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Cytotoxic mechanism of *Piper gaudichaudianum* Kunth essential oil and its major compound nerolidol

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ABSTRACT

Piper gaudichaudianum Kunth is used in popular medicine as anti-inflamatory and against liver disorders. One of the most studied components of the plant is the essential oil for which chemical analysis revealed (*E*)-nerolidol as major compound. Recently, we have shown that *P. gaudichaudianum* essential oil possesses strong cytotoxic effects in mammalian V79 cells. The aim of this study was to analyze the cytotoxicity and mutagenicity of *P. gaudichaudianum* essential oil and nerolidol using *Saccharomyces cerevisiae* as model study. Treatment of the XV185-14c and N123 strains with essential oil and nerolidol led to cytotoxicity but did not induce mutagenicity. Our results revealed an important role of base excision repair (BER) as the *ntg1*, *ntg2*, *apn1* and *apn2* mutants showed pronounced sensitivity to essential oil and nerolidol. In the absence of superoxide dismutase (in *sod1*Δ mutant strain) sensitivity to the essential oil and nerolidol increased indicating that this oil and nerolidol are generating reactive oxygen species (ROS). The ROS production was confirmed by DCF-DA probing assay in Sod-deficient strains. From this, we conclude that the observed cytotoxicity to *P. gaudichaudianum* essential oil and nerolidol is mainly related to ROS and DNA single strand breaks generated by the presence of oxidative lesions.

1. Introduction

Piper gaudichaudianum Kunth is a plant that belongs to the Piperaceae family. This family is composed of 7 genera and approximately 1100 species. The genus *Piper* (Piperaceae) is well known for its aromatic herbs, which are largely distributed in tropical and subtropical regions of the world. Some Piper species are also employed in folk medicine as analgesics for pain management, toothaches and wound treatment (Guerrini et al., 2009). *P. gaudichaudianum* Kunth is widely distributed in the Brazilian Atlantic forest, from the Northeast to southern of Brazil, and reaching Argentina and Paraguay. This plant is popularly known by the names "Iaborandi", "Jaborandi", "Paripaioba" and "Pariparoba" (Di Stasi and Hiruma-Lima, 2002).

According to popular medicine, the leaves infusion and fresh leaves of *P. gaudichaudianum* are used to relieve toothache, while the fresh roots are used as anti-inflammatory and against liver disorders (Di Stasi and Hiruma-Lima, 2002). Other previously described biological activities using different plant material from this species include fungicidal and larvicidal effects (Lago et al., 2004; Morais et al., 2007) as well as anti-inflammatory and analgesic activities of leaf extracts (Di Stasi and Hiruma-Lima, 2002; Moreira et al., 2001). Furthermore, the essential oil of *P. gaudichaudianum* leaves has anti-inflammatory and larvicidal activity (Morais et al., 2007). We have recently demonstrated that this essential oil has strong cytotoxic, genotoxic and mutagenic effects in mammalian V79 cells, and these effects are likely related to its oxidative potential (Péres et al., 2009).

The previously published studies on *P. gaudichaudianum* have presented analyses of its leaves and its essential oil phytochemical

Abbreviations: BER, *Saccharomyces cerevisiae* strains defective in base excision repair; Can, canavanine; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethylsulfoxide; GC × GC/TOF-MS, Comprehensive two dimensional gas chromatography/time-of-flight mass spectrometer analyses; GX X GC, Comprehensive two-dimensional gas-chromatography; H_2O_2, hydrogen peroxide; His, histidine; Hom, homoserine; HR, *Saccharomyces cerevisiae* strains defective in homologous recombination; Lys, lysine; MM, minimal medium; NER, *Saccharomyces cerevisiae* strains defective in nucleotide excision repair; NHEJ, *Saccharomyces cerevisiae* strains defective in non-homologous end-joining; 4-NQO, 4 nitroquinoline-oxide; OH·, hydroxyl radical; PRR, *Saccharomyces cerevisiae* strains defective in post-replication repair; ROOH, hydroperoxides; ROS, reactive oxygen species; SC, synthetic complete medium; TLS, *Saccharomyces cerevisiae* strains defective in translesion synthesis; TIC, typical two-dimensional separation/total ion chromatogram; YPD, Complete liquid medium.

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profile, reporting triterpenes, flavonoids and alkaloids as the major constituent compounds. Among the terpenes identified were (*E*)-nerolidol, d-limonene, linalool, β-caryophyllene, α-humulene and cymene (Andrade et al., 1998; Péres et al., 2009; Poser et al., 1994; Rorig and Poser, 1991).

(*E*)-nerolidol, one of the major sesquiterpenes present in the essential oil of *P. gaudichaudianum* that was identified previously by our research group (Péres et al., 2009), is used to enhance flavor and aroma, is approved by the U.S. Food and Drug Administration, and has been studied as a topical skin penetration enhancer for the transdermal delivery of therapeutic drugs (Lapczynski et al., 2008; Williams and Barry, 2004). Studies related to the biological activity of nerolidol showed an antifungal effect against *Microsporum gypseum* (Lee et al., 2007), activity against L3 larvae of *Anisakis* type I (Navarro-Moll et al., 2011), and antimalarial (Lopes et al., 1999), antileishmanial and antiulcer activities (Arruda et al., 2005; Klopell et al., 2007). In addition, (*E*)-nerolidol and α-humulene have been shown to be cytotoxic on renal cell adenocarcinoma ACHN, the hormone-dependent prostate carcinoma LNCaP, the amelanotic melanoma C32 and the MCF-7 breast cancer cell line (Legault et al., 2003; Loizzo et al., 2007a,b; Sylvestre et al., 2007).

In view of the absence of knowledge about the exact cytotoxic mechanisms of *P. gaudichaudianum* essential oil, the aim of the present study was to investigate the cytotoxic effects of the essential oil and compound nerolidol (a racemic mixture of *cis* and *trans* isomers) using *Saccharomyces cerevisiae* strains deficient in the major DNA repair proteins. Induced mutagenesis was also tested in the haploid XV185-14c and N123 *S. cerevisiae* strains. The oxidative potential of the essential oil and nerolidol was also estimated by cytotoxic assay with superoxide dismutase and catalase yeast deficient strains and 2′,7′-dichlorofluorescein (DCF) fluorescent assay, which detects intracellular ROS generation. In addition, we performed a more detailed chemical analysis of *P. gaudichaudianum* essential oil by comprehensive two dimensional gas chromatography/time-of-flight mass spectrometer analyses (GC × GC/TOF-MS).

2. Materials and methods

2.1. Chemicals

Amino acids (L-histidine, L-threonine, L-methionine, L-tryptophan, L-leucine, L-lysine, L-arginine), 4 nitroquinoline-oxide (4-NQO), hydrogen peroxide (H_2O_2), L-canavanine, nitrogen bases (adenine and uracil), 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Yeast extract, bacto-peptone, bacto-agar, yeast nitrogen base with no amino acids and glucose were acquired from Difco Laboratories (Detroit, MI, USA). Nerolidol (a racemic mixture of cis and trans isomers) used in the analytical and biological assays was also acquired from Sigma–Aldrich.

2.2. Sample

Leaves from *P. gaudichaudianum* were collected in Riozinho (Rio Grande do Sul province, Brazil) in February, 2010. The voucher specimen was identified and deposited at the Herbarium of the Department of Botany, Federal University of Rio Grande do Sul, Porto Alegre, Brazil (Voucher No.: ICN 128412). The leaves of *P. gaudichaudianum* were air-dried and the oil was obtained by hydrodistillation process for 4 h, using a Clevenger type apparatus, in accordance with the method recommended by British Pharmacopoeia (2011), producing 0.55% (w/v) of essential oil. The distilled oil was dried over anhydrous sodium sulfate and stored in closed dark vials at 4 °C until use. The oil was yellow and had a distinct sharp odor. The essential oil and the standard nerolidol were diluted (1:100 v/v) in hexane prior to GC × GC/TOF-MS.

2.3. Analysis by GC × GC/TOF-MS

The GC × GC/TOF-MS analyses were performed using an Agilent 6890GC system with an Pegasus III TOFMS analyzer (LECO Corporation, St. Joseph, MI, USA). The primary GC column was DB-5 (methyl silicon wit 5% of phenyl substituted groups)

Table 1
Saccharomyces cerevisiae strains used in this study. BER: base excision repair; NER: nucleotide excision repair; HR: homologous recombination; NHEJ: non-homologous end-joining; TLS: translesion synthesis.

