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Antifungal and antioxidant activities of the phytomedicine pipsissewa, *Chimaphila umbellata*

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Abstract

Bioassay-guided fractionation of *Chimaphila umbellata* (L.) W. Bart (Pyrolaceae) ethanol extracts led to the identification of 2,7-dimethyl-1,4-naphthoquinone (chimaphilin) as the principal antifungal component. The structure of chimaphilin was confirmed by ^{1}H and ^{13}C NMR spectroscopy. The antifungal activity of chimaphilin was evaluated using the microdilution method with *Saccharomyces cerevisiae* (0.05 mg/mL) and the dandruff-associated fungi *Malassezia globosa* (0.39 mg/mL) and *Malassezia restricta* (0.55 mg/mL). Pronounced antioxidant activity of *C. umbellata* crude extract was also identified using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, suggesting this phytomedicine has an antioxidant function in wound healing. A chemical-genetic profile was completed with chimaphilin using ~4700 *S. cerevisiae* gene deletion mutants. Cellular roles of deleted genes in the most susceptible mutants and secondary assays indicate that the targets for chimaphilin include pathways involved in cell wall biogenesis and transcription.

Keywords: *Chimaphila umbellata*; *Malassezia*; Antifungal mode of action; Antioxidant activity; Chemical-genetic profile; 2,7-Dimethyl-1,4-naphthoquinone

1. Introduction

New antifungal drugs with distinct modes of action need to be identified because of increasing incidence of fungal resistance to existing antibiotics (Loeffler and Stevens, 2003). Plant secondary metabolites have great potential as a source of effective antifungal agents (Duarte et al., 2004). As examples, plant-derived compounds such as hydroquinones and naphthoquinones (lapachol, juglone), sesquiterpenes (cinnamodial, capsidiol), and alkaloids, such as berberine, have shown diverse antimicrobial activities, including antifungal activities. An advantage to the approach of using ethnobotanical leads to identify compounds with antimicrobial activity is that many of these remedies have been used by traditional healers for thousands of years with few or no adverse effects.

Chimaphila umbellata (L.) W. Bart (Pyrolaceae), commonly known as pipsissewa or umbellate wintergreen, has been used by First Nations Peoples of eastern Canada as a traditional medicine for infections, inflammations of various kinds, kidney stones, gonorrhea, stomachache, backache, and coughs; it has also been used as a blood

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purifier, diuretic and astringent (Arnason et al., 1981; Chevalier, 1996; Marles et al., 2000). Some of the trends seen in this pattern of usage suggest that antimicrobial activity is exerted. Jones et al. (2000), in a survey of potential antifungal phytomedicines used by First Nations Peoples of eastern Canada, reported that extracts of *C. umbellata* had the greatest antifungal activity of all the materials analyzed. *C. umbellata* contains significant amounts of several known biologically active compounds, including arbutin, sitosterol, ursolic acid and chimaphilin (**1**) (Foster and Duke, 1990). Thus, *C. umbellata* is an excellent candidate for further investigation as a topical antifungal agent. In this paper, we use bioassay-guided isolation to determine that chimaphilin (**1**) is the main antifungal principle in *C. umbellata*. We evaluated the antifungal activity of chimaphilin (**1**) against fungi involved in causing the scalp condition commonly referred to as dandruff. Antioxidant activity was also examined with *C. umbellata* extracts, since it is relevant to the healing of skin injuries. A chemical-genetic screen of ~4700 *Saccharomyces cerevisiae* gene deletion mutants indicated that chimaphilin (**1**) interferes with cell wall, mitochondrial, transcription and other functions. Two secondary assays were performed that further supported the inhibitory effects of chimaphilin (**1**) on cell wall and transcription functions.

2. Results and discussion

About 22 g of dried extract was recovered from an 80% EtOH extract of 100 g of dried *C. umbellata*. After fractionation using solvents of different polarity, only the hexane fraction (0.676 g) showed significant antifungal activity. The hexane fraction was resolved into 14 UV–visible bands by thin layer chromatography. We evaluated antifungal activity of each band with and without UV light exposure since enhanced activities can occur through UV-induced type I and type II reactions (Wainwright, 1998). Only the first and second (i.e., the least polar) bands had antifungal activity based on disc diffusion assays with *S. cerevisiae*, and inhibitory activities did not significantly differ with or without UV light exposure (Table 1). Additional antifungal compound was obtained by column chromatography from 1.26 g of hexane extract to yield 120 mg of yellow needle-like crystals. A second column chromatography procedure was performed to purify 48.7 mg of the antifungal compound, identified as 2,7-dimethyl-1,4-naphthoquinone ($C_{12}H_{10}O_2$, chimaphilin (**1**)).

Table 1
Inhibition zone diameters (mm) from disc diffusion assays with *S. cerevisiae* S288c and *C. umbellata* extracts

Fraction[a]	UV+	UV−
Crude Extract (2.0 mg/disc)	9.0	8.0
Hexane (2.0 mg/disc)	17.0	12.0
TLC 1 (0.6 mg/disc)	26.5	20.5
TLC 2 (0.6 mg/disc)	17.0	17.5
Berberine (2.0 mg/disc)	18.5	15.0
Carrier solvent	0	0

[a] Fractions include crude and hexane extracts, and TLC bands 1 and 2, along with positive (berberine) and negative (carrier solvent) controls, as described in text. Treatments were with (UV+), or without (UV−) ultraviolet irradiation (10 W/m^2, 320–400 nm) for 2 h.

The identification of the antifungal compound as chimaphilin (**1**) was achieved by ^{1}H and ^{13}C NMR spectroscopic analyses