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MICROBIOLOGY

Antifungal chemosensitization through induction of oxidative stress: A model for control of candidiasis based on the *Lippia origanoides* essential oil

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Abstract: In this work, evaluated the antifungal chemosensitizing effect of the *Lippia origanoides* essential oil (EO) through the induction of oxidative stress. The EO was obtained by hydrodistillation and analyzed by GC-MS. To evaluate the antifungal chemosensitizing effect through induction of oxidative stress, cultures of the model yeast *Saccharomyces cerevisiae* Δ*ycf1* were exposed to sub-inhibitory concentrations of the EO, and the expression of genes known, due be overexpressed in response to oxidative and mutagenic stress was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) method. Carvacrol and thymol were identified as the main components. The EO was effective in preventing or reducing the growth of the microorganisms tested. The gene expression profiles showed that EO promoted changes in the patterns of expression of genes involved in oxidative and mutagenic stress resistance. The combined use of the *L. origanoides* EO with fluconazole has been tested on *Candida* yeasts and the strategy resulted in a synergistic enhancement of the antifungal action of the azolic chemical product. Indeed, in association with EO, the fluconazole MICs dropped. Thus, the combinatorial use of *L. origanoides* EO as a chemosensitizer agent should contribute to enhancing the efficiency of conventional antifungal drugs, reducing their negative side effects.

Key words: *Candida, chemosensitization, essential oil, Lippia origanoides,* oxidative stress.

INTRODUCTION

Candida species are responsible for a high number of cases of opportunistic fungal infections. Immunocompromised patients, such as HIV-infected individuals, transplant recipients, and cancer patients are particularly vulnerable and may die mainly due to invasive opportunistic fungal infections (Binder & Lass-Flörl 2011). Currently, in addition to the high toxicity and the limited spectrum of activity displayed by some classes of commercials antifungal, along with the inappropriate or excessive use of antibiotics, which have contributed to the development of resistance to major classes of antifungal drugs available, the constant search for new and effective compounds that can be used as an alternative therapy is required (Agarwal et al. 2010).

In this sense, essential oils (EOs) have displayed antifungal activity against cutaneous and systematic mycotic agents and are potential sources of new antimicrobial compounds, representing an alternative for the treatment of infectious diseases. At low concentrations, EOs can also act as antifungal chemosensitizers, conferring a new strategy in which the co-application of these

compounds along with a conventional antimicrobial drug increases the effectiveness of the drug (Campbell et al. 2012).

The EO of *Lippia origanoides* Kunth (Verbenaceae), known in northern Brazil as "Sálvia do Marajó", was reported to display antimicrobial activity, which was attributed to the presence of thymol and carvacrol in their chemical composition (Santos et al. 2004, Oliveira et al. 2007). Carvacrol and thymol are isomeric monoterpenes found in EOs of plants showing strong antifungal activity, among others against *Candida* spp. (Ahmad et al. 2011, Khan et al. 2015). However, in sub-inhibitory concentrations, the response of a living cell to essential oils compounds at the molecular level is not known yet. Thus, further exploration of the subject is necessary.

The first step in this study was to determine the susceptibility of *Candida albicans*, *Candida parapsilosis,* and *Candida tropicalis* yeast species to *L. origanoides* EO carvacrol chemotype, collected in Pará State, Brazil. The second step was to analyze the chemosensitizer effects of a sub-inhibitory concentration of this EO, by exploring oxidative and mutagenic stress responses, using *Saccharomyces cerevisiae* as model yeast. Besides the advantage of being a single-celled microorganism of rapid growth, the complete genome of *S. cerevisiae* is known and mutated strains with known sensitivity to oxidative stress-inducing agents are available. In addition, *S. cerevisiae* is most closely related to *Candida albicans*, the major opportunistic fungal pathogen (Hughes 2002). We used the Δ*ycf1* mutant strain of *S. cerevisiae* as a model organism for exploring the effect of sub-inhibitory concentrations of EO from *L. origanoides* on the antioxidant defense system, and more precisely the ability of this EO to trigger the onset of the response of genes involved in the neutralization of the oxidative stress. Indeed, this mutant is hypersensitive to drugs and cadmium for it is lacking a vacuolar ATP-binding cassette transporter allowing the vacuolar transfer and accumulation of glutathione-conjugated drugs and cadmium (Li et al. 1997). To assess the potential of *L. origanoides* EO to promote chemosensitization, *S. cerevisiae* Δ*ycf1* cells were exposed to EO, and the changes in the patterns of expression of genes of the oxidative and mutagenic stress response (*GSH1*, *KAR2, PRX1,* and *RNR3*) were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). These genes were selected because they are well known to be upregulated in situations during which the oxidative and mutagenic status of the cell is increased. Promoting the onset of oxidative and mutagenic stress in yeast will be of great importance to aid in the development of new antifungal drugs in combinatorial use with EOs.

MATERIALS AND METHODS

General Experimental Procedures

Plant material

The aerial parts (leaves and thin stems) of *L. origanoides* were collected in the Amazonian basin, close to the city of Santarém, Pará, Brazil, in July 2012. The intermediate geographical position of the specimens was determined using the Global Positioning System (GPS), resulting in the coordinates 02°30'870''S and 54°56'416''W, at an altitude of approximately 52 m above the sea level. The botanical material was identified morphologically by Dra. Fátima Regina Gonçalves Salimena from the Department of Botany of the Institute of Biological Sciences, Federal University of Juiz de Fora, Brazil, where a voucher specimen (CESJ 64029) has been deposited.