Strain	Genotype	DNA repair pathway affected	Source
BY4741 (WT)	*MATα*; *his*3Δ1; leu2Δ0; *met*15Δ0; *ura*3Δ	–	Euroscarf[a]
apn1Δ	BY4741; with *apn1*::*kanMX4*	BER	Euroscarf
rad1Δ	BY4741; with *rad1*::*kanMX4*	NER	Euroscarf
rad10Δ	BY4741; with *rad10*::*kanMX4*	NER	Euroscarf
rad18Δ	BY4741; with *rad18*::*kanMX4*	PRR	Euroscarf
rad30Δ	BY4741; with *rad30*::*kanMX4*	TLS	Euroscarf
rad50Δ	BY4741; with *rad50*::*kanMX4*	NHEJ	Euroscarf
rad52Δ	BY4741; with *rad52*::*kanMX4*	HR	Euroscarf
rev1Δ	BY4741; with *rev1*::*kanMX4*	TLS	Euroscarf
rev3Δ	BY4741; with *rev3*::*kanMX4*	TLS	Euroscarf
ku70Δ	BY4741; with *ku70*::*kanMX4*	NHEJ	Euroscarf
SJR751 (WT)	*MATα*; *ade*2-101$_{oc}$; *his*3Δ200; *ura*3ΔNco; *lys*2Δ*Bgl*; *leu*2-R	–	RL Swanson[b]
ntg1Δ	SJ751; with *ntg1*::LEU2	BER	RL Swanson
ntg2Δ	SJ751; with *ntg2*::*his*G	BER	RL Swanson
ntg1Δ ntg2Δ	SJ751; with *ntg1*::LEU2 *ntg2*::*his*G	BER	RL Swanson
ntg1Δ ntg2Δ apn1Δ	SJ751; with *ntg1*::LEU2 *ntg2*::*his*G *apn1*::*HIS3*	BER	RL Swanson
ntg1Δ ntg2Δ apn1Δ rad1Δ	SJ751; with *ntg1*::LEU2 *ntg2*::*his*G *apn1*::HIS3 *rad1*::*his*G	BER/NER	RL Swanson
ntg1Δ ntg2Δ apn1Δ rev3Δ	SJ751; with *ntg1*::LEU2 *ntg2*::*his*G *apn1*::HIS3 *rev3*::*kanMX4*	BER/TLS	RL Swanson
ntg1Δ ntg2Δ apn1Δ rad52Δ	SJ751; with *ntg1*::LEU2 *ntg2*::*his*G *apn1*::HIS3 rad52::*URA3*	BER/HR	RL Swanson
EG103 (SOD-WT)	MATα; leu2-3 112his3-Δ1 trp1-289 ura3-52	–	E Gralla[c]
EG118 (*sod1*Δ)	like EG103, except *sod1*::URA3	–	E Gralla
EG110 (*sod2*Δ)	like EG103, except *sod2*::TRP1	–	E Gralla
EG133 (sod1Δ sod2Δ)	like EG103, except *sod1*::URA3 e *sod2*::TRP1	–	E Gralla
EG223 (*ctt1*Δ)	like EG103, except *ctt1*::TRP1	–	E Gralla
EG (sod1Δ ctt1Δ)	like EG103, except *sod1*::URA3 e *ctt1*::TRP1	–	E Gralla
XV185-14c	*MATα*; *ade*2-2; *arg*4-17; *his*1-7; *lys*1-1; *trp*5-48; *hom*3-10	–	RC Von Borstel[d]
N123	MATa; his1-7	–	JAP Henriques[e]

[a] Strains obtained from Euroscarf, Frankfurt, Germany.
[b] RL Swanson, Atlanta, Georgia, USA.
[c] E Gralla, Los Angeles, California, USA.
[d] RC Von Borstel, Edmonton, Alberta, Canada.
[e] JAP Henriques, Porto Alegre, Rio Grande do Sul, Brazil.

Table 2
Fifty six main volatile components identified in the essential oil of *Piper gaudichaudianum* leaves using GC × GC-TOFMS.

Peak	T_R 1st D* (min)	T_R 2nd D** (min)	Compound name	Library formula	Library CAS≠	Library molecular weight	Library similarity	Library reverse match factor	Percent response
1	6.70	2.35	Limonene	$C_{10}H_{16}$	138-86-3	136	883	886	0.05
2	7.63	2.72	Linalool	$C_{10}H_{18}O$	22564-99-4	154	874	876	0.04
3	9.63	3.72	Terpineol	$C_{10}H_{18}O$	10482-56-1	154	857	882	0.01
4	15.90	0.04	α-Copaene	$C_{15}H_{24}$	3856-25-5	204	908	910	0.01
5	16.50	0.21	β-Elemene	$C_{15}H_{24}$	515-13-9	204	916	939	0.71
6	17.30	0.37	α-Gurjunene	$C_{15}H_{24}$	489-40-7	204	887	890	0.14
7	17.63	0.23	α-Santalene	$C_{15}H_{24}$	512-61-8	204	882	883	0.33
8	17.63	0.58	Aristolene	$C_{15}H_{24}$	27862-07-3	204	841	850	0.02
9	17.77	0.66	*E*-caryophyllene	$C_{15}H_{24}$	87-44-5	204	966	966	7.51
10	18.10	0.60	β-Copaene	$C_{15}H_{24}$	18252-44-3	204	894	897	0.20
11	18.23	0.54	γ-Elemene	$C_{15}H_{24}$	339154-91-5	204	862	862	0.06
12	18.30	0.25	Trans-α-bergamotene	$C_{15}H_{24}$	26560-14-5	204	893	901	0.23
13	18.57	0.69	Aromadendrene	$C_{15}H_{24}$	489-39-4	204	927	927	2.92
14	19.03	0.90	Trans-muurola-3,5-diene	$C_{15}H_{24}$	189165-77-3	204	787	820	0.06
15	19.30	1.02	α-Humulene	$C_{15}H_{24}$	6753-98-6	204	923	924	21.32
16	19.43	0.64	β-Santalene	$C_{15}H_{24}$	511-59-1	204	903	903	1.08
17	19.57	0.98	Alloaromadendrene	$C_{15}H_{24}$	25246-27-9	204	928	933	3.28
18	19.90	0.98	Drima-7,9(11)-diene	$C_{15}H_{24}$	0-00-0	204	899	899	0.34
19	20.10	0.94	γ-Gurjunene	$C_{15}H_{24}$	22567-17-5	204	821	821	0.23
20	20.17	0.96	γ-Muurolene	$C_{15}H_{24}$	30021-74-0	204	861	863	0.85
21	20.23	0.67	γ-Curcumene	$C_{15}H_{24}$	28976-68-3	204	799	800	0.12
22	20.37	2.35	Germacrene D	$C_{15}H_{24}$	23986-74-5	204	827	914	0.03
23	20.70	1.22	β-Selinene	$C_{15}H_{24}$	17066-67-0	204	919	919	3.43
24	21.10	1.20	α-Selinene	$C_{15}H_{24}$	473-13-2	204	925	927	2.67
25	21.17	1.38	Bicyclogermacrene	$C_{15}H_{24}$	24703-35-3	204	923	923	13.16
26	21.23	1.13	α-Muurolene	$C_{15}H_{24}$	31983-22-9	204	853	855	0.73
27	21.50	0.68	E,E-α-farnesene	$C_{15}H_{24}$	502-61-4	204	928	930	1.31
28	21.57	1.21	δ-Amorphene	$C_{15}H_{24}$	189165-79-5	204	854	854	0.21
29	21.57	1.40	Germacreno A	$C_{15}H_{24}$	28387-44-2	204	835	837	0.12
30	21.70	0.95	β-Curcumene	$C_{15}H_{24}$	28976-67-2	204	818	820	0.52
31	21.90	1.37	γ-Cadinene	$C_{15}H_{24}$	39029-41-9	204	893	893	1.27
32	22.10	1.52	7-Epi-α-Selinene	$C_{15}H_{24}$	123123-37-5	204	812	812	0.16
33	22.30	1.32	δ-Cadinene	$C_{15}H_{24}$	483-76-1	204	901	903	1.47
34	22.43	1.49	Zonarene	$C_{15}H_{24}$	95910-36-4	204	820	888	0.01
35	22.70	1.07	γ-E-bisabolene	$C_{15}H_{24}$	53585-13-0	204	851	851	0.21
36	22.97	1.45	α-Cadinene	$C_{15}H_{24}$	82468-90-4	204	846	846	0.15
37	23.23	2.44	α-Colacorene	$C_{15}H_{20}$	21391-99-1	200	884	884	0.16
38	23.57	1.84	Elemol	$C_{15}H_{26}O$	639-99-6	222	839	840	0.27
39	23.97	1.93	Germacreno B	$C_{15}H_{24}$	15423-57-1	204	882	886	0.37
40	24.03	1.30	*E*-nerolidol	$C_{15}H_{26}O$	142-50-7	222	931	919	22.06
41	24.50	1.87	Ledol	$C_{15}H_{26}O$	577-27-5	222	824	850	0.04
42	25.03	2.55	Spathulenol	$C_{15}H_{24}O$	77171-55-2	220	867	879	1.97
43	25.23	2.69	Caryophyllene oxide	$C_{15}H_{24}O$	1139-30-6	220	861	865	0.01
44	25.30	2.65	Globulol	$C_{15}H_{26}O$	51371-47-2	222	810	810	0.16
45	26.23	2.36	Viridiflorol	$C_{15}H_{26}O$	552-02-3	222	822	833	0.18
46	26.50	2.97	Humulene epoxide II	$C_{15}H_{24}O$	19888-34-7	220	835	836	2.27
47	26.77	2.30	1,10-Di-epi-cubenol	$C_{15}H_{26}O$	73365-77-2	222	824	824	0.09
48	27.63	2.91	Epoxy-allo alloaromadendrene	$C_{15}H_{24}O$	85760-81-2	220	758	818	0.00
49	27.77	3.04	Caryophylla-4(14),8(15)-dien-5-α-ol	$C_{15}H_{24}O$	147235-15-2	220	867	870	0.15
50	28.03	3.22	α-Muurolol	$C_{15}H_{26}O$	19912-62-0	222	732	903	0.02
51	28.63	2.87	α-Cadinol	$C_{15}H_{26}O$	481-34-5	222	816	818	0.26
52	28.70	3.19	Intermedeol	$C_{15}H_{26}O$	5945-72-2	222	862	862	0.45
53	29.37	2.23	α-Bisabolol	$C_{15}H_{26}O$	15352-77-9	222	827	827	0.18
54	29.90	3.30	Z-α-Trans-bergamotol	$C_{15}H_{24}O$	88034-74-6	220	764	770	0.09
55	30.17	2.34	α-Bisabolol	$C_{15}H_{26}O$	515-69-5	222	734	874	0.02
56	30.43	3.30	Z,E-farnesol	$C_{15}H_{26}O$	3790-71-4	222	785	786	0.12
									93.86

* T_R 1st D – Retention times on the first dimension.
** T_R 2st D – Retention times on the second dimension.

