RESEARCH ARTICLE

Induction of oxidative stress as a possible mechanism of the antifungal action of three phenylpropanoids

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Abstract

The increasing incidence of hospital-acquired infections caused by drug-resistant pathogens, host toxicity, the poor efficacy of drugs and high treatment costs has drawn attention to the potential of natural products as antifungals in mucocutaneous infections and combinational therapies. Moreover, cellular and subcellular targets for these compounds may provide better options for the development of novel antifungal therapies. Eugenol, methyl eugenol and estragole are phenylpropanoids found in essential oil. They are known to possess pharmacological properties including antimicrobial activity. Induction of oxidative stress characterized by elevated levels of free radicals and an impaired antioxidant defence system is implicated as a possible mechanism of cell death. An insight into the mechanism of action was gained by propidium iodide cell sorting and oxidative stress response to test compounds in *Candida albicans*. The extent of lipid peroxidation (LPO) of cytoplasmic membranes was estimated to confirm a state of oxidative stress. Activity levels of primary defence enzymes and glutathione were thus further determined. Whereas these compounds cause fungal cell death by disrupting membrane integrity at minimum inhibitory concentrations (MIC), sub-MIC doses of these compounds significantly impair the defence system in *C. albicans*. The study has implications for understanding microbial cell death caused by essential oil components eliciting oxidative stress in *Candida*. The formation of membrane lesions by these phenylpropanoids thus appears to be the result of free radical cascade-mediated LPO.

Introduction

There are several problems with the currently available antifungal drugs, including undesirable side effects, low efficacy and susceptibility to the development of drug resistance. This has a profound effect on human health and well-being. Although the infections caused by non-*albicans* *Candida* species have increased, *Candida albicans* is still the most common fungal pathogen and has been established as the predominant cause of virtually all types of candidiasis (Odds, 1988; Calderone, 2002). The increase in the prevalence of fungal infections has intensified the need for new and effective antifungals.

Natural products provide an unparalleled source of chemical scaffolds with diverse biological activities and have a profound impact on antimicrobial drug discovery. Estragole (1-allyl-4-methoxybenzene), methyl eugenol [1,2-dimethoxy 4-(2-propenyl) benzene] and eugenol [2-ethoxy-4-(2-propenyl) phenol] are essential oil components belonging to a group of naturally occurring phenylpropanoids (Fig. 1). We have earlier reported the fungicidal activity of eugenol, methyl eugenol and estragole (Ahmad *et al.*, 2010b; Khan *et al.*, 2010a, b) and have shown that they are negligibly toxic to human erythrocytes (Ahmad *et al.*, 2010b; Khan *et al.*, 2010c). However, fungal cell death is reported to be mediated either by the formation of lesions of the plasma membrane (PM) or the alteration of membrane permeability (Pinto *et al.*, 2009; Khan *et al.*, 2010a). Although the antifungal activity of these compounds is attributed to the inhibition of ergosterol biosynthesis, the

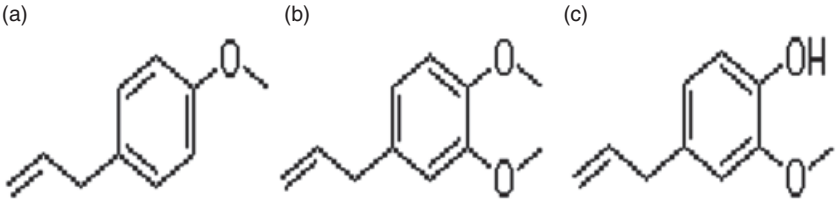

Fig. 1. Chemical structures of estragole (a), methyl eugenol (b) and eugenol (c).

cause of the resulting extensive membrane damage is still not completely clear (Pinto *et al.*, 2009; Ahmad *et al.*, 2010b; Khan *et al.*, 2010a). Studies into the fungicidal action of these compounds are needed, as there is at present limited information about their vast antimicrobial effects. One of the possible mechanisms of cell death is induction of oxidative stress, characterized by elevated levels of free radicals and an impaired antioxidant defence system of enzymatic and nonenzymatic components. Stress responses are important for the survival of the initial contact of fungal pathogens with the host immune system. They are also critical for the establishment of the disease (Missall *et al.*, 2004; Brown *et al.*, 2009) and the development of drug resistance in *C. albicans* (Cannon *et al.*, 2007). Understanding stress responses to natural compounds may aid us in formulating and developing novel and more effective antifungal combinations.