Essential oil extraction

The extraction was performed according to the protocol described by Sarrazin et al. (2015). For this, aerial parts of *L. origanoides* were triturated and submitted to the hydrodistillation process, using a Clevenger-type apparatus for 3 h. A sample of the fresh material (200 g) was immersed in distilled water at a ratio of 1:10 (w:v). The oil was dried over anhydrous sodium sulfate and the yield was calculated based on the plant's dry weight. The procedure was performed in triplicate, giving a yield of 1.7 % of EO.

Gas chromatography-mass spectrometry analysis

The analysis of the EO was carried out by gas chromatography-mass spectrometry (GC-MS) using an Agilent apparatus, (model 6890, plus series GC system), equipped with a mass selective detector (Agilent, MSD 5973), and an autosampler (Agilent, 7863). The analysis was realized in an apolar capillary column BD-5ms (J & W Scientific, Folsom, CA, USA) (60 m × 0.25 mm, 0.25 mm film thickness) fused-silica capillary column. The oven temperature was settled at 50 °C (5 min in isothermal mode), raised at 4 °C min^{-1} to 150 °C (2 min in isothermal mode), and then held for 20 min at 250 °C, (held isothermal for 5 min) and increasing to 10 °C min^{-1} to 275 °C (constant during 15 min) and injector temperature of 250 °C. Helium was the carrier gas at a constant flow of 0.6 mL min^{-1}, with an inlet pressure of 16.5 psi. Samples (1 µL) were diluted in hexane and injected in the split mode (1 µL), with split flow adjusted to yield 30:1 ratio. Mass spectra were obtained by electron impact at 70 eV of energy. The temperatures of the ionization chamber and the transfer line were maintained at 230-285 °C, respectively. The quantitative data regarding the volatile constituents were obtained by peak-area normalization using a GC 6890 Plus Series coupled to an FID Detector, operated under similar conditions to the GC-MS system. The retention index was calculated for all the volatiles constituents using a homologous series of C8-C30 n-alkanes (Sigma-Aldrich), according to the linear equation of Van den Dool & Kratz (1963).

Microorganisms' cultivation and determination of the antifungal activity of *L. origanoides* EO

Candida yeasts and inoculum standardization

Antifungal activity was performed using three yeast strains: *C. albicans* (CCCD - CC001), *C. tropicalis* (CCCD - CC002), and *C. parapsilosis* (CCCD - CC003). The strains were purchased in lyophilized form (Cefar Diagnostic, São Paulo - Brazil), rehydrated in 1 mL of Sabouraud Dextrose Broth - SDB (Himedia, Mumbai - India) at 27 ± 2 °C for 48 h. The inoculi were prepared by the direct inoculation of isolate colonies in 1mL of sterile saline solution and adjusted to the 0.5 standard of the McFarland scale, corresponding to 2 to 5 × 10^6 CFU mL^{-1} (NCCLS/CLSI - National Committee for Clinical Laboratory Standards 2002).

Determination of the antifungal activity of L. origanoides EO on Candida yeasts using the disk diffusion method

This test, based on a standardized methodology (Bauer et al. 1966), was used to evaluate the inhibitory spectrum of the EO against the analyzed microorganisms. The inoculi were seeded on Sabouraud dextrose agar (SDA) medium solidified in Petri dishes. After that, filter paper discs (6 mm-diameter)

containing 10µL of the undiluted EO were pressed lightly against the surface of the agar. After 30 min at room temperature, the dishes were incubated in a bacteriological oven at 27 ± 2 °C for 48 h. At the end of the test period, the diameter of the inhibition zone formed over the agar culture was measured in millimeters. The tests were performed in triplicate.

Determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of L. origanoides EO on Candida yeasts

A standardized methodology was used (NCCLS 2004). A serial doubling dilution of the *L. origanoides* EO (10 to 0.07 µL mL^{-1}) was prepared using Tween 80 solution (0.5%) as solvent (Sigma-Aldrich, São Paulo - Brazil). The tests were performed in 96 well plates, where each well received 90 µL of the specific concentration of the EO, 90 µL of Sabouraud dextrose broth (SDB) medium, and 20 µL of the inoculum. The cultures were incubated at 27 ± 2 °C for 24 h. To test the possible growth inhibition triggered by the EO, 20 µL of a 0.02% solution of resazurin in water (Vetec, Rio de Janeiro - Brazil) was added, and the cultures were further incubated for 3 h. Then, the color within wells was read visually, and the MIC was indicated by the weaker concentration of the EO resulting in a blue coloration of the corresponding wells. More precisely, the inhibition of yeast growth was revealed by the lack of reduction of resazurin to refazurin and the permanence of the blue color of resazurin. On the contrary, the shift of color from blue to red indicated the presence of live microorganisms. The MIC values were defined by the lowest concentration of the EO that inhibited the growth of the microorganism. The wells that showed no apparent growth were selected to evaluate the MFC, which was determined by the absence of microbial growth on plates containing SDA medium. MFC is defined as the lowest concentration of the EO for which 99.99% or more of the initial inoculum was killed. For comparative purposes, the standard drug Fluconazole (Himedia) was used as positive control. The tests were performed in triplicate.

Antifungal chemosensitizing potential of sub-inhibitory concentrations of L. origanoides essential oil

The antifungal chemosensitizing potential of sub-inhibitory concentrations of *L. origanoides* essential oil (0.125 and 0.25 µL mL^{-1}) was also determined using a microdilution assay. Briefly, 20 µL of the essential oil was added to 96 well plates containing 20 µL of antifungal (Fluconazole) in serial dilutions ranging from 2.1 to 0.01 mg mL^{-1} and inoculated after that with 160 µL of cell suspension of yeasts with approximately 1.5 x 10^6 CFU mL^{-1}. The plates were incubated at 27 °C for 24 h. The inhibition of growth was confirmed using resazurin reduction in a liquid medium assay as described above. All assays were performed in triplicate.