(60 m × 0.25 mm × 0.1 μm) and secondary column used was DB-17MS (methyl silicon wit 50% of phenyl substituted groups) (2.15 m × 0.18 mm × 0.18 μm), both from J&W-Agilent (Folsom, CA, USA). The primary oven temperature program was 105 °C (0.2 min)–2 °C/min–200 °C, the secondary oven with 10 °C offset. Modulation time was 4 s, with a 1.6 s hot pulse and 0.4 s cold pulse in each stage. The carrier gas used was Helium (from White Martins, 99.999% purity) with constant flow rate of 1.0 mL/min, injector with split rate of 1:50, temperature for the injector, the transfer line and the ion source were set at 250 °C, 250 °C and 250 °C, respectively. The acquisition rate 100 s/s and mass range of *m/z* 50-450. The components were quantified by LECO Corp ChromaTOFTM version 3.1. All peaks with signal-to-noise ratio higher than 100 were found in the raw GC × GC chromatogram. The workstation automatically gave the parameters such as similarity and reverse of peaks through comparing them with the compounds in the library. The results were combined in a peak table. The NIST/EPA/NIH Mass Spectral Library Version 2.0 was used in this work.

2.4. Yeast strains and media

The relevant genotypes of the *S. cerevisiae* strains used in this work are indicated in Table 1. Yeast media used were the complete liquid medium (YPD), minimal medium (MM) and synthetic complete medium (SC) described in Burke et al. (2000). For mutagenesis of the XV185-14c strain, the omission media, lacking lysine (SC-lys), histidine (C-his) or homoserine (SC-hom), was supplemented with 0.1 mg lysine, histidine, or methionine, respectively, per 100 mL of MM (Von Borstel et al., 1971). For the determination of forward mutagenesis in strain N123, plates lacking arginine were supplemented with 60 μg/mL canavanine (SC + can).

2.5. Yeast growth conditions and treatment

Stationary- and exponential phase cultures were used, and the cell concentration and budding percentage was performed by microscopic counts using a Neubauer chamber. The XV185-14c yeast strain was used to determine the concentrations of the *P. gaudichaudianum* essential oil and nerolidol to be tested in this study, based on the recommendations of the *OECD Guidelines for the Testing of Chemicals* (Genetic Toxicology: *Saccharomyces cerevisiae*, Gene Mutation Assay: No. 480). For all treatments, DMSO stock solutions of *P. gaudichaudianum* and nerolidol were prepared immediately prior to use so that the DMSO concentration in the treatment never exceeded 0.2%. The appropriate concentrations were obtained by dilution of stock solutions in distilled water. The solvent controls included in the tests were found to be negative. The 4-NQO (0.5 μg/mL) and H_2O_2 (4 mM) were used as positive control in the mutagenic and DCF-DA assays, respectively.

2.6. Detection of reverse and frameshift mutations in the XV185-14c haploid strain

Suspensions of 2×10^8 cells/mL in stationary or exponential growth phases were incubated in phosphate-buffered saline solution (PBS; Na_2HPO_4, KH_2PO_4 and KCl; 20 mM; pH 7.4) for mutagenic evaluation under non-growth conditions, or in fresh synthetic media for mutagenic evaluation under growth conditions, with different concentrations of *P. gaudichaudianum* essential oil or nerolidol (0.5, 1, 10, 25, 50 and 100 μg/mL) at 30 °C for 18 h. Survival was determined on SC (3–5 days, 30 °C) and mutation induction (LYS, HIS or HOM revertants) on the appropriate omission media (7–10 days, 30 °C). While *his1-7* is a non-suppressible missense allele and reversions result from mutation at the locus itself (Snow, 1978), *lys1-1* is a suppressible ochre nonsense mutant allele (Hawthorne, 1969) that can be reverted either by a locus-specific mutation or by a forward mutation in a suppressor gene (Von Borstel et al., 1971). The two mutation types at the *lys1-1* locus were differentiated according to Schuller and von Borstel (1974). It is believed that *hom3-10* contains a frameshift mutation due to its response to a range of diagnostic mutagens (Von Borstel et al., 1971).

2.7. Detection of forward mutations in the N123 strain

Yeast cells in stationary or exponential growth phase (2×10^8 cells/mL) were treated for 18 h at 30 °C with *P. gaudichaudianum* essential oil or nerolidol at increased concentrations varying from 0.5 to 100 μg/mL. After treatment, appropriate dilutions of cells were plated onto SC plates to determine cell survival and were then plated onto SC media supplemented with canavanine (60 μg/mL) in order to determine the presence of a forward mutation in the *CAN1* locus. Plates were incubated in the dark at 30 °C for 3–5 days before assaying survival and counting the revertant colonies.

2.8. Survival assays

The relative sensitivity of the DNA repair pathway-defective strains was assayed by treating 1×10^8 cells/mL exponential cultures in PBS with various concentrations of *P. gaudichaudianum* essential oil or nerolidol (0.5, 1, 10, 25, 50 and 100 μg/mL), followed by incubation with aeration by rotary shaking at 30 °C for 18 h. The strains deficient in superoxide dismutase and/or catalase were treated with *P. gaudichaudianum* essential oil or nerolidol in PBS at 30 °C for 1 h with agitation. To determine colony-forming ability, suitable aliquots were plated on YPD. Plates were incubated at 30 °C for 2–4 days before the surviving colonies were counted.

2.9. Determination of reactive oxygen species (ROS) generation

The DCFH-DA assay was used to estimate the intracellular generation of ROS. This assay is a reliable method for measuring intracellular ROS such as H_2O_2, hydroxyl radical ($OH^{\cdot}$), and hydroperoxides (ROOH). The dye is a non-polar compound that readily penetrates into cells. Intracellular peroxides oxidize DCFH-DA to a highly fluorescent compound (2′,7′-dichlorofluorescein; DCF). After treatment with *P. gaudichaudianum* essential oil or nerolidol for 1 h at 30 °C, SOD-WT, *sod1*Δ and *sod2*Δ yeast cells were washed and incubated with 10 μM DCFH-DA for 30 min at 37 °C in the dark. The H_2O_2 (4 mM) was used as positive control. After incubation, the cells were washed with PBS and analyzed within 30 min using FACS Calibur (Becton Dickinson, San Jose, CA, United States). The DCFH-oxidation was measured using 488 nm excitation and 530/30 nm band pass emission filters. As a rule, 10,000 cells were counted in each experiment. The Cell Quest software (Becton Dickinson) was used to calculate the mean fluorescence. Data were expressed as percentages of control values.

2.10. Statistics

All experiments were independently repeated at least three times, with triplicate samples for each treatment. Data were expressed as means ± standard deviation (S.D.) values. Statistical analyses of the data were performed using ANOVA One-Way, and the means were compared using Dunnett's multiple comparison test. *P*-values less than 0.05 were considered to be significant.

3. Results

3.1. Analysis of *P. gaudichaudianum* essential oil and nerolidol by GC × GC/TOF-MS

In the GC × GC system, compounds are separated by volatility difference on the first dimension non-polar column, and by polarity on the second medium-polar column. The composition of the volatile fraction obtained from *P. gaudichaudianum essential oil* leaves using the GC × GC/TOF-MS technique is summarized in Table 2. Identification based on NIST/EPA/NIH library mass spectra search using similarity and reverse factors was above 731 and 769, respectively. This technique is based on the application of two GC columns coated with different stationary phases connected in series through a special interface called modulator. The modulator is the heart of the instrument because it ensures that the separation is both comprehensive and multidimensional (Welke et al., 2012). The addition of the TOFMS provides a sensitive detector with full-scan MS capability and a high data density in the 2D separation space. The combination of GC × GC/TOF-MS has been shown to be very useful for many complex samples (Von Muhlen et al., 2008). Fifty six volatile compounds from the essential oil of *P. gaudichaudianum* leaves were identified. The essential oil is characterized by high percentages of sesquiterpenes. In this study, the percentages of (*E*)-nerolidol and α-humulene found in the essential oil were 22.06% and 21.32%, respectively.

A typical two-dimensional separation/total ion chromatogram (TIC) of the essential oil is shown in Fig. 1 and the nerolidol chromatogram (a mixture of *cis*- and *trans*-nerolidol from Sigma–Aldrich) is shown in Fig. 2A. The chromatographic peak data consist of first dimension retention times, second dimension retention times and peak volume (TIC). The GC × GC chromatogram is constructed as a rasterized image of the TIC computed from each secondary chromatogram. Fig. 2B compares the mass spectrum of the blod (peak) at 24.03 min in the sample of essential oil, the sample of standard and the mass spectrum of (*E*)-nerolidol from the NIST/EPA/NIH library mass spectra and confirmed that in *P. gaudichaudianum* essential oil has only *trans*-nerolidol, according to retention time.