This study was conducted to illustrate the mechanism of action of three phenylpropanoids and to explore the oxidative stress response in *C. albicans* to sub-minimum inhibitory concentrations (MIC) of these natural compounds. The effect of these compounds on cytoplasmic membrane was studied by a flow cytometric (FCM) evaluation of the uptake of propidium iodide (PI) by the fungal cells. Activities of primary scavenging enzymes, mainly superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx), and the levels of the reduced tripeptide glutathione (GSH) were thus determined following treatment with these essential oil components at lower concentrations. Considering the universal role played by lipid peroxidation (LPO) products [thiobarbituric acid-reactive substances (TBARS)] in the formation of membrane lesions, the extent of LPO of cytoplasmic membrane was evaluated after treatment with subinhibitory concentrations of test compounds.

Materials and methods

Growth and materials

Candida albicans ATCC 90028 cells were grown in YPD media containing (w/v) 1% yeast extract, 2% peptone and 2% dextrose and maintained on YPD plates with 2.5% w/v agar. NADP, NADPH, oxidized and reduced forms of GSH, eugenol and methyl eugenol were purchased from Sigma and estragole from Aldrich. All the other chemicals and media constituents were purchased from Himedia (India).

MIC

The MIC was defined as the lowest concentration of eugenol, methyl eugenol or estragole that causes a 90% decrease in absorbance (MIC_{90}) compared with that of the control (without the test compound). MIC_{90} was determined *in vitro* in liquid media using the microbroth dilution method against *C. albicans* ATCC 90028 as described by NCCLS M27-A2 (NCCLS, 2002).

FCM analysis

FCM analysis was performed using PI, which penetrates cells with severe membrane lesions only, showing increased red fluorescence (Pina-Vaz *et al.*, 2001). Cells (10^6 mL^{-1}) were incubated at 35 °C up to the mid-exponential phase and then treated with different concentrations of test compounds (100 μg mL^{-1}, MIC, 4 MIC) along with positive (without a test compound/drug) and negative [2 μg mL^{-1} amphotericin B (AmB)] controls. The suspensions were centrifuged, washed and resuspended in phosphate-buffered saline (PBS). A 5-μL aliquot of PI solution in PBS was added to the cell suspensions to obtain a final concentration of 1 μg PI mL^{-1}. The samples were then incubated in the dark at 35 °C for 30 min. Unstained cells were always included as autofluorescence controls (Khan *et al.*, 2010a). Cell-associated fluorescence was measured using a FACS-Calibur flow cytometer (Becton Dickinson Biosciences) using a blue argon laser of 488 nm, 15 mW, and the results were analysed using CELLQUEST PRO software (Becton Dickinson Biosciences). Fluorescence in the FL2 channel (log red fluorescence, long-pass 650-nm filter) for PI was acquired and recorded, using logarithmic scales, for a minimum of 10 000 events per sample. For data analysis, quadrants were adjusted in raw data density plots for fluorescence intensity of control samples to include a maximum of 5% of the cells in the upper right quadrant and then used in the analysis of the remaining samples to quantify the percentages of cells showing altered fluorescence in comparison with the drug-free controls. Values are expressed as the mean ± SEM of all three respective categories (Fig. 2).