Determination of sub-inhibitory concentration and genes' expression analysis

S. cerevisiae yeast strain and growth media

For the determination of sub-inhibitory concentration and genes' expression analysis, we used the *S. cerevisiae* yeast strain Δycf1 (YDR1356) as an experimental model. The yeast strain was kindly provided by Dr. Daniel Brèthes (French National Centre for Scientific Research - Institut de Biochimie et Génétique Cellulaire - France). Complete medium YPD (yeast extract peptone dextrose) containing 0.5% yeast extract, 2% bacto-peptone, and 2% glucose was used for routine growth of yeast cells.

Sub-inhibitory concentration determination of L. origanoides EO on S. cerevisiae cells

This experiment was performed to determine the sub-inhibitory EO concentration prior to realizing the chemosensitization assay. Overnight cultures, obtained by inoculation of isolate colonies, were grown in 1mL of YPD broth medium at 27 ± 2 °C with shaking at 225 rpm. After dilution in 25 mL of sterile YPD broth, the growth was monitored by optical density at 600 nm (OD600). Cultures were distributed into Falcon tubes (1 mL/tube, OD600 = 0.3). Subsequently, stock solution of EO diluted in Tween 80 (0.5%) was added to the specified final concentrations of 5.0, 2.0, 0.5, 0.25 and 0.125 µL mL^{-1}. Apart from the untreated cell control, the other control treatments, including Tween 80 (0.5%), were the following: an essential oil-free exposition, and cadmium chloride (final concentration of 10 and 20 µM). Cadmium was used as a reference treatment because it is a toxicant well known to induce strong oxidative stress in yeast (see Vido et al. 2001). After 3 h of exposure, cell growth was measured by turbidimetry at 600 nm. The IC_{50} value was obtained by linear regression analysis of the dose-response curves.

Quantitative real-time PCR assay

The expression of the oxidative stress response gene was determined after exposure of yeast to two sub-inhibitory concentrations of EO (0.25 and 0.125 µl mL^{-1}). Yeast cells, grown overnight (OD_{600} = 3.8), were added to 25 mL YPD broth medium, and re-incubated for 3 h at 27 ± 2 °C with shaking at 225 rpm. Growth was monitored by OD_{600}. Aliquots of 4 mL (OD_{600} = 0.3) were exposed to the EO and incubated for 3 h under the same conditions as above. Cd^{+2} was used as a reference compound (final concentrations of 10 and 20 µM). Cells were then collected by centrifugation and processed for RNA extraction. The Absolutely RNA RT-PCR Miniprep kit (Agilent, Stratagene) was used, according to the manufacturer's instructions with the following modification: 100 µL of the lysis buffer containing guanidine thiocyanate and 0.7 µL β-mercaptoethanol were subsequently added an equal volume of glass beads (Ø 0.5 mm, Sartorius, glass beads BBI-854701) and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Cells were then disrupted by vortexing 5x for 30 seconds with the station of 30 seconds in ice between each vortex sessions to avoid excessive warming. The elution volume was 30 µL and the concentration of RNA was quantified using a nanodrop spectrometer (Epoch, Biotek). RNA purity was checked and met the following requirements: A260/A280>1.7 and A260/A230>1.5. The integrity of the 18S and 26S ribosomal bands was checked on a 1% agarose-formaldehyde gel. First-strand cDNA was synthesized from 5 µg total RNAs using the AffinityScript Multiple Temperature cDNA Synthesis kits (Agilent, Stratagene) using 3 µL of random primers (0.1 µg $µL^{-1}$), 1 µL of AffinityScript Multiple Temperature RT, 2 µL of 10x AffinityScript RT buffer, dNTP (25 mM each) and RNase free water in a final volume of 20 µL. The retro-transcription was performed by incubating the reactions for 60 min at 42 °C. Specific primer pairs were determined using the LightCycler probe design software (Roche) and matched the coding sequence of the target genes. The GenBank accession numbers and the corresponding primer pairs are summarised in Table SI. Real-time qPCR reactions were performed using an Mx3000P QPCR System (AGILENT, Stratagene). Each 25 µL reaction contained 1 µL of reverse-transcribed product template, 12.5 µL of 2x SYBR Green QPCR Master mix (Agilent), 2µL of the gene-specific primer pairs (at a concentration of 300 nM each), and 9.5 µL of H_2O. The program used was: one cycle at 95 °C for 10 min and then 50 amplification cycles

at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Standard curves were generated using 10-fold dilutions of a cDNA template on the LightCycler apparatus and using each couple of gene-specific primers (one standard curve per couple). Each dilution was assayed in triplicate for each couple of primers. Each standard curve was made by plotting the Ct against the log of the starting quantity of template for each dilution. The equation for the regression line and the r-value were calculated. From that equation, the slope of the standard curve was deduced and used to calculate the PCR efficiency, E, for each couple of primers, as follows: $E = 10^{-1}/\text{slope}$. The measured hybridization efficiencies were 2.45, 2.07, 1.77, and 1.97 for *GSH1*, *KAR2*, *PRX1,* and *RNR3* genes respectively. The yeast 18S ribosomal RNA was used as reference, and the following probes were designed from the GenBank accession number Z75578: 5'-TCAACACGGGGAAACTCACC-3' for the forward primer, corresponding to position 1191-1210, and 5'-AACCAGCAAATGCTAGCACCA-3' for the reverse primer, corresponding to position 1370-1350. The measured hybridization efficiency for this couple of primers was 2.01. Relative quantification of each gene expression level was normalized to the yeast 18S ribosomal gene expression and calculated based on the Pfaffl method (Pfaffl 2001). The Pfaffl formula can be rearranged as follows:

$$\underbrace{\frac{E^{\Delta Ct\,(sample-control)}_{(ref)}}{E^{\Delta Ct(sample-control)}_{(target)}}}_{D} = \underbrace{\frac{E^{Ct\,(sample)}_{(ref)}}{E^{Ct\,(sample)}_{(target)}}}_{A} \; x \; \underbrace{\frac{E^{Ct\,(control)}_{(target)}}{E^{Ct\,(control)}_{(ref)}}}_{1/B}$$

Where **D** is the differential expression of the target gene, i.e. the expression of the target gene in the test sample relative to that in the control sample, **A** is the relative expression of the target gene in the test sample, and **B** is the relative expression of the target gene in the control sample, and both A and B are expressions relative to that of the reference gene; Ct is the number of PCR cycles needed to enter in the exponential phase of amplification; E(ref) and E(target) are the hybridization efficiencies of couples of primers specific to the reference and target genes, respectively. Most often, only the differential expression, D, is displayed in articles. In the present article, we decided it useful to display the genes' expression relative to the reference gene in the test, A, and control samples, B. For each gene, the mean value of the relative expression level, and the associated standard error (n=3) were determined. The reaction specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SybrGreen fluorescence level during gradual heating of the PCR products from 60 to 95 °C. Samples were run in duplicate in optically clear 96-well plates (ABgene, Thermo Fisher Scientific, USA). All qPCR experiments were performed according to the MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines (Bustin et al. 2009).

Statistical analysis

The results of the antifungal assay and expression values were expressed as means ± standard error of the mean (SEM) and statistical significance was determined by One-way analysis of variance (ANOVA) followed by Tukey's test, with the level of significance set at $p < 0.05$ using the program GraphPad Prism 3.0, with a 95% confidence interval. The IC50 value was obtained by linear regression analysis of the dose-response curves generated from the absorbance data with the statistical package Microsoft Excel and expressed as the mean ± standard deviation (SD) of experiments done in triplicate.

RESULTS AND DISCUSSION

Chemical composition of L. origanoides EO

The analysis of the EO of *L. origanoides,* obtained by hydrodistillation, was carried out by GC and GC-MS. Table I shows the chromatographic results, expressed as area percentages.

A total of 99.0% of the chemical compounds were identified. The GC-MS analysis showed that the major constituents of the oil were oxygenated monoterpenes with 76.8%. The main volatile compounds were carvacrol (46.1%) and thymol (11.8%), totaling about 58% of the oil of *L. origanoides.* Other constituents in significant quantities were *p*-cymene (9.6%) and *p*-methoxy thymol (8.4%). Oliveira et al. (2007) reported the composition of *L. origanoide*s EO from Oriximiná, Pará, Brazil, in which carvacrol was the main constituent (38%), followed by thymol (18%) and *p*-cymene (10%). In other studies, the concentration of carvacrol in the EO of the *L. origanoides* leaves varied between 33.5 to 42.9% (Santos et al. 2004, Stashenko et al. 2010). The oil produced from a specimen existing in Venezuela showed that thymol (62%), *p*-cymene (9%), carvacrol (8%), and γ-terpinene (6%) were the major compounds (Rojas et al. 2006). On the other hand, Ribeiro et al. (2014) suggested a new chemotype for *L. origanoides*, characterized by an EO rich in (E)-methyl cinnamate (with a peak of 52.4% in August) and (E)-nerolidol (with a peak of 29.2% in December). *Lippia schomburgkiana*, a synonymous species of *L. origanoides*, existing in Maranhão, Brazil, also showed another different type with the predominance of 1.8-cineol (64.1%) (da Silva et al. 2009). These data show that *Lippia origanoides* EO can show a qualitative and quantitative variation based on genetic variations and the influence of environmental conditions existing in sampling sites.

Antifungal activity assay of L. origanoides EO on different Candida strains

We evaluated the inhibitory spectrum of the EO against *C. albicans, C. parapsilosis,* and *C. tropicalis.* These strains showed a high degree of sensitivity as evidenced by the large inhibition zones around the disks (Table II). EO concentrations between 0.31 to 1.25 μL mL^{-1} (MIC) were able to completely inhibit the growth of yeast strains, while 0.62 to 5 μL mL^{-1} (MFC) induced fungicidal activity. Fluconazole, commonly used against *Candida* infections, showed MIC values between 0.12 to 1.05 mg mL^{-1}. Thus, we demonstrated that the EO of *L. origanoides* exhibited fungistatic or fungicidal activity against three *Candida* strains.

Fungi are eukaryotic organisms, and for that reason, they generally present more difficult therapeutic problems than bacterial infections (Ismail et al. 2008). In particular, *Candida* species are inherently resistant or rapidly acquire resistance to antifungal drugs, justifying the search for new therapeutic strategies (Ahmad et al. 2011). Many EOs have biological activity both *in vitro* and *in vivo*, which has justified research in traditional medicine focused on the characterization of their antimicrobial activity (Agarwal et al. 2010). The antifungal activity of EOs with high carvacrol and thymol concentration has been reported on *Candida* species (Khan et al. 2015). Santos et al. (2004) and Oliveira et al. (2007) screened microorganisms for sensitivity to *L. origanoides*, collected in Piauí and Pará states in Brazil, respectively, and found that the EO possessed antimicrobial activity against Gram-positive, Gram-negative bacteria, and fungi. The authors attributed the antimicrobial activity to the major compounds in the EO, carvacrol, and thymol, but the MIC, MFC, and possible interactions with standard drugs had not been determined.