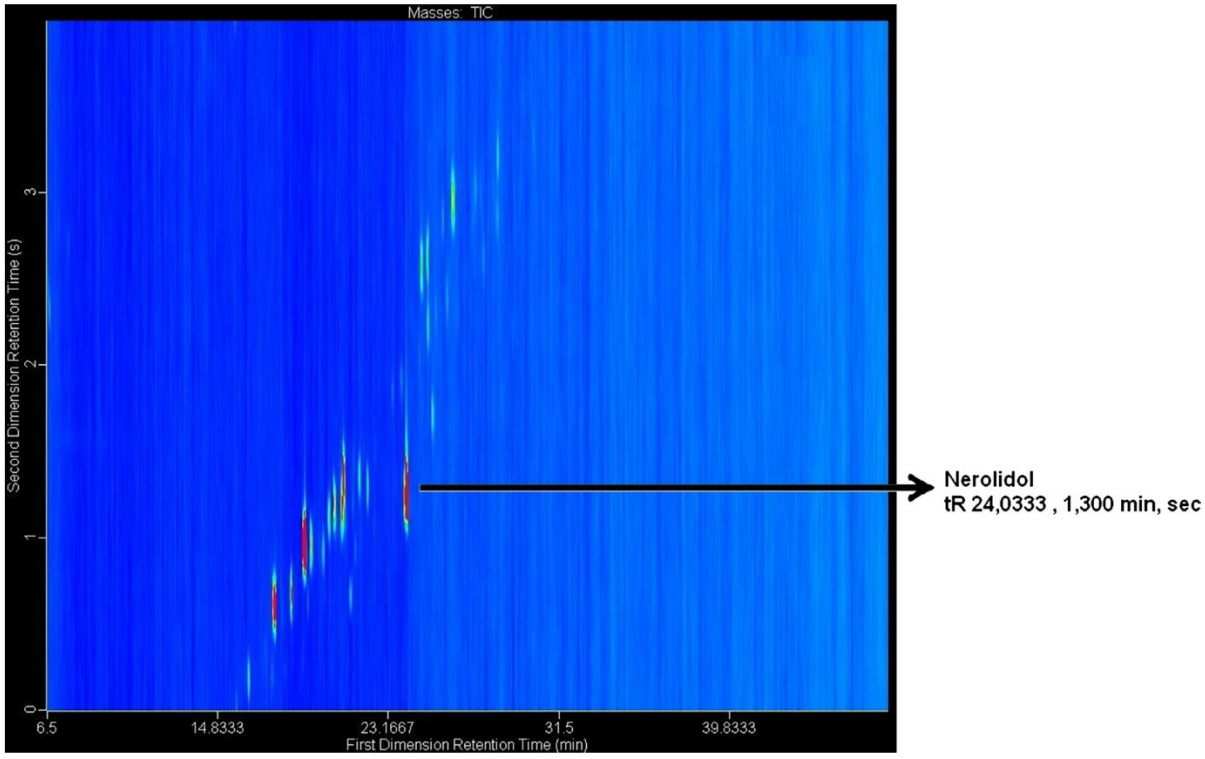

Fig. 1. GC × GC-TOFMS total ion current chromatogram (TIC) data color plot of *P. gaudichaudianum* essential oil. Detail of a 2D-GC chromatogram from 6.5 to 45 min.

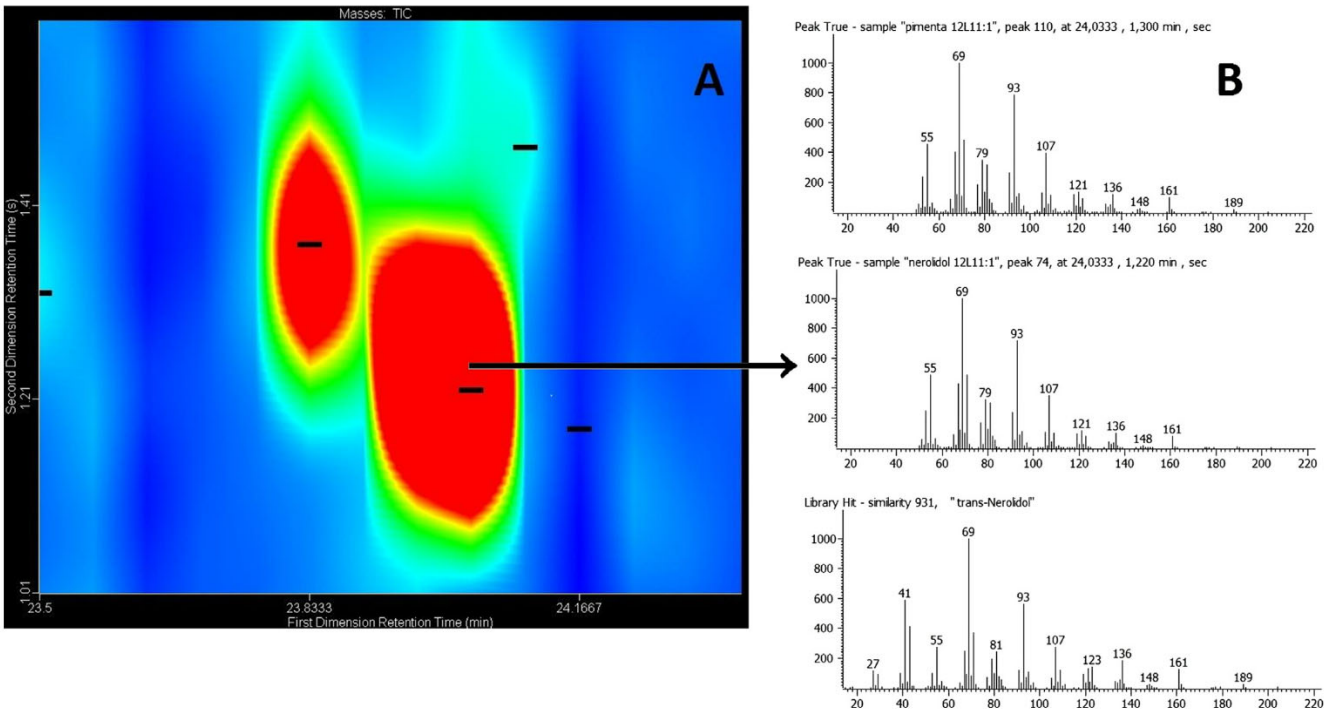

Fig. 2. Expansion of a part of the GC × GC contour plot of standard nerolidol (a racemic mixture of *cis* and *trans* isomers). (A) Detail of a 2D-GC chromatogram from 23.5 to 25 min. (B) Spectra of a majority peak in the sample, spectra of an identification peak in the standard and in the NIST library, respectively. The experimental conditions are given in the text.

3.2. Cytotoxicity and detection of induced mutations

As shown in Table 3, *P. gaudichaudianum* essential oil induced dose-dependent cytotoxic effects both in stationary and exponential phase cultures in the XV185-14c strain, with a significant effect at the concentrations of 50 and 100 μg/mL. The essential oil induced a significant increase in mutation frequencies only at the *lys1* locus at the highest concentration and in cultures treated at the

exponential phase of growth. Nerolidol was cytotoxic in XV185-14c at concentration of 25, 50 and 100 μg/mL and did not demonstrate any induction of mutagenicity at the three loci evaluated (Table 4). To complement this study, we used the N123 strain to evaluate the cytotoxicity as well as the induction of forward mutations. In this strain, we did not observe any mutagenic effect for either the essential oil (Table 5) or nerolidol (Table 6), but the cytotoxic effects were pronounced after nerolidol treatment. The treatments under growth conditions were always more cytotoxic for both samples, independent of the tested strain. At the highest concentration (100 μg/mL) used, nerolidol induced cytotoxicity that was at least five times higher than that induced by the essential oil of *P. gaudichaudianum*, regardless of the strain tested.

3.3. Cytotoxicity effects in S. cerevisiae DNA repair-defective strains

Due to the high toxicity observed, we decided to evaluate the interaction of DNA repair pathways in response to the *P. gaudichaudianum* essential oil and nerolidol-induced DNA damage. Therefore, we performed survival assays to compare the sensitivities of single, double, triple and quadruple mutants defective in BER, NER, HR, NHEJ, PRR and TLS. Among the BER-deficient strains, *P. gaudichaudianum* essential oil displayed increased cytotoxicity in the single mutant *ntg2*Δ and the double mutant *ntg1*Δ*ntg2*Δ when compared to the isogenic wild-type SJR751 strain (Fig. 3A). Nerolidol induced cytotoxicity to *ntg1*Δ, *ntg1*Δ*ntg2*Δ, and *ntg1*Δ*ntg2*Δ*apn1*Δ mutants increased compared to the isogenic SJR751 (Fig. 3B), as did the cytotoxicity in the single mutant *apn1*Δ in relation to the isogenic wild-type BY4741 strain (Fig. 3B and Fig. 3C). The Ntg1 and Ntg2 proteins are DNA N-glycosylase and apurinic/apyrimidinic (AP) lyase, respectively, and the Apn1 protein is a major apurinic/apyrimidinic endonuclease with 3′-repair diesterase action (Boiteux and Guillet, 2004; Doetsch et al., 2001). The *rad1*Δ and *rad10*Δ strains, which lack the 5′- and 3′-endonucleases of NER (Prakash and Prakash, 2000), showed an intermediate sensitivity to *P. gaudichaudianum* essential oil (Fig. 4A). These single mutants had the same nerolidol sensitivity as the wild-type BY4741 strain (Fig. 4B).

Among the double-strand repair defective strains, the essential oil induced a moderate sensitivity in the *rad50*Δ mutant (Rad50 protein is a subunit of the MRX complex involved in both HR and NHEJ) and did not induce sensitivity in *rad52*Δ (HR) or *ku70*Δ (NHEJ) mutants (Fig. 5A). The *rad50*Δ, *rad52*Δ and *ku70*Δ mutants as well as the isogenic BY4741 strain showed dose-dependent sensitivity to Nerolidol (Fig. 5B and C).

Deletion of the TLS and PRR proteins had no influence on the sensitivity to *P. gaudichaudianum* essential oil (Fig. 6A). Interestingly, nerolidol exhibited a remarkable resistance in several of these mutants, as observed in Fig. 6B: *rev1*Δ, which is deficient in the deoxycytidyl transferase that forms a complex with DNA polymerase ζ in TLS (Lawrence, 2004); *rad30*Δ, which lacks polymerase eta, which is involved in TLS during post-replication repair, catalyzing the synthesis of DNA opposite cyclobutane pyrimidine dimers and other lesions (Ai et al., 2011); and *rad18*Δ, which is deficient in the E3 ubiquitin ligase that forms a heterodimer with Rad6p to monoubiquitinate PCNA in PRR (Prakash et al., 2005). The absence of Rev3, the catalytic subunit of polymerase ζ (Lawrence, 2004), also had a small effect on the sensitivity to nerolidol (Fig. 6B and Fig. 6C).

To evaluate the overlap of different DNA repair pathways, the mutants without DNA repair proteins from BER, NER, HR or TLS were used. *P. gaudichaudianum* essential oil induced slight cytotoxic effects in the quadruple mutants *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ, while the nerolidol induced slight

Table 3
Reversion of point mutation for (*his*1-7), ochre allele (*lys*1-1), and frameshift mutations (*hom*3-10) in haploid XV185-14c strain of *S. cerevisiae* after *P. gaudichaudianum* essential oil treatment at 30 °C for 18 h.