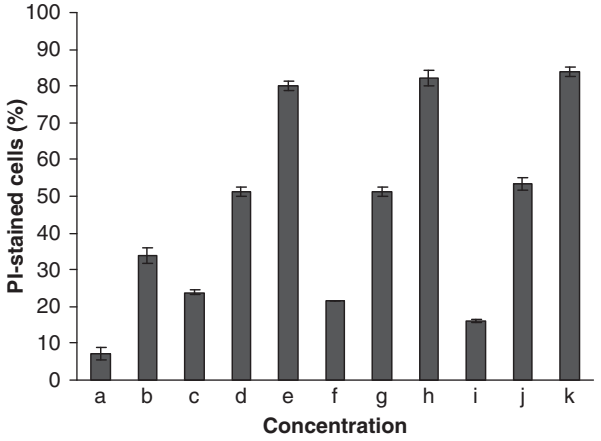

Fig. 2. Percentage of PI-stained cells analysed by flow cytometry without (a) and with exposure to amphotericin B (b), 100 μg mL^{-1} estragole (c), 200 μg mL^{-1} estragole (d); 800 μg mL^{-1} estragole (e); 100 μg mL^{-1} methyl eugenol (f); 350 μg mL^{-1} methyl eugenol (g); 1400 μg mL^{-1} methyl eugenol (h); 100 μg mL^{-1} eugenol (i); 500 μg mL^{-1} eugenol (j); and 2000 μg mL^{-1} eugenol (k) for a period of 30 min.

PM preparation for LPO

PMs used to estimate LPO were isolated from *C. albicans* ATCC 90028 using the method of Niimi *et al.* (2004), with minor modifications. Cells were grown in YPD liquid medium in the presence of 0, 10, 50 and 100 μg mL^{-1} of the phenylpropanoids (estragole, methyl eugenol or eugenol) at 30 °C with shaking at 200 r.p.m. up to the mid-log phase ($OD_{600\,nm} \approx 1.25$) and were then harvested. The cells were disrupted using a Braun MSK cell homogenizer. The homogenizing buffer contained 2 mM EDTA, 20% glycerol (v/v), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 50 mM Tris, pH 7.5. The homogenate was centrifuged at 1957 ***g*** for 10 min. After two washings, PM fractions were pelleted from the supernatant by centrifugation at 54 782 ***g*** (Beckman Ultracentrifuge Optima-L-90K U.C.) for 45 min. The PMs were suspended in 20% glycerol (v/v), 0.5 mM EDTA, 0.5 mM PMSF and 10 mM HEPES, pH 7.0. They were washed once by centrifugation and then stored at − 80 °C.

Preparation of cell-free extract

Cell-free extracts of treated and control *C. albicans* ATCC 90028 were prepared according to the method described by Jethwaney *et al.* (1997), with minor modifications. Cells (1 g wet weight) grown in YPD medium to the mid-exponential phase in the presence of 0, 10, 50 and 100 μg mL^{-1} of test phenylpropanoids, were suspended in 2 mL grinding medium (250 mM sucrose, 10 mM Tris HCl, pH 7.5, 1 mM PMSF) and 2-g glass beads (0.45 mm). The suspension was disrupted mechanically in a cell homogenizer (Braun MSK) by agitating it for nine cycles of 10 s each at 4000 vibrations min^{-1}. The homogenate was collected and centrifuged at 1000 ***g*** for 5 min at 4 °C to remove unbroken cells and glass beads and further centrifuged for 40 min at 15 000 ***g*** at 4 °C. The supernatant was used as a cell-free extract. Enzymes were assayed using a Systronics UV-VIS 117 (India) spectrophotometer.

LPO

The formation of malondialdehyde due to peroxidation was measured using the thiobarbituric acid method of Bernheim *et al.* (1948), with modifications. A 0.2-mL aliquot of PM was added to 2 mL of the reaction mixture containing 1.8 mL phosphate buffer (0.1 M, pH 7.4). The reaction mixture was incubated at 37 °C in a water-bath shaker for 1 h. The reaction was terminated by adding 1.0 mL of 10% trichloroacetic acid, followed by the addition of 1.0 mL of 0.67% thiobarbituric acid. All the tubes were kept in a boiling water bath for 20 min. The tubes were then cooled on ice and centrifuged at 2500 ***g*** for 10 min. The absorbance of the resulting supernatant, containing TBARS, was measured at 432 nm against a reagent blank at 25 °C. The results were expressed as nmol TBARS formed mg^{-1} protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm.