Table I. Chemical composition of the essential oil of *Lippia origanoides* Kunth sampled in the Santarém region.

Constituents	$RI_{Calc.}$ [a]	$RI_{Lit.}$ [a]	Oil %
(*Z*)-Hexen-3-ol	854	859	0.2
α-Thujene	926	924	0.8
α-Pinene	934	932	0.4
1-Octen-3-ol	976	974	0.2
Myrcene	990	988	1.2
α-Terpinene	1016	1014	0.7
p-Cymene	1025	1020	9.6
Limonene	1026	1024	0.2
1,8-Cineole	1032	1026	0.7
γ-Terpinene	1056	1054	1.7
Linalool	1098	1095	2.8
Umbellulone	1169	1167	0.4
Terpinen-4-ol	1176	1174	0.9
Thymol methyl ether	1234	1232	1.6
Thymol isomer (MW=150)	1282	-	0.4
Thymol	1292	1289	11.8
Carvacrol	1299	1298	46.1
Thymol acetate	1351	1349	0.6
Carvacrol acetate	1372	1370	1.1
Geranyl acetate	1382	1379	0.4
(*E*)-Caryophyllene	1418	1416	2.1
trans-α-Bergamotene	1434	1432	0.1
α-Humulene	1455	1452	0.2
p-Methoxythymol	1487	1484	8.4
β-Bisabolene	1506	1505	0.2
trans-γ-Bisabolene	1531	1529	0.5
p-Methoxycarvacrol (tent.)	1555	-	1.6
Caryophyllene oxide	1584	1582	2.2
2-Phenylethyl tyglate	1587	1584	0.2
Humulene epoxide II	1611	1608	0.2
α-Bisabolol	1686	1685	0.2
Not identified sesquiterpenes			1.3
Monoterpene hydrocarbons			14.6
Oxygenated monoterpenes			76,8
Sesquiterpenes hydrocarbons			3.1
Oxygenated sesquiterpenes			2,6
Others			0.6
Total identified			99.0

[a]: $RI_{calc.}$ and $RI_{Lit.}$: retention indices calculated and compared to those found in the literature.

Table II. Antifungal potential of the essential oil of *L. origanoides* against *Candida* species.

Microorganisms	*Lippia origanoides* essential oil			Flu
	DD [a] (10 µL)	MIC (µl mL^{-1})	MFC (µl mL^{-1})	MIC (mg mL^{-1})
C. albicans	44.1 ± 0.3	0.62	5	1.05
C. tropicalis	30.6 ± 0.2	1.25	2.5	1.05
C. parapsilosis	24.0 ± 0.1	0.31	0.62	0.12

[a] Diameter of the inhibition of growth expressed in mm.
DD - disk diffusion; MIC - Minimum Inhibitory Concentration; MFC - Minimum Fungicidal Concentration; Flu – Fluconazole.
The inhibition of yeast growth was revealed by the lack of reduction of resazurin to refazurin.

Disruption of membrane integrity and ergosterol biosynthesis caused by thymol and carvacrol has been implicated as a mode of antimicrobial action against different bacterial and *Candida* species (Ahmad et al. 2011). In *S. cerevisiae,* it has been demonstrated that the surface of cells treated with thymol was significantly damaged (Bennis et al. 2004). Contrary to that, it has been proposed that, rather than causing non-specific lesions of membranes, thymol activated specific signaling pathways in yeast causing cytosolic Ca^{2+} bursts and transcription responses similar to Ca^{2+} stress and nutrient starvation (Rao et al. 2010). At sub-inhibitory concentrations, changes to the transcriptome and proteome during exposure can reveal how the cell responds to the compound, and up-regulation of genes involved in certain metabolic, or biosynthesis pathways can be indicative of which cell structures or processes are affected (Rao et al. 2010). Fungi require well-defined regulation of expression of antioxidation systems, not only for protection from host defense responses but also for maintaining redox homeostasis needed for normal fungal growth, and because of that pivotal role, destabilization of antioxidation systems can be an effective way to control fungal pathogens (Kim et al. 2011). Thus, besides evaluating the antifungal activity of the EO from *L. origanoides* by conventional methods, we illustrated the chemosensitizing effect of sub-inhibitory concentrations of this oil on the antioxidant defense system, using *S. cerevisiae* as an experimental yeast model.

Growth inhibition effect of L. origanoides EO on S. cerevisiae yeast cells

This test was conducted to determine and select the sub-inhibitory concentrations of EO that will be further used in the chemosensitization assay. *S. cerevisiae* Δ*ycf*1 mutated cells were exposed to five different concentrations of EO of *L. origanoides* (ranging in decreasing order from 5 to 0.125 µL mL^{-1}) for 3 hours, and the growth inhibition was measured. The *S. cerevisiae* yeast susceptibility to *L. origanoides* EO was compared to that of Cd^{2+}, a well-known inducer of oxidative stress (Table III). The optic density of the untreated cells was 0.84 ± 0.01.