Agent	Treatment	Survival (%)	LYS1/10^7 survivors[b]	HIS1/10^7 survivors[a]	HOM3/10^7 survivors[a]
Treatment of stationary-phase cells under non-growth conditions					
NC[c]	0	100.00 (231)	1.66 ± 0.44 (37)	1.65 ± 0.167 (43)	0.94 ± 0.25 (21)
4-NQO[d]	0.5 μg/mL	59.46 ± 3.46 (137)***	22.84 ± 0.34 (311)***	35.12 ± 3.23 (426)***	21.56 ± 3.59 (287)***
Essential oil	1 μg/mL	96.81 ± 2.37 (221)	1.83 ± 0.76 (40)	2.13 ± 0.55 (43)	1.06 ± 0.34 (22)
	10 μg/mL	89.30 ± 2.10 (209)	1.82 ± 0.43 (37)	2.42 ± 0.31 (45)	1.32 ± 0.47 (26)
	25 μg/mL	73. 07 ± 0.53 (169)	2.57 ± 0.72 (42)	2.61 ± 0.34 (45)	1.66 ± 0.34 (28)
	50 μg/mL	62.20 ± 1.67 (143)*	3.44 ± 1.02 (47)	2.99 ± 1.04 (45)	1.93 ± 0.19 (27)
	100 μg/mL	46.12 ± 5.83 (106)**	4.89 ± 0.93 (53)	3.85 ± 1.41 (38)	2.21 ± 0.20 (24)
Treatment of exponential-phase cells under non-growth conditions					
NC[c]	0	100. 00 (191)	1.64 ± 0.15 (32)	1.46 ± 0.26 (25)	1.42 ± 0.53 (21)
4-NQO[d]	0.5 μg/mL	49.59 ± 2.60 (93)***	19.04 ± 5.76 (207)***	29.82 ± 3.31 (277)***	25.13 ± 5.15 (24)***
Essential oil	1 μg/mL	89.96 ± 1.43 (173)	2.17 ± 0.64 (35)	1.93 ± 0.34 (29)	1.77 ± 0.59 (23)
	10 μg/mL	78.60 ± 1.89 (149)	2.69 ± 0.166 (39)	2.40 ± 0.38 (32)	1.56 ± 0.16 (27)
	25 μg/mL	73.19 ± 0.98 (141)	3.05 ± 0.33 (40)	2.15 ± 0.65 (30)	2.08 ± 0.35 (27)
	50 μg/mL	56. 91 ± 3.62 (105)*	4.27 ± 0.83 (40)	3.02 ± 0.76 (27)	2.17 ± 0.75 (24)
	100 μg/mL	22. 15 ± 2.53 (43)***	6.97 ± 0.27 (31)*	4.75 ± 1.16 (19)	5.29 ± 0.69 (25)
Treatment under growth conditions					
NC[c]	0	100.00 (409)	1.76 ± 0.45 (70)	1.49 ± 0.32 (59)	1.21 ± 0.29 (47)
4-NQO[d]	0.5 μg/mL	36.88 ± 2.57 (149)***	39.9 ± 2.56 (454)***	25.32 ± 3.91 (386)***	27.86 ± 2.88 (245)***
Essential oil	1 μg/mL	83.41 ± 2.17 (342)	3.02 ± 1.10 (95)	1.74 ± 0.58 (65)	1.5 ± 0.68 (49)
	10 μg/mL	75.67 ± 3.28 (306)	4.02 ± 1.00 (117)	1.98 ± 0.94 (68)	1.68 ± 0.41 (51)
	25 μg/mL	64.61 ± 0.34 (264)	5.33 ± 1.65 (134)	2.43 ± 0.90 (71)	1.67 ± 0.44 (46)
	50 μg/mL	42.15 ± 2.59 (170)**	9.63 ± 1.68 (161)*	2.83 ± 1.00 (52)	2.25 ± 1.14 (42)
	100 μg/mL	12.20 ± 2.97 (48)***	22.34 ± 2.97 (105)**	5.42 ± 0.50 (26)	3.79 ± 0.78 (19)

Data represented as mean ± S.D. from three independent experiments (values in parentheses in columns 3–6 refers to number of colonies); significantly different in relation to the negative control group at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA Dunnett's Multiple Comparison Test.

[a] Locus-specific revertants.
[b] Locus non-specific revertants (forward mutation).
[c] Negative control (solvent; DMSO).
[d] Positive control: 4-NQO treatment at 0.5 μg/ml.

Table 4
Reversion of point mutation for (*his*1-7), ochre allele (*lys*1-1), and frameshift mutations (*hom*3-10) in haploid XV185-14c strain of *S. cerevisiae* after nerolidol treatment at 30 °C for 18 h.

Agent	Treatment	Survival (%)	LYS1/10^7 survivors[b]	HIS1/10^7 survivors[a]	HOM3/10^7 survivors[a]
Treatment of stationary-phase cells under non-growth conditions					
NC[c]	0	100.00 (269)	1.40 ± 0.59 (31)	2.35 ± 1.14 (55)	1.87 ± 1.00 (34)
4-NQO[d]	0.5 μg/mL	56.56 ± 2.36(153)***	16.87 ± 2.22 (247)***	20.30 ± 2.05 (305)***	14.18 ± 2.00 (209)***
Nerolidol	1 μg/mL	91.98 ± 2.09(246)	1.76 ± 0.74 (31)	2.87 ± 1.12(63)	14.18 ± 2.00 (33)
	10 μg/mL	79.26 ± 4.88 (210)	2.42 ± 0.84 (40)	3.92 ± 1.46 (75)	2.15 ± 0.91 (33)
	25 μg/mL	70.19 ± 4.33 (189)*	2.16 ± 0.53 (37)	4.34 ± 1.16 (79)	1.97 ± 0.47 (23
	50 μg/mL	43.90 ± 4.52(121)***	1.16 ± 0.99 (14)	4.33 ± 0.91 (66)	1.77 ± 0.03 (12)
	100 μg/mL	8.98 ± 2.38 (106)***	0.24 ± 0.22 (5)	5.41 ± 0.99 (32)	0.42 ± 0.20 (2)
Treatment of exponential-phase cells under non-growth conditions					
NC[c]	0	100. 00 (194)	1.63 ± 0.28 (32)	1.49 ± 0.29 (29)	1.33 ± 0.08 (26)
4-NQO[d]	0.5 μg/mL	54.40 ± 4.18 (106)***	16.06 ± 4.29 (164)***	20.04 ± 5.81(203)***	13.68 ± 2.12 (145)***
Nerolidol	1 μg/mL	86.59 ± 2.26 (169)	2.33 ± 0.14 (39)	2.23 ± 0.58 (37)	1.60 ± 0.30 (27)
	10 μg/mL	80.67 ± 2.13 (157)	2.59 ± 0.11 (41)	2.53 ± 0.76 (39)	1.17 ± 0.15 (18)
	25 μg/mL	64.71 ± 2.10 (126)*	2.66 ± 0.16 (34)	3.24 ± 0.57 (40)	0.94 ± 0.16 (11)
	50 μg/mL	35.33 ± 4.01 (69)***	3.59 ± 1.08 (24)	4.85 ± 1.03 (32)	0.96 ± 0.42 (6)
	100 μg/mL	5.22 ± 1.35 (11)***	4.23 ± 2.20 (5)	5.09 ± 0.95 (6)	0.64 ± 1.11 (1)
Treatment under growth conditions					
NC[c]	0	100.00 (309)	1.00 ± 0.33 (45)	1.62 ± 0.79 (59)	1.0 ± 0.42 (28)
4-NQO[d]	0.5 μg/mL	38.63 ± 2.38 (120)***	17.76 ± 6.20 (239)***	20.11 ± 1.71 (386)***	14.72 ± 3.06 (170)***
Nerolidol	1 μg/mL	87.07 ± 0.923 (270)	1.59 ± 0.42 (49)	2.01 ± 0.87 (65)	1.14 ± 0.50 (28)
	10 μg/mL	76.56 ± 1.54 (237)*	1.78 ± 0.39 (51)	2.27 ± 0.59 (68)	1.46 ± 0.40 (33)
	25 μg/mL	58.30 ± 3.62 (180)**	2.07 ± 0.47 (46)	2.77 ± 1.01 (71)	1.85 ± 0.57 (20)
	50 μg/mL	34.01 ± 5.22 (102)***	1.62 ± 0.74 (38)	3.68 ± 0.66 (52)	2.26 ± 1.17 (22)
	100 μg/mL	2.51 ± 1.99 (7)***	1.11 ± 1.92 (3)	4.86 ± 1.59 (26)	4.05 ± 0.63 (3)

Data represented as mean ± S.D. from three independent experiments (values in parentheses in columns 3–6 refers to number of colonies); significantly different in relation to the negative control group at $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by one-way ANOVA Dunnett's Multiple Comparison Test.
[a] Locus-specific revertants.
[b] Locus non-specific revertants (forward mutation).
[c] Negative control (solvent; DMSO).
[d] Positive control: 4-NQO treatment at 0.5 μg/ml.

Table 5
Induction of forward mutation (*CAN1*) in N123 strain of *S. cerevisiae* after *P. gaudichaudianum* essential oil treatment at 30 °C for 18 h.