SOD

SOD activity was measured by the inhibition of pyrogallol autoxidation as described by Marklund & Marklund (1974). The enzyme SOD rapidly dismutates univalently reduced oxygen, the superoxide anion radical, and converts it to hydrogen peroxide (H_2O_2). For each sample, a control was taken in which 2.9 mL Tris buffer (50 mM, pH 8.5) was added to 0.1 mL pyragallol (20 mM). Both the solutions were freshly prepared at the time of the assay. In the test sample, 0.1 mL of cell-free extract was added to pyragallol and Tris buffer. After an induction period of 90 s, the inhibition of pyrogallol was monitored every 30 s for 3 min at a wavelength of 420 nm. Pyrogallol autoxidation per 3 mL assay mixture was calculated as follows:

$$\text{Unit of SOD mL}^{-1}\text{ of sample} = (A - B/A \times 50 \times 100) \times 10\,(\text{dilution factor})$$

where A is the difference in absorbance per 1 min in the control and B the difference in absorbance per 1 min in the test samples. Results were expressed in U mg^{-1} protein.

Catalase

Catalase activity was assayed using the method of Claiborne (1985). The assay mixture consisted of 1.99 mL phosphate buffer (0.05 M, pH 7.0), 1.0 mL of H_2O_2 (0.0019 M) and 10 μL cell-free extract in a total volume of 3.0 mL in a quartz cuvette. The decrease in absorbance due to the disappearance of H_2O_2 was recorded at 30-s intervals up to 3 min at

230 nm at 25 °C. Catalase activity was calculated in terms of μmol H_2O_2 consumed min^{-1} mg^{-1} protein using an extinction coefficient of 0.081×10^{-1} M^{-1} cm^{-1}.

Reduced GSH

Cytosolic GSH was determined using the method of Jollow *et al.* (1974), with slight modifications. A 1.0-mL aliquot of the cell-free extract was precipitated with 1 mL of 4% sulphosalicylic acid. The samples were then kept for 1 h at 4 °C and centrifuged at 1200 ***g*** for 15 min at 4 °C. The assay mixture contained 0.1 mL of the above supernatant, 2.7 mL of phosphate buffer (0.01 M, pH 7.4) and 0.2 mL of freshly prepared 5,5′-dithiobis-2-nitrobenzene (40 mg in 10 mL of 0.1 M phosphate buffer pH 7.4) in a total volume of 3 mL. The yellow colour developed due to the formation of 5-thio-2-nitrobenzoate was measured immediately at 412 nm at 25 °C. Results were expressed in μmol GSH mg^{-1} protein.

GPx

The specific activity of GPx was measured according to the method described by Mohandas *et al.* (1984). The reaction mixture consisted of 1.53 mL of phosphate buffer (0.05 M, pH 7.0), 0.1 mL of 1 mM EDTA, 0.1 mL of 1 mM NaN_3, 0.1 mL of 1 mM GSH, 0.1 mL of 0.2 mM NADPH and 0.01 mL of 0.25 mM H_2O_2. Cell-free extract (100 μL) was added to the reaction mixture for a final volume of 2.0 mL. The activity was measured in terms of a decrease in $A_{340\,nm}$ suggestive of the disappearance of NADPH at 30-s intervals for 3 min at 25 °C. The enzyme activity was calculated as μmol NADPH oxidized min^{-1} mg^{-1} protein using a molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1}.

Estimation of protein

Soluble protein was determined using the Coomassie Blue method of Bradford (1976) using bovine serum albumin as a standard.

Statistical analysis

Each experiment was performed three times and in triplicate. The results obtained were expressed in terms of mean ± SEM. Statistical analyses were performed using GRAPH PAD INSTAT software, and a *P*-value of < 0.05 was considered significant.

Results

MIC

MIC_{90} of eugenol, methyl eugenol and estragole against *C. albicans* ATCC 90028 was 500, 350 and 200 μg mL^{-1}, respectively.