The IC_{50} of *L. origanoides* EO on the *S. cerevisiae* yeast Δ*ycf*1 mutant was determined to be 0.45 ± 0.05 µL mL^{-1}, corresponding closely to the effect of 20 µM of Cd^{2+} that resulted in 54% inhibition of growth. Therefore, *L. origanoides* EO well exerted a potent fungicidal activity against *S. cerevisiae* in keeping with that against *Candida* spp. The sub-inhibitory concentrations of *L. origanoides* EO were selected to be 0.25 and 0.125 µL mL^{-1}, corresponding to growth inhibitions of 33 and 4%, respectively,

Table III. Effect of the essential oil of *L. origanoides* on the cell growth of the *S. cerevisiae* Δ*ycf*1 model mutant.

Treatment EO of *L. origanoides* µL mL^{-1}	[a] Growth	[b] Growth inhibition (%)
5	0.23 ± 0.02 ***	69
2	0.28 ± 0.09 ***	62
0.5	0.34 ± 0.04 ***	54
0.25	0.50 ± 0.05 ***	33
0.125	0.72 ± 0.15	4
Cd^{2+} [20 µM]	0.34 ± 0.09 ***	54
Cd^{2+} [10 µM]	0.72 ± 0.06	4
Tween 80 (0.5%)	0.75 ± 0.15	0

[a]: after 3 h of exposure, the cell growth was measured by turbidimetry - reading the absorbance at 600 nm (mean ± SD, *n* = 3).
[b]: inhibition percent in relation to control cells treated with Tween 80 (0.5 %).
***(*p* < 0.001): for comparison between EO treatments and control Tween 80 (0.5%) treatment.**
Untreated cells: OD = 0.84 ± 0.01.

being known that the observed 4% of growth inhibition at 0.125 µL mL^{-1} was not statistically significant. According to Agarwal et al. (2003), the concentration of the drug is critical because the effect on gene expression may not be detectable if the drug concentration is too low, and secondary drug effects could mask the primary responses if the test concentration is too high.

Induction of the oxidative stress response genes in the S. cerevisiae Δycf1 mutant exposed to L. origanoides EO

The *L. origanoides* EO effectiveness in chemosensitization was assessed by exploring the oxidative stress response in *S. cerevisiae* by measurement of *GSH1, KAR2, PRX1,* and *RNR3* genes' expression. The effects of that EO were first compared with that of cadmium, a toxic metal known to induce oxidative stress in yeast, and second, a blend of cadmium and EO was assessed to see whether that oil could exacerbate the toxic effects of Cd^{2+} and vice-versa. The relative genes' expressions are displayed in Table IV.

At a concentration of 10 µM, Cd^{2+} exerted no inducing effect, and at 20 µM Cd^{2+} a slight and non-significant trend to upregulation was observed. This means that at those concentrations and in that mutant, the growth inhibitory effect of Cd^{2+} was not linked to the onset of oxidative stress. This is most probably due because, at low doses, the endoplasmic reticulum is the primary target of cadmium toxicity in yeast, before the induction of oxidative stress that takes place at higher doses (Gardarin et al. 2010). The EO was able to significantly stimulate the antioxidant gene response since *PRX1* and *RNR3* genes were 4- and 5-times upregulated at a concentration of 0.125 µL mL^{-1}, whereas *GSH1* and *RNR3* genes were 4- and 3-times upregulated at a concentration of 0.25 µL mL^{-1} as compared to control. This means that the *L. origanoides* EO can trigger mutagenic damage at those concentrations. The addition to EO-containing cultures of Cd^{2+} exacerbated and amplified the upregulation of the 4 scrutinized antioxidant stress genes. All 4 tested genes were significantly upregulated as compared to control cultures in presence of both xenobiotic compounds (but *RNR3* when cultures contained 0.25 µL mL^{-1} of EO and 10 µM Cd^{2+}). The maximal answer was reached at concentrations of 0.125 µL mL^{-1} EO

Table IV. Effects of *L. origanoides* EO on the antioxidant defense system of the *S. cerevisiae* Δ*ycf*1 model mutant.

Treatment	Genes' relative expression			
	GSH1	KAR2	PRX1	RNR3
Control (Tween 80)	46 ± 2	87 ± 20	43 ± 10	42 ± 7
Cd^{2+} (10 µM)	22 ± 12	45 ± 34	25 ± 17	32 ± 23
Cd^{2+} (20 µM)	85 ± 43	229 ± 112	114 ± 55	114 ± 59
EO (0.125 µL mL^{-1})	222 ± 107	312 ± 110	197 ± 56 §	220 ± 71 §
EO (0.125 µL mL^{-1}) + Cd^{2+} (10 µM)	302 ± 48 §	475 ± 84 §	320 ± 63 §	251 ± 48 §
EO (0.125 µL mL^{-1}) + Cd^{2+} (20 µM)	672 ± 183 §	666 ± 65 §	420 ± 83 §	521 ± 112 §
EO (0.25 µL mL^{-1})	218 ± 19 §	181 ± 38	137 ± 48	141 ± 35 §
EO (0.25 µL mL^{-1}) + Cd^{2+} (10 µM)	251 ± 31 §	224 ± 30 §	181 ± 22 §	108 ± 58
EO (0.25 µL mL^{-1}) + Cd^{2+} (20 µM)	283 ± 52 §	443 ± 107 §	264 ± 33 §	318 ± 88 §

The 18S ribosomal RNA was used as reference. All values were multiplied by 1000 to facilitate reading. The values are expressed as mean ± SEM (*n* = 3).
§ (*p* < 0.05) Significant differences between the relative expression values in the presence of EO compared to that of control.

and 20 µM Cd^{2+}. Under such conditions, *GSH1*, *KAR2*, *PRX1*, and *RNR3* were upregulated 14-, 7-, 9-, and 12-times, respectively.