Agent	Treatment	Survival (%)	Mutants (10^7 survivors)
Treatment of stationary-phase cells under non-growth conditions			
NC[a]	0	100.00 ± (57)	5.22 ± 2.68 (30)
4-NQO[b]	0.5 μg/mL	39.91 ± 1.69 (26)***	78.50 ± 5.72 (169)***
Essential oil	1 μg/mL	98.35 ± 0.59 (56)	5.41 ± 3.36 (32)
	10 μg/mL	94.98 ± 3.57 (54)	5.59 ± 3.27 (28)
	25 μg/mL	91.78 ± 1.81 (52)	6.33 ± 3.87 (33)
	50 μg/mL	80.46 ± 5.33 (47)	7.17 ± 4.58 (32)
	100 μg/mL	68.52 ± 3.30 (39)**	8.79 ± 6.10 (34)
Treatment of exponential-phase cells under non-growth conditions			
NC[a]	0	100.00 (37)	5.32 ± 1.93 (21)
4-NQO[b]	0.5 μg/mL	30.08 ± 5.30 (12)***	81.79 ± 4.70 (94)***
Essential oil	1 μg/mL	95.91 ± 2.65 (36)	5.59 ± 2.04 (21)
	10 μg/mL	89.36 ± 3.33 (35)	5.94 ± 1.63 (20)
	25 μg/mL	84.70 ± 1.19 (32)	6.346 ± 2.36 (21)
	50 μg/mL	79.74 ± 2.96 (30)	6.49 ± 0. 219 (20)
	100 μg/mL	59.53 ± 3.65 (23)**	6.29 ± 3.14 (15)
Treatment under growth conditions			
NC[a]	0	100.00 (45)	4.32 ± 1.84 (19)
4-NQO[b]	0.5 μg/mL	28.00 2.37 (13)***	96.95 ± 1.10 (121)***
Essential oil	1 μg/mL	96.32 ± 2.97 (43)	4.63 ± 2.19 (20)
	10 μg/mL	93.86 ± 2.91 (42)	5.72 ± 2.70 (24)
	25 μg/mL	88.27 ± 3.38 (39)	5.51 ± 3.05 (21)
	50 μg/mL	69.36 ± 2.74 (31)	6.72 ± 2.43 (21)
	100 μg/mL	48.10 ± 1.27 (22)**	8.55 ± 2.09 (19)

Data represented as mean ± S.D. from three independent experiments (values in parentheses in columns 3 and 4 refers to number of colonies); significantly different in relation to the negative control group at $^*P < 0.05$; $^{**}P < 0.01$/ $^{***}P < 0.001$ by one-way ANOVA Dunnett's Multiple Comparison Test.
[a] Negative control (solvent; DMSO).
[b] Positive control: 4-NQO treatment at 0.5 μg/ml.

cytotoxic effects in *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ and pronounced effects on the *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ strain (Fig. 7).

3.4. Cytotoxicity in strains deficient in superoxide dismutase and/or catalase

To analyze the possible involvement of the essential oil and nerolidol in the generation of ROS, we used the strains deficient in superoxide dismutase and/or catalase. The essential oil exhibited a small amount of cytotoxicity in the *sod1*Δ single mutant (lacks cytoplasmic superoxide dismutase) (Fig. 8A), and nerolidol exhibited little cytotoxicity in the *sod1*Δ and *sod2*Δ single mutants (lacks mitochondrial superoxide dismutase) as well as the double mutant *sod1*Δ*sod2*Δ (Fig. 8B).

3.5. Effects of P. gaudichaudianum essential oil and nerolidol on ROS generation

The results of the DCF assay indicated that *P. gaudichaudianum* essential oil and nerolidol caused an increase in the intracellular levels of ROS. The relative DCF fluorescence intensities measured after treatment showed that at 100 μg/mL, *P. gaudichaudianum* essential oil induces an ROS increase in SOD-WT and *sod1*Δ strains (Table 7). The nerolidol was also able to significantly increase the ROS level in all tested strains (SOD-WT, *sod1*Δ and *sod2*Δ at the highest concentration Table 7).

4. Discussion

(*E*)-Nerolidol is one of the major sesquiterpenes present in the essential oil of *P. gaudichaudianum* that was identified in a sample collected in 2006 by our research group. When GC-MS was used, 47 compounds were tentatively identified, and the major compounds were (*E*)-nerolidol (22.4%), α-humulene (16.5%), (*E*)-caryophyllene (8.9%) and bicyclogermacrene (7.4%) (Péres et al., 2009).While, we identified 56 volatile compounds (Table 2) with

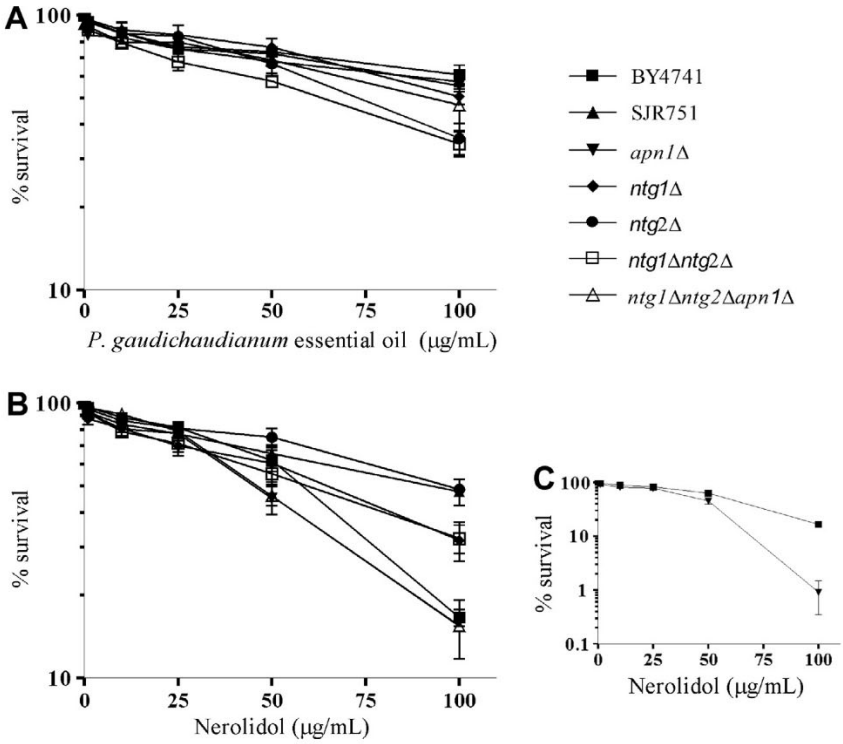

Fig. 3. Survival of *S. cerevisiae* strains deficient in BER after treatment with *P. gaudichaudianum* essential oil (A) and nerolidol (B). In detail, the BY4741 and *apn1*Δ strains after nerolidol treatment (C). The survival of the parental strain (BY4741 and SJR751) is compared to that of variants defective for the BER proteins Apn1, Ntg1 and Ntg2.

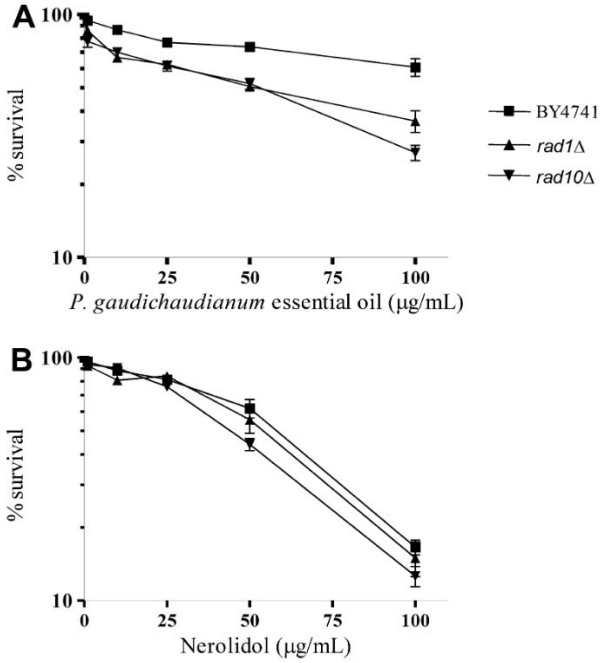

Fig. 4. Survival of *S. cerevisiae* strains deficient in NER after treatment with *P. gaudichaudianum* essential oil (A) and nerolidol (B). The survival of the parental strain (BY4741) is compared to that of variants defective for the NER endonucleases Rad1 and Rad10.

the same major compounds, however, in different concentrations as shown in Table 2. The analysis of GC-MS has some widely known limitations, such as cases in which coelutions are unavoidable and frequent in complex samples, which renders the spectra unsuitable for qualitative purposes, or for species in very low con-

Table 6
Induction of forward mutation (*CAN1*) in N123 strain of *S. cerevisiae* after nerolidol treatment at 30 °C for 18 h.

Agent	Treatment	Survival (%)	Mutants (10^7 survivors)
Treatment of stationary-phase cells under non-growth conditions			
NC[a]	0	100.00 (91)	2.22 ± 0.70 (27)
4-NQO[b]	0.5 μg/mL	33.48 ± 3.02 (31)***	64.74 ± 3.20 (198)***
Nerolidol	1 μg/mL	89.16 ± 2.29 (82)	2.46 ± 0.93 (25)
	10 μg/mL	77.12 ± 3.30 (71)	3.18 ± 1.04 (24)
	25 μg/mL	64.34 ± 2.92 (58)*	3.22 ± 1.28 (22)
	50 μg/mL	39.94 ± 3.39 (38)***	3.63 ± 1.37 (13)
	100 μg/mL	11.41 ± 3.64 (12)***	3.62 ± 1.79 (4)
Treatment of exponential-phase cells under non-growth conditions			
NC[a]	0	100.00 (64)	3.00 ± 0.58 (19)
4-NQO[b]	0.5 μg/mL	26.83 ± 2.71 (18)***	60.33 ± 1.59 (105)***
Nerolidol	1 μg/mL	87.77 ± 1.63 (57)	2.89 ± 0.64 (17)
	10 μg/mL	75.39 ± 2.75 (49)	3.35 ± 1.03 (16)
	25 μg/mL	57.49 ± 0.17 (37)**	4.07 ± 0.88 (14)
	50 μg/mL	38.09 ± 2.52 (25)***	5.13 ± 0.83 (13)
	100 μg/mL	7.03 ± 0.89 (5)***	4.84 ± 0.26 (2)
Treatment under growth conditions			
NC[a]	0	100.00 (107)	2.90 ± 0.48 (30)
4-NQO[b]	0.5 μg/mL	20.49 ± 1.45 (22)***	80.55 ± 4.95 (175)***
Nerolidol	1 μg/mL	86.08 ± 2.25 (92)	3.29 ± 0.65 (29)
	10 μg/mL	73.14 ± 4.17 (79)	3.80 ± 0.68 (29)
	25 μg/mL	64.21 ± 4.12 (69)*	3.75 ± 1.12 (24)
	50 μg/mL	34.41 ± 3.74 37)***	3.90 ± 0.78 (13)
	100 μg/mL	6.62 ± 1.39 (8)***	4.84 ± 1.24 (3)

Data represented as mean ± S.D. from three independent experiments (values in parentheses in columns 3 and 4 refers to number of colonies); significantly different in relation to the negative control group at $^{*}P < 0.05$; $^{**}P < 0.01$/ $^{***}P < 0.001$ by one-way ANOVA Dunnett's Multiple Comparison Test.