FCM analysis of cell integrity

Flow cytometry was used to investigate the effect of the test phenylpropanoids on the membrane integrity of fungal cells. PI is a nucleic acid-binding fluorescent probe commonly used to evaluate the effect of drugs on cell membranes. Cells with severe membrane lesions leading to inherent loss of viability will internalize PI, resulting in an increase in red fluorescence. The PI penetration in yeast cells treated with AmB (negative control) and in cells treated with three concentrations of test compounds along with the positive control (without compound/drug) is shown in Figs 2 and 3. The damaging effect of test compounds was greater than that of AmB. The extent of damage to membrane integrity and therefore to PI uptake was found to be a function of MIC. Differences in the corresponding damage by the respective inhibitory concentrations of all three compounds were much smaller. Estragole, methyl eugenol and eugenol impair membrane integrity in 50–51% cells at their respective MICs. However, at their respective 2 × MICs, the cell leakage was observed to be 78–82%. It is important to note that at 100 μg mL^{-1}, maximum internalization of PI was elicited by estragole, followed by methyl eugenol and then eugenol. However, the order was found to be altered (methyl eugenol > eugenol > estragole) when the PI uptake was seen as a function of MIC (100 μg mL^{-1} = 1/2 MIC estragole, 1/3 MIC methyl eugenol and 1/5 MIC eugenol).

Response to oxidative stress

The three test phenylpropanoids modulate oxidative stress parameters in *C. albicans*, namely LPO, the activity of primary defence enzymes, namely SOD, catalase and GPx, and the levels of the nonenzymatic defence component GSH, as described in Table 1. The above studies were performed with 10, 50 and 100 μg mL^{-1} concentrations of test phenylpropanoids. LPO increased 2.7–3.8-fold in the presence of 10–100 μg mL^{-1} estragole (Table 1). At 10 μg mL^{-1}, the most destructive compound proved to be estragole. Methyl eugenol-treated cells showed a 1.98–5.52-fold increase in LPO. Eugenol-treated cells were increased 1.76–4.93-fold compared with the control cells. The sequence of the pro-oxidant nature of these compounds was thus methyl eugenol > eugenol > estragole.

SOD activity was increased significantly, most profoundly by methyl eugenol, followed by eugenol and estragole. A 1.57–1.83-fold increase in activity was observed in estragole-treated cells. There was a 1.51–2.11-fold increase post-treatment with methyl eugenol and a 1.64–2.11-fold increase with eugenol. A significant increase in catalase activity was observed in cells treated with methyl eugenol (2.8–3.27-fold). In the other two cases, the activity increased only 1.99-fold with estragole and 1.69-fold with eugenol. This increase in catalase activity indicates increased peroxide formation