GSH1, *KAR2*, *PRX1*, and *RNR3* are good biomarker genes of oxidative and mutagenic stress in *S. cerevisiae*.

When *S. cerevisiae* cells were submitted to a 10 mM cadmium challenge for 1 h, the protein *GSH1, KAR2* and *PRX1* were more than 10-, 20- and 5-fold more abundant than in the untreated control cells (Vido et al. 2001). Cadmium itself is well known to induce oxidative stress in various organisms and among other *S. cerevisiae* (Liu et al. 2005, Muthukumar & Nachiappan 2010). The expression of the *RNR3* gene was found to be up-regulated more than 10-fold after treatments of cells with mutagenic agents (Endo-Ichikawa et al. 1995, 1996).

GSH1 encodes the first enzyme of glutathione biosynthesis: *g*-glutamylcysteine synthetase (Jamieson 1998). GSH1 is possibly the most abundant redox-scavenging molecule in yeast cells. This molecule participates in the primary non-enzymatic defense system, and the maintenance of GSH homeostasis is particularly important for protection against cellular damage caused by oxidative stress (Dormer et al. 2000, Khan et al. 2015). Thus, the 6- and 14-fold increased *GSH1* gene expression observed upon exposition to 0.125 µL mL^{-1} *L. origanoides* EO and either 10 or 20 µM Cd^{2+}, respectively, is likely an adaptive response against the onset of oxidative stress.

RNR3 codes the ribonucleotide reductase (RNR), an enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides needed for DNA synthesis (Elledge et al. 1992). The ribonucleotide reductase gene (*RNR3*) is a known DNA damage-responsive gene (Endo-Ichikawa et al. 1995, 1996). Carcinogenic and genotoxic agents, ranging from DNA alkylating agents, oxidative chemicals, and radiations, were able to induce *RNR3* expression at a sublethal dose. In contrast, both non-mutagenic and non-genotoxic chemicals tested were unable to induce *RNR3* expression (Jia et al. 2002). EOs from medicinal plants demonstrated significant *RNR3* gene induction, equivalent to that caused by hydrogen peroxide at equitoxic doses. EO-induced cytotoxicity involved oxidative stress,

as evidenced by the protection observed in the presence of ROS inhibitors such as glutathione and catalase (Bakkali et al. 2005). Thus, the 5- and 12-fold increased *RNR3* gene expression observed upon exposition to 0.125 µL mL^{-1} *L. origanoides* EO without or with 20 µM Cd^{2+}, respectively, is indicating a mutagenic effect probably linked to the onset of oxidative stress.

Peroxiredoxins (Prxs) are antioxidant enzymes that act as peroxidases reducing hydrogen peroxide (H_2O_2) and hydroperoxides to water or the corresponding alcohol, respectively (Bang et al. 2012). The *S. cerevisiae* Prx1p is located in mitochondria, and it is overexpressed when cells use the respiratory pathway, as well as in response to oxidative stress conditions (Pedrajas et al. 2000). In addition, a mutation in the *PRX1* gene sensitizes cells to H_2O_2, to lethal heat shock, and to cadmium in an oxygen-dependent manner (Greetham & Grant 2009). Thus, the 4- and 9-fold increased *PRX1* gene expression observed upon exposition to 0.125 µL mL^{-1} *L. origanoides* EO without or with 20 µM Cd^{2+}, respectively, is indicating an adaptive response against the onset of oxidative stress taking place in mitochondria.

In yeast, the *KAR2* gene encodes a molecular BiP chaperone that belongs to a family of proteins expressed in the endoplasmic reticulum (ER) of all eukaryotic cells. BiP chaperones are involved in functions essential to cell viability, such as polypeptide translocation into the ER lumen, protein folding, and protein degradation (Haas 1994, Simons et al. 1995). The Kar2/BiP chaperone is an important sensor of reactive oxygen species that changes its activity when these harmful chemicals are present and helps to protect the cell from damage (Wang et al. 2014). Thus, the 5- and 7-fold increased *KAR2* gene expression observed upon exposition to 0.125 µL mL^{-1} *L. origanoides* EO and either 10 or 20 µM Cd^{2+}, respectively, is likely an adaptive response against the onset of oxidative stress within the luminal part of the ER.

Antifungal chemosensitization of sub-inhibitory concentrations of *L. origanoides* EO in association with fluconazole on *Candida* yeasts

Since the combined treatment of *S. cerevisiae* Δ*ycf*1 mutant with *L. origanoides* EO and Cd^{2+} exacerbated and amplified the upregulation of antioxidant stress genes, we decided to test whether the combined use of that EO with fluconazole could enhance in a synergistic way the antifungal action of that latter chemical. Low concentrations of *L. origanoides* essential oil (0.125 and 0.25 µL mL^{-1}) were used, well below the MIC for *Candida* species, since for example the concentrations used represented one-fifth and one-tenth of the MIC for *C. tropicalis*. The presence of low concentrations of *L. origanoides* essential oil (0.125 and 0.25 µL mL^{-1}), promoted a significant reduction of the fluconazole concentrations required to prevent the growth of microorganisms, illustrating the chemosensitization ability of that EO, i.e. enhancing the activity of a standard antifungal drug. For example, on *C. albicans*, the MIC of fluconazole in combination with 0.125 and 0.2 µL mL^{-1} concentrations of essential oil was found to be 0.03 and 0.01mg mL^{-1} versus 1.05 mL^{-1} when acting alone (Table V). Similarly, against *C. tropicalis* and *C. parapsilosis*, both concentrations of EO lowered the fluconazole MIC 35 and 12-fold, respectively. The concentrations of EO which were active in combinations with fluconazole did not present any effect when used alone, even in the case of *C. parapsilosis* treated with 0.25 µL mL^{-1} of the EO, a concentration 20% lower than the MIC.