[a] Negative control (solvent; DMSO).

[b] Positive control: 4-NQO treatment at 0.5 μg/ml.

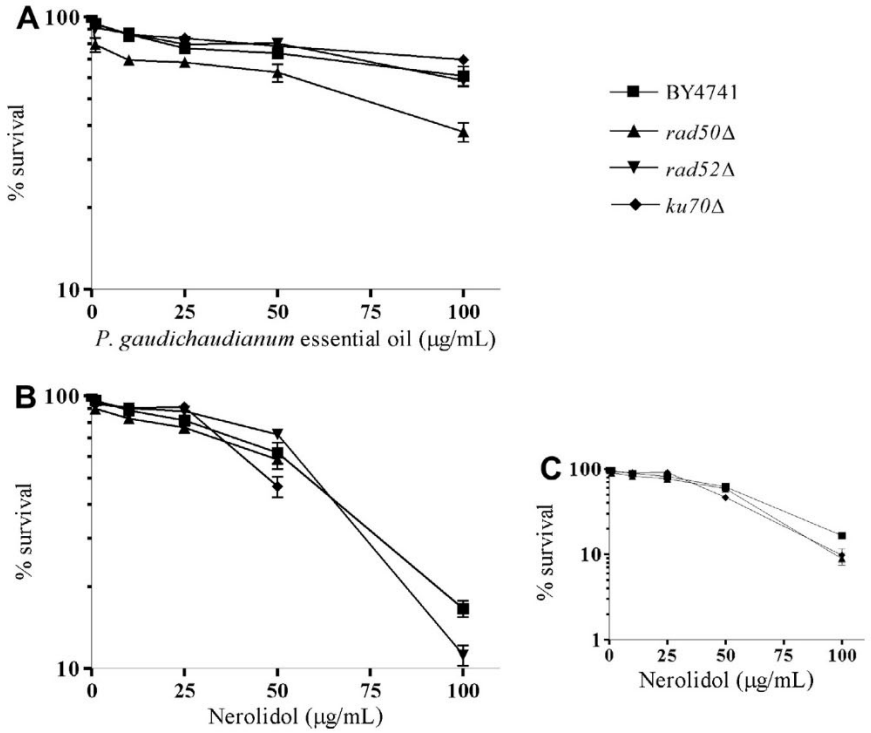

Fig. 5. Survival of *S. cerevisiae* strains deficient in HR and NHEJ after treatment with *P. gaudichaudianum* essential oil (A) and nerolidol (B). In detail, the BY4741, *rad50*Δ and *ku70*Δ strains after nerolidol treatment (C). The survival of the parental strain (BY4741) is compared to that of variants defective for the HR protein Rad52 and NHEJ proteins Rad50 and Ku70.

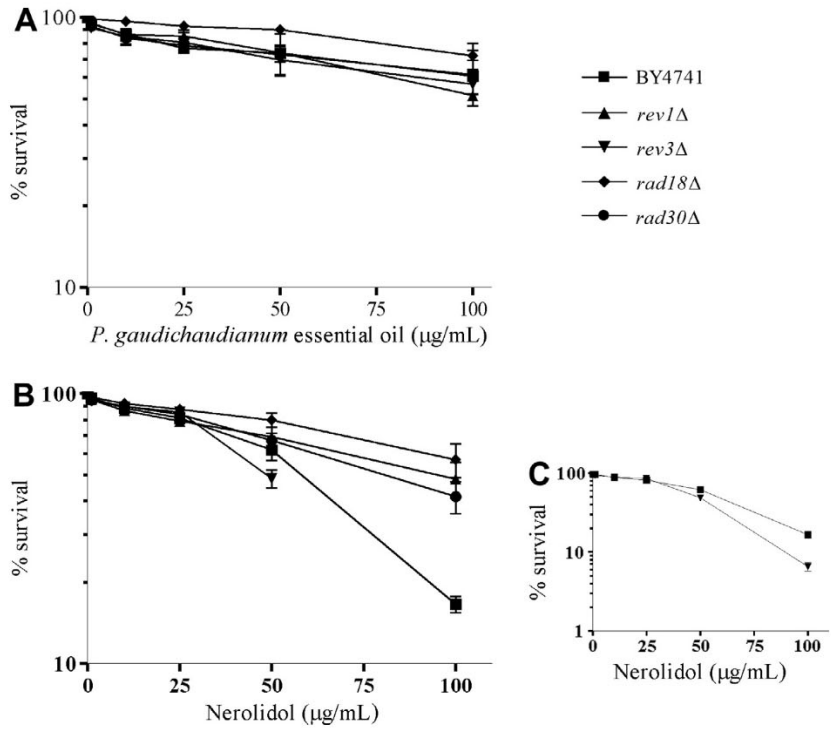

Fig. 6. Survival of *S. cerevisiae* strains deficient in TLS after treatment with *P. gaudichaudianum* essential oil (A) and nerolidol (B). In detail, the BY4741 and *rev3*Δ strains after nerolidol treatment (C). The survival of the parental strain (BY4741) is compared to that of variants defective for the TLS proteins Rev1, Rev3, Rad18 and Rad30.

centrations. In addition, positive identification may not be achieved even with good quality spectra, notably for compounds having several highly similar structural isomers, presenting comparable fragmentation patterns (Pedroso et al., 2011). Such problems can be avoided by using comprehensive two-dimensional GC (GC × GC) (Tranchida et al., 2010). Using this method, our results showed that the (*E*)-nerolidol is the major compound present in *P. gaudichaudianum* essential oil (Fig. 2 B). The differences in

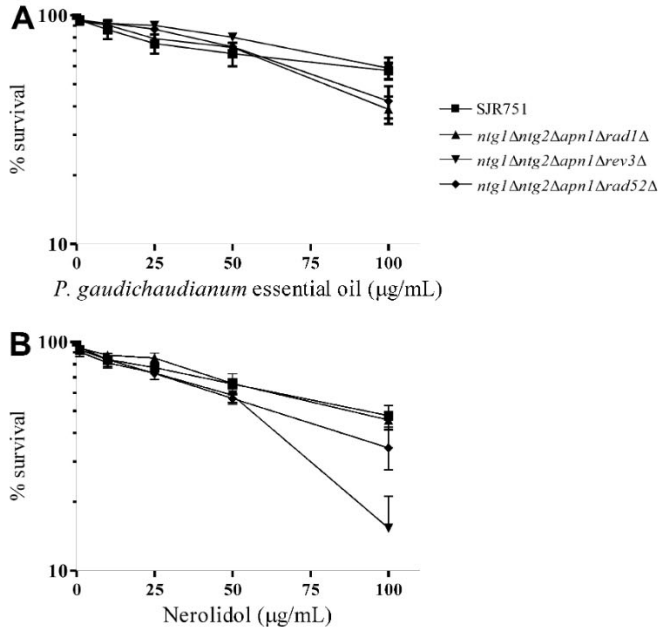

Fig. 7. Survival of *S. cerevisiae* quadruple mutant strains deficient in BER, HR, NER and TLS after treatment with *P. gaudichaudianum* essential oil (A) and nerolidol (B). The survival of the parental strain (SJR751) is compared to that of variants defective for the BER proteins Apn1, Ntg1 and Ntg2, the NER protein Rad1, the HR protein Rad52, the NER endonuclease Rad1 and the TLS protein Rev3.

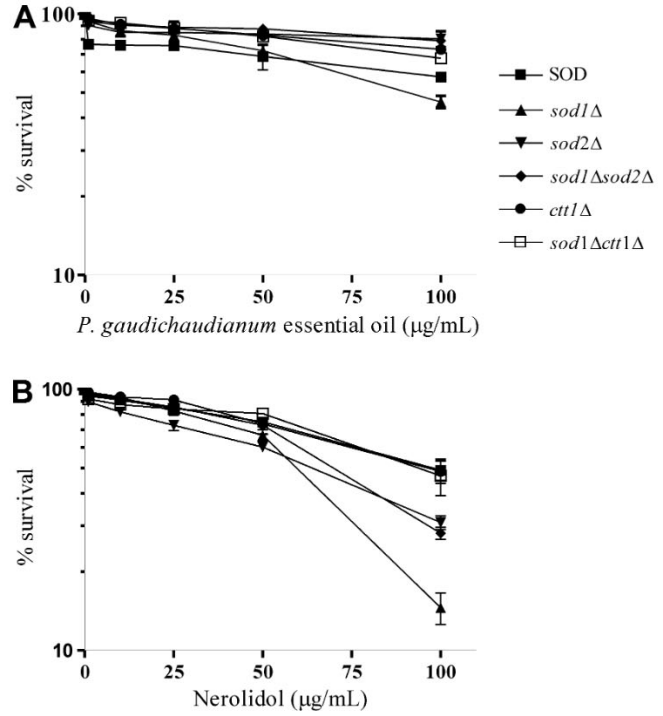

Fig. 8. Survival of *S. cerevisiae* single and double mutant strains deficient in superoxide dismutase and/or catalase after treatment with *P. gaudichaudianum* essential oil (A) and nerolidol (B). The survival of the parental strain (SOD) is compared to that of variants defective for the superoxide dismutase proteins Sod1 and Sod2 and the catalase protein Ctt1.

concentrations of major compounds are mainly dependent on seasonal and climatic conditions (Péres et al., 2009).