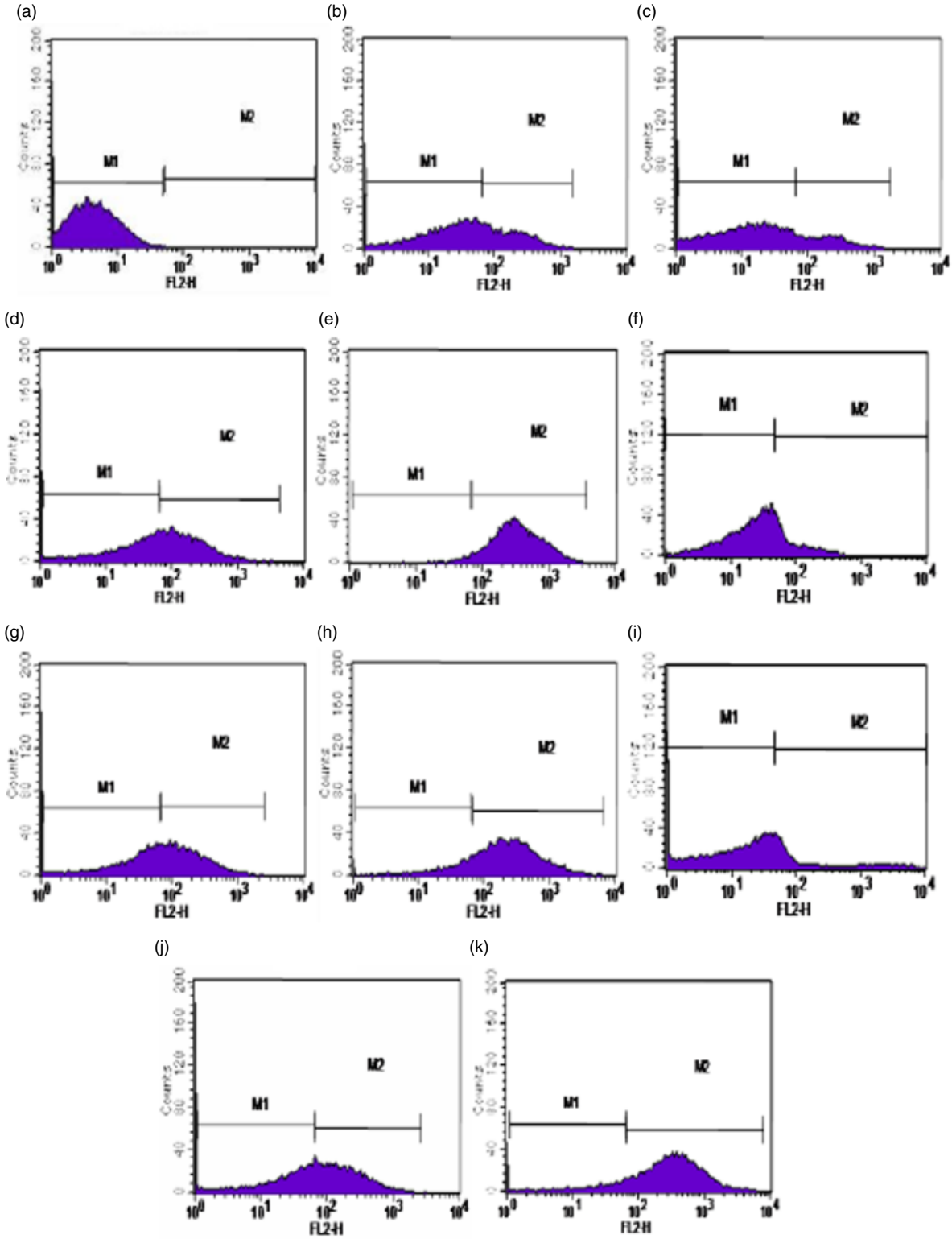

Fig. 3. Histograms of *Candida albicans* ATCC 90028 for samples treated with increasing concentrations of estragole, methyl eugenol and eugenol; untreated control cells (a); cells treated with AmB (b); 100 μg mL^{-1} of estragole (c); 200 μg mL^{-1} of estragole (d); 800 μg mL^{-1} of estragole (e); 100 μg mL^{-1} of methyl eugenol (f); 350 μg mL^{-1} of methyl eugenol (g); 1400 μg mL^{-1} of methyl eugenol (h); 100 μg mL^{-1} of eugenol (i); 500 μg mL^{-1} of eugenol (j); and 2000 μg mL^{-1} of eugenol (k). The histograms show in the log-mode the relative fluorescence intensities of *C. albicans* cells marked with PI. By setting the gate M1, morphologically intact cells observed with a higher relative fluorescence intensity are marked, whereas in the gate M2, dead, nonviable cells are marked.

Table 1. Oxidative stress response to estragole, methyl eugenol and eugenol in *Candida albicans* ATCC 90028

		Estragole (μg mL^{-1})			Methyl eugenol (μg mL^{-1})			Eugenol (μg mL^{-1})		
Oxidative stress parameters	Control†	10	50	100	10	50	100	10	50	100
LPO (nmol TBARS mg^{-1} protein)	233.338 ± 19.398	629.221 ± 7.138*	669.415 ± 21.976*	887.283 ± 53.346*	462.036 ± 25.056*	963.418 ± 21.197*	1288.235 ± 75.835*	411.139 ± 48.76*	679.375 ± 54.406*	1150.494 ± 62.688*
SOD (U mg^{-1} protein)	22.4782 ± 1.55	35.3731 ± 1.288*	39.6648 ± 1.305*	41.1772 ± 2.564*	33.8416 ± 1.286*	39.3483 ± 1.014*	47.4993 ± 1.618*	36.8205 ± 1.688*	41.1245 ± 1.372*	47.3820 ± 1.206*
Catalase (μmol H_2O_2 min^{-1} mg^{-1} protein)	5.6210 ± 0.676	6.9167 ± 0.378*	8.7396 ± 0.357*	11.1693 ± 0.328*	15.7427 ± 0.571*	16.8296 ± 0.538*	18.3642 ± 0.499*	5.8137 ± 0.563	7.3167 ± 0.380*	9.5145 ± 0.354*
GPx (μmol NADPH oxidized min^{-1} mg^{-1} protein)	9.7712 ± 0.055	21.166 ± 0.753*	9.8333 ± 0.656*	9.3935 ± 0.472*	23.9556 ± 0.651*	14.8611 ± 0.898*	9.4906 ± 0.773*	39.1807 ± 0.746*	24.1393 ± 0.460*	13.0643 ± 0.751*
GSH (μmol mg^{-1} protein)	0.3853 ± 0.034	0.3156 ± 0.05*	0.2316 ± 0.044*	0.1015 ± 0.019*	0.3406 ± 0.024*	0.3242 ± 0.028*	0.1974 ± 0.039*	0.3174 ± 0.027*	0.3106 ± 0.032*	0.1981 ± 0.028*