Inhibition of microorganisms' growth by EOs relies on different mechanisms of action. The fungal cell death can be mediated either by increasing cell membrane ionic permeability, inhibition of germ tube formation, or alteration of ergosterol biosynthesis (Khan et al. 2010). The possible mechanism

Table V. Antifungal activity assay of sub-inhibitory concentrations of *L. origanoides* EO in association with Fluconazole against *Candida* species.

Microorganisms	MIC (mg mL^{-1})		
	Flu	Flu + EO 0.125 µl mL^{-1}	Flu + EO 0.25 µl L-1
C. albicans	1.05	0.03	0.01
C. tropicalis	1.05	0.03	0.03
C. parapsilosis	0.12	0.01	0.01

MIC - Minimum Inhibitory Concentration; Flu – Fluconazole.
Each concentration of combined treatment was assessed in triplicate.
The inhibition of yeast growth was revealed by the lack of reduction of resazurin to refazurin.

of cell death by induction of oxidative stress, characterized by elevated levels of free radicals (ROS), has been identified (Khan et al. 2011). The anti-*Candida albicans* activity of *Anethum graveolens* EO was causally linked with induction of endogenous ROS (Chen et al. 2013). The consequences of the pro-oxidant activity of EO or its compounds result in damage to biomolecules such as DNA, proteins, and lipids, and the consequent cellular death (Pedrajas et al. 2000, Aruoma 2003). The combinatorial therapy involving plant metabolites with antifungal drugs would be an effective complementary approach for the treatment of infections caused by drug-resistant *Candida*, and special attention has been given to natural compounds possessing chemosensitizing activity (Doke et al. 2014). In *C. albicans*, the exposure to carvacrol and thymol, for example, promoted changes in the enzymatic and non-enzymatic defense systems, suggesting their potency in inducing oxidative stress at low concentrations (Khan et al. 2015). A chemosensitizing agent does not necessarily require a great degree of antimicrobial potency to be effective; the co-application can enhance the effectiveness of commercial fungicide, debilitating the ability of a pathogen to develop resistance. The aim of the chemosensitization process, especially using natural compounds, is to decrease dosage levels of commercial drugs, with consequent lower costs and risks of negative side effects (Kim et al. 2011, Dzhavakhiya et al. 2012). Redox-active natural compounds that destabilize the fungal antioxidative system could act as potent chemosensitizing agents when co-applied with oxidative stress drugs, such amphotericin B (Kim et al. 2011, 2012) and miconazole (Bink et al. 2011). The co-application of some conventional industrial fungicides (triazoles and strobilurins), with certain phenolic acids or benzo analogs, which target cellular oxidative stress-response systems, enhanced the antifungal activity of these fungicides against pathogenic yeast and filamentous fungi causing invasive mycoses in humans or postharvest decay in agricultural products (Dzhavakhiya et al. 2012). Natural phenolic compounds were synergistic and enhanced the activity of commercial antifungal drugs against yeast strains of *Candida* and *Cryptococcus neoformans* (Faria et al. 2011). The combinations of carvacrol with azole antifungal were found synergistic against the growth of *C. albicans*. On the other hand, the treatment with thymol and azole together did not have any interaction (Doke et al. 2014).

The azole compounds have emerged as the principal drugs used in the treatment of *Candida* infections and particularly fluconazole, which targets the ergosterol biosynthesis pathway (a lipid present in fungal membranes) and remains among the most common antifungal drug. However, the

prolonged use of fluconazole has contributed to the development of drug resistance in *C. albicans* and other species (Guo et al. 2009).

The use of essential oils as antimicrobial agents offers a low risk in the development of resistance due to the presence of different chemical compounds that can act through different mechanisms of action, and thereby prevent the adaptation process (Daferera et al. 2003). A growing number of papers have begun to appear over the past decade showing that certain natural products, relatively non-toxic to humans, increase antifungal activity when co-administered with a commercial antifungal agent (Campbell et al. 2012). In this study, the gene expression analysis obtained on the model yeast *S. cerevisiae* supports the hypothesis that low concentrations of *L. origanoides* EO promote antifungal chemosensitization by induction of oxidative stress.

CONCLUSION

The antimicrobial potential of the EO of *L. origanoides* from the western Amazon was confirmed against *Candida* species. At sub-inhibitory concentrations, this oil promoted the over-expression of oxidative stress-resistance genes encoding proteins located in the cytoplasm (GSH1p), mitochondria (PRX1p), and the ER (KAR2p/BIP), as well as the induction of the DNA damage-responsive gene RNR3. The pro-oxidant effect of this EO was magnified and potentiated in combination with cadmium. Based on that proven pro-oxidative property, the combined use of that EO with Fluconazole could be tested on three different yeast species of the genus *Candida*, and that strategy resulted in a synergistic enhancement of the antifungal action of that azolic chemical. These results confirm the potential use of *L. origanoides* EO as a chemosensitizer agent that may contribute to enhancing the efficacy of conventional antifungal drugs, reducing negative side effects, and preventing the emergence of drug-resistant mutant strains.

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SUPPLEMENTARY MATERIAL

Table SI.

How to cite

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