellow oil. Yield: 85%. ESI (m/z): 169 $[M + H]^+$ Anal. calcd for $C_{10}H_{16}O_2$: C 71.39, H 9.59. Found C 71.43, H 9.56. Spectroscopic data are in agreement with literature (Elgendy and Khayyat 2008; Yarovaya et al. 2002).

(*Z*)-3-methyl-5-(3,3-dimethyloxiran-2-yl)pent-2-enal 10

Yellow oil. Yield: 91%. ESI (m/z): 169 $[M + H]^+$. Anal. calcd for $C_{10}H_{16}O_2$: C 71.39, H 9.59. Found C 71.44, H 9.58. Spectroscopic data are in agreement with literature (Elgendy and Khayyat 2008; Yarovaya et al. 2002).

(*E*)-3-methyl-3-(2-(3,3-dimethyloxiran-2-yl)ethyl) oxirane-2-carbaldehyde 11

Prepared as described for 6,7 epoxides using an excess of MCPBA 77% and following oxidation of alcohol with a solution of Py-SO_3 in DMSO. Yellow oil. Yield: 86%. ESI (m/z): 184 $[M + H]^+$. Anal. calcd for $C_{10}H_{16}O_3$. C 65.19, H 8.75. Found C 65.27, H 8.72. Spectroscopic data are in agreement with literature (Escande et al. 2015)

(*Z*)-3-methyl-3-(2-(3,3-dimethyloxiran-2-yl)ethyl) oxirane-2-carbaldehyde 12

Prepared as described for 6,7 epoxides using an excess of MCPBA 77% and following oxidation of alcohol with a solution of Py-SO_3 in DMSO. Yield: 81%. ESI (m/z): 184 $[M + H]^+$. Anal. calcd for $C_{10}H_{16}O_3$. C 65.19, H 8.75. Found C 65.25, H 8.74. Spectroscopic data are in agreement with literature (Escande et al. 2015).

(*S*)-3-methyl-5-(3,3-dimethyloxiran-2-yl)pentanal 16

Prepared as described for 6,7 epoxides. Yield: 95%. ESI (m/z): 171 $[M+H]^+$. $[\alpha]_D^{20}$: +45(c 0.6, $CHCl_3$). Spectroscopic data are in agreement with literature (Yarovaya et al. 2002).

(*R*)-3-methyl-5-(3,3-dimethyloxiran-2-yl)pentanal 17

Prepared as described for 6,7 epoxides. Yield: 96%. ESI (m/z): 171 $[M + H]^+$. $[\alpha]_D^{20}$: −43 (c 0.6, $CHCl_3$). Spectroscopic data are in agreement with literature (Yarovaya et al. 2002).

General procedure for the synthesis of compounds 18 and 19

A mixture of 5% Pd/C (15.6 mg, 5 mol %), citronellal (151.2 g, 0.98 mmol), and ethyl acetate (12 mL) was stirred at room temperature for 2 h under an H_2 atmosphere. The reaction mixture was filtered through celite and the filtrate was concentrated under vacuum to afford 20 as colorless liquid.

(*S*)-3,7-Dimethyloctanal 18

Yield: 99%. ESI (m/z): 157 [M + H]+. $[\alpha]_D^{20}$: −74 (c 0.2, $CHCl_3$). Anal. calcd for C $_{10}H_{20}O$. C 76.86, H 12.90. Found: C 76.63, H 12.66. Spectroscopic data are in agreement with literature (Mikami et al. 2011).

(*R*)-3,7-Dimethyloctanal 19

Yield: 99%. ESI (m/z): 157 [M + H]+. $[\alpha]_D^{20}$: +76 (c 0.2, $CHCl_3$). Anal. calcd for C $_{10}H_{20}O$. C 76.86, H 12.90. Found: C 76.63, H 12.60. Spectroscopic data are in agreement with literature (Mikami et al. 2011).

Cell line, cell culture, and treatment

The human pancreatic cancer MIA PaCa-2 cell line and human B-lymphoma DeFew cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Life Technologies, Grand Island, NY, USA), penicillin and streptomycin (P/S, 100 U ml^{-1}) (Lonza, Walkersville, MD, USA) and 2.5% of horse serum (Gibco). The DeFew cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% P/S. HUVECs were purchased from Promocell (Heidelberg, Germany). All experiments were performed on low-passage cell cultures. Cells were grown in endothelial growth medium-2 (EBM-2, FBS 2%, VEGF, R3-IGF-1, hEGF, hFGF, hydrocortisone, ascorbic acid, heparin, and GA-1000) (Clonetics, Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA). All cell lines were grown at 37 °C in a 5% CO_2 atmosphere.

Determination of cell viability

The viability of cells was determined by MTT test to detect functional mitochondria in living cells (Mosmann 1983). Functional mitochondria can transform MTT to formazan salts, which can be measured with a spectrophotometer. The proportion of viable cells in treated samples was compared with control samples. MIA PaCa-2 cells (5×10^3/well) and DeFew (20×10^4/well) cells were plated on 96-well microtiter plates to a final volume of 200 µl. After 72 h of incubation with different concentrations of tested compounds (12.5, 25, 50, 100, 200, 400 µM) at 37 °C, 25 µl of MTT (5 mg/ml) were added to each well and the cells were incubated for an additional 3 h. Thereafter, cells were lysed with 100 µl of a solution containing 50% (v/v) N,N-dimethylformamide, 20% (w/v) SDS (pH 4.5) to allow solubilization of dark blue crystals. The optical density of each well was measured with a microplate spectrophotometer (TitertekMultiskan MCC/340, Titertek Berthold, Huntsville, AL) equipped with a 620 nm filter. Cells viability in response to treatment with compounds was calculated as % viable cells = (OD treated/OD control)×100.

Determination of half maximal inhibitory concentration (IC_{50}) of drugs

The half maximal inhibitory concentration is a measure of the effectiveness of a compound in inhibiting biochemical processes and biological functions. According to the in vitro MTT assay, the IC_{50} represents the concentration of the tested drugs that is required for 50% inhibition of the cell viability. Based on the obtained data using in vitro MTT assay, the IC_{50} values was calculated separately at 72 h after that MIA PaCa-2 cells, DeFew and HUVECs cells were exposed to the agents. To determine the IC_{50} values, the concentration range used of each compounds was from 12.5–400 µM. IC_{50} values were then calculated from cell

viability dose–response curves (linear regression analysis) and defined as the concentration resulting in 50% inhibition in cells survival as compared to controls (cells treated with DMSO 0.4%). The stock solutions of the compounds made in DMSO was 100 mM. The final DMSO concentration at the highest compounds concentrations was 0.4%. This DMSO concentration did not affect cell viability.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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