Each experiment was performed three times and in triplicate. Results obtained were expressed in terms of mean ± SEM.
†Control represents cells not treated with the test compounds.
*Significant reduction compared with controls ($P < 0.05$) after Student's *t* test.

by phenylpropanoids, the production being maximal in methyl eugenol.

Significant amounts of GSH depletion were observed with all the tested concentrations of phenylpropanoids. GSH depletion was greater in cells treated with estragole than in those treated with the other phenylpropanoids: a reduction of 18–74% with estragole, 12–49% with methyl eugenol and 18–49% with eugenol. The GSH depletion response of cells to methyl eugenol and eugenol was similar. GPx activity in cells treated with different concentrations of eugenol resulted in an initial 4.01-fold increase at 10 μg mL^{-1}, followed by a significant decrease with increasing concentrations of the compound. A similar response was also observed in the other two cases: an initial 2.17-fold increase with estragole and a 2.45-fold increase with methyl eugenol at 10 μg mL^{-1}, followed by a significant decrease with increasing concentrations of the compounds.

Discussion

Studies on the antifungal activity of essential oil components have been restricted to membrane damage only, as can be seen from the extensive research carried out in the past few decades. However, a concrete mechanism for their antimicrobial action has yet to be explained. Eugenol and methyl eugenol are potent fungicidals against fluconazole-sensitive and -resistant *Candida* isolates, with MIC ranges of 475–500 and 340–350 μg mL^{-1}, respectively (Ahmad *et al.*, 2010a). Estragole, however, has been shown to be more potent in a MIC range of 125–200 μg mL^{-1} against *Candida* isolates (Khan *et al.*, 2010a). The penetration of PI into the cells was facilitated by the presence of all three phenylpropanoids at their respective inhibitory and subinhibitory concentrations (Figs 2 and 3). At a sub-MIC concentration of 100 μg mL^{-1}, methyl eugenol and eugenol showed very convincing PI incorporation. Although estragole showed the maximum PI uptake of all the three compounds by *C. albicans*, it did not deliver the expected impairment of membrane as projected from its half MIC (MIC 200 μg mL^{-1} for ATCC 90028). This result suggests an appreciable potency of both eugenol and methyl eugenol even at lower concentrations.

Oxidative stress, associated with elevated reactive oxygen species (ROS), is one of the most sensitive predictors of cell death. LPO is one of the most important expressions of oxidative stress induced by ROS. ROS readily react with unsaturated lipids and produce polar lipid hydroperoxides that can cause increased membrane fluidity by disturbing hydrophobic phospholipids (Aikens & Dix, 1993; van Ginkel & Sevanian, 1994). *Candida albicans* contains about 70% polyunsaturated lipids (Georgopapadakou *et al.*, 1987) and hence the involvement of lipid peroxides and their products leading to the structural and functional deformity of the

membrane is highly likely. Accumulation of TBARS including lipid hydroperoxides and aldehydes is representative of lipid damage. This accumulation increased considerably in cytoplasmic membranes in cells treated with the three compounds (Table 1), indicating significant induction of oxidative stress. Both methyl eugenol and eugenol cause enormous damage to the lipids in comparison with estragole (Table 1), suggesting that they are more potent than estragole. This result complements the findings of fluorescence-activated cell sorting (FACS) analysis of PI incorporation. Another fungal membrane component, ergosterol, besides being crucial to maintain and regulate membrane structural and functional integrity, also inhibits LPO (Wiseman *et al.*, 1993). All three phenylpropanoids completely inhibit ergosterol biosynthesis in *Candida* (Ahmad *et al.*, 2010b; Khan *et al.*, 2010c). As shown in the present study, the phenylpropanoids therefore facilitate LPO in two ways: firstly, by inhibiting ergosterol biosynthesis, and secondly, by inducing oxidative stress. As a result, the membrane becomes highly permeable and incorporates PI.