In order to gain further insight into the biological properties of *P. gaudichaudianum* essential oil and nerolidol, we evaluated the response of strains defective in DNA repair as well as the mutagenic and oxidative properties of these components using the yeast *S. cerevisiae* as a model organism.

P. gaudichaudianum essential oil showed cytotoxic effects in the XV185-14c and N123 strains but induced mutagenesis only at the *lys1* locus at the highest concentration. The nerolidol cytotoxic effects were more pronounced compared to those of the essential oil in these strains, and it did not induce any type of mutagenesis. In our previous work with V79 cultured mammalian cells, we have shown, using the alkaline version of the comet assay, that *P. gaudichaudianum* essential oil induces significant single DNA strand breaks and alkali-labile sites, however it does not induce DNA double strand breaks. Also, it presented mutagenic effects as shown by micronucleus assay. However, these effects appeared to be indirect, occurring as a result of ROS generation, since a lipid peroxidation increase was detected in these cells after treatment with the essential oil (Péres et al., 2009). The absence of nerolidol (a racemic mixture of *cis* and *trans* isomers) mutagenic effects are in accordance with Gonçalves et al. (2011), who demonstrated that nerolidol does not have a mutagenic effect using the *Salmonella typhimurium* TA100 and TA98 strains in the presence or absence of metabolic activation systems, although nerolidol did induce cytotoxicity in these strains. Additionally, in mouse cells, nerolidol induces clastogenicity but very weak genotoxicity (Pículo et al., 2011).

To determine whether the essential oil and nerolidol exert direct or indirect effects on DNA, we evaluated the sensitivity of DNA repair defective *S. cerevisiae* strains to these compounds. Our results indicate that the *ntg2*Δ single mutant and *ntg1*Δ*ntg2*Δ double mutant display sensitivity after the *P. gaudichaudianum* essential oil treatment, the *ntg1*Δ and *apn1*Δ single mutants, the *ntg1*Δ*ntg2*Δ double mutant and the *ntg1*Δ*ntg2*Δ*apn1*Δ triple mutant showed increased sensitivity to nerolidol Fig. 3); the proteins that are absent in these strains belong to the BER pathway. The BER primarily involves the repair of oxidative DNA damage, which encompasses the small helix non-distorting base lesion and abasic sites (Lindahl and Wood, 1999; You et al., 1999). Our data indicate that BER enzymes involved in the repair of lesions are induced, most likely indirectly, by *P. gaudichaudianum* essential oil and nerolidol.

There is an overlap between BER and NER with respect to DNA damage processing, such as during the repair of thymine glycol and 8-oxoguanine oxidative lesions (Boiteux and Guillet, 2004). Our results indicate that the NER-deficient strains *rad1*Δ and *rad10*Δ have an intermediary sensitivity to *P. gaudichaudianum* essential oil Fig. 4A), which could indicate the involvement of these pathways in the repair of lesion induced by the essential oil. Nerolidol did not induce increased sensitivity in strains lacking some of the NER proteins Fig. 4B). Although a previous study reported that nerolidol showed pro-oxidant activity (Ruberto et al., 2000), in support of the sensitivity of the BER strains, the repair pathway induced by this sesquiterpene seems to be slightly different from that induced by *P. gaudichaudianum* essential oil. The complex composition of the essential oil can explain this difference because in addition to nerolidol, it also contains other sesquiterpenes, such as α-humulene (21.32%), bicyclogermacrene (13.16%) and (E)-caryophyllene (7.51%). Alfa-humulene, e.g., showed cytotoxic activity on the human prostate carcinoma LNCaP, human prostate cancer PC-3, adenocarcinomic human alveolar basal epithelial A-549, colorectal adenocarcinoma DLD-1 and melanoma M4BEU tumor cells and its mechanism of action appears to be associated with glutathione (GSH) depletion and ROS production (Legault et al., 2003; Loizzo et al., 2007a).

Interestingly, the double-strand repair defective strains did not demonstrate pronounced sensitivity to either *P. gaudichaudianum* essential oil or nerolidol (Fig. 5), confirming that the essential oil and nerolidol do not induce double-strand break formation, as

Table 7
Effects of *P. gaudichaudianum* essential oil and nerolidol on the ROS production in SOD strains.

Treatment	Concentration	DCF fluorescence (% of control) in SOD-WT strain[c]	DCF fluorescence (% of control) in *sod1*Δ strain[c]	DCF fluorescence (% of control) in *sod2*Δ strain[c]
NC[a]		100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
H_2O_2[b]	4 mM	141.35 ± 2.83*	185.95 ± 9.93**	197.55 ± 19.43**
P. gaudichaudianum essential oil	1 μg/mL	105.53 ± 9.58	108.26 ± 16.30	107.21 ± 10.20
	10 μg/mL	111.52 ± 12.14	122.81 ± 18.89	110.01 ± 7.62
	25 μg/mL	112.88 ± 5.56	124.64 ± 14.24	112.86 ± 0.79
	50 μg/mL	115.01 ± 8.01	127.35 ± 1.91	108.45 ± 15.37
	100 μg/mL	128.63 ± 5.51*	130.74 ± 0.34*	119.98 ± 14.94
Nerolidol	1 μg/mL	115.47 ± 10.34	122.56 ± 16.31	109.46 ± 7.01
	10 μg/mL	114.45 ± 10.28	137.91 ± 14.23	110.97 ± 6.26
	25 μg/mL	123.90 ± 27.27	130.47 ± 11.07	112.95 ± 6.48
	50 μg/mL	125.95 ± 2.308	131.51 ± 7.79	125.40 ± 12.09
	100 μg/mL	140.21 ± 12.79*	173.20 ± 12.59**	147.69 ± 7.43*

Data are expressed as percentages of control values. Data significant in relation to negative control at $^{*}P < 0.05$; $^{**}P < 0.01$ by One Way Anova followed by Dunnett's Multiple comparison Test.
[a] Negative control (solvent; DMSO).
[b] Positive control: H_2O_2 treatment at 5 mM.
[c] Mean and standard deviation of three independent experiments.

described in Péres et al. (2009), and this seems also be true for nerolidol.

While the deletion of TLS and PRR proteins had no influence on sensitivity to *P. gaudichaudianum* essential oil, the *rev1*Δ, *rad30*Δ and *rad18*Δ single mutants exhibited remarkable resistance to nerolidol (Fig. 6), suggesting that these pathways can participate in the cytotoxic potential of nerolidol. Because Rad30 (Pol η) can promote error-free replication and the Rev1 protein, which functions in TLS together with Pol ζ, can act in error-free or error-prone pathways (Prakash et al., 2005; Andersen et al., 2008), these results could explain the absence of mutagenesis in XV185-14c and N123 strains treated with nerolidol (Tables 4 and 6); however, more research is needed to affirm that nerolidol enables error-free TLS.

The sensitivity showed by the *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ and *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ mutants to *P. gaudichaudianum* essential oil (Fig. 7A) is likely to be due to the absence of Ntg2 because when Ntg2 is absent, the sensitivity increases, which is what occurred with the single *ntg2*Δ and double *ntg1*Δ*ntg2* mutants. These data support the involvement of this enzyme in the repair of damage induced by *P. gaudichaudianum* essential oil. Likewise, the Ntg1 and Apn1 enzymes appear to be necessary to remove the lesions induced by nerolidol, as the *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ and *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ quadruple mutants showed sensitivity to nerolidol (Fig. 7B), as did the *ntg1*Δ and *apn1*Δ single mutants, the *ntg1*Δ*ntg2*Δ double mutant and the *ntg1*Δ*ntg2*Δ*apn1*Δ triple mutant.

When testing the abilities of *P. gaudichaudianum* essential oil and nerolidol to generate ROS, we found that the absence of the superoxide dismutase enzyme increases the sensitivity to the essential oil and nerolidol (Fig. 8). This effect was more significant in the *sod1*Δ strain after nerolidol treatment (Fig. 8A). The Sod1, copper- and zinc-containing superoxide dismutases provide the primary defense against superoxide damage by catalytically removing it through a disproportionation reaction, which involves redox cycling at the active copper site (Longo et al., 2008). Additionally, using the DCF assay, we confirmed that *P. gaudichaudianum* essential oil and nerolidol are able to produce ROS (Table 7), but the type of ROS produced remains to be determined.

Bakkali et al. (2005, 2006) have demonstrated using yeast as a model study that ROS and hydrogen peroxide are involved in the cytotoxic effects as well as in cytoplasmic petite induction (loss of mitochondria or mitochondrial dysfunction) of essential oils from different medicinal plants. It has also been demonstrated that nerolidol decreases HepG2 viability by interfering with the membrane, increasing ROS and decreasing ATP levels (Ferreira et al., 2012). One possible cytotoxic mechanism to essential oils in general involves damage in mitochondrial membranes, changing the electron flow through the electron transport chain, with increase in ROS production (Bakkali et al., 2008). Therefore, the decrease in yeast cell survival shown here could also be related to energy depletion as consequence of mitochondrial damage. The increase in ROS which oxidize and damage DNA explains the induction of single-strand breaks and activation of BER machinery. However, in the case of *P. gaudichaudianum* essential oil, if its major recognized mode of action involved ROS formation, resulting in toxic effect, other mechanisms, including direct DNA ligation or DNA topoisomerase inhibition should also be investigated, in function of the sensitivity observed in NER deficient strains.

5. Conclusion

This study showed that *P. gaudichaudianum* essential oil and nerolidol (a racemic mixture of *cis* and *trans* isomers) are not mutagenic but do induce significant cytotoxic effects in *Saccharomyces cerevisiae* that are related to ROS generation and the formation of single-strand breaks. Moreover, *trans-nerolidol* is the major compound responsible for the biological effects induced by *P. gaudichaudianum* essential oil.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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