It is reasonable to envisage the involvement of oxidative stress in the antifungal activity of these phenylpropanoids, as the beneficial health effects of eugenol and related compounds are shown to be attributed mainly to free radical scavenging, metal chelating and modulating cell signalling pathways (Yeh *et al.*, 2008; Kaur *et al.*, 2010); however, the antimicrobial effects in this context are not fully characterized. Although all aerobically growing organisms are continuously exposed to reactive oxidants, oxidative stress occurs only when the concentration of the oxidants increases beyond the antioxidant capacity of the cells (Fridovich, 1998). The induction of strong SOD and catalase activity in response to enhanced levels of ROS plays an important role in the defence mechanisms against oxidative stress in aerobic organisms (Angelova *et al.*, 2005). SOD is the primary defence enzyme for scavenging free radicals in *Candida* (Martchenko *et al.*, 2004). The phenylpropanoids under investigation increase SOD activity in the same order (i.e. methyl eugenol > eugenol > estragole) observed with LPO. Detoxification of H_2O_2 is a fundamental aspect of the cellular antioxidant responses in which catalase plays a major role, producing H_2O and O_2. The activity of catalase was found to be increased significantly by all three phenylpropanoids, with methyl eugenol showing evidence of the highest increase, followed by eugenol and estragole.

The generation of peroxides and hydroperoxides stimulates the cell antioxidant armoury, including GPx, catalase and GSH (reduced form), to counteract damaging effects. GSH is particularly vital for the normal cellular function and constitutes one of the physiologically important mechanisms to curtail the progression of cell damage. The decreased level of GSH, followed by the treatment of *Candida* cells with the test compounds (Table 1) was observed to be significant. It has been reported that eugenol has the ability to form phenoxyl radical, which, upon peroxidation, further forms reactive eugenol quinone methide (Thompson *et al.*, 1989, 1998), whose stability controls the degree of cytotoxicity (Fujisawa *et al.*, 2002). GSH depletion, observed in the present study (Table 1), thus appears to be a consequence of the reactivity of quinone methide towards *C. albicans* (Thompson *et al.*, 1989; Thompson & Bolton, 2009). It is therefore reasonable to speculate that reactive metabolite(s) of these phenylpropanoids might be involved in their antimicrobial activity.

The activity of GPx increased with the treatment of $10\,\mu g\,mL^{-1}$ of test compounds, but on increasing their concentrations, a sudden decline in the activity was observed (Table 1). An apparent decline after a strong increase can be explained by the decreased levels of GSH induced by increasing concentrations of phenylpropanoids; being a substrate, it is a limiting factor of GPx activity. The secondary explanation for the decreased GPx activity could be the enzyme's inactivation by the test compounds or its decreased production under the influence of test compounds. A decrease in GPx activity results in an increase in the concentration of H_2O_2 in treated cells, which in turn may stimulate an increased activity of catalase, as observed in the present study.

Methyl eugenol and eugenol induced the greatest damage to lipids observed as an increase in LPO and internalization of PI by FACS analysis. Both the compounds thus produce ROS, perhaps a phenoxyl radical, to induce high levels of oxidative stress in *C. albicans*. The oxidative stress induced by estragole appears to be mediated primarily by the declining levels of GSH. The present study shows that extensive LPO causes disintegration of the membrane when *Candida* cells are exposed to three naturally occurring phenylpropanoids: methyl eugenol, eugenol and estragole. To our knowledge, this study is the first to implicate the induction of oxidative stress as a possible mechanism of the fungicidal effects of phenylpropanoids.

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Authors' contribution

A.K. and A.A. contributed equally to this work.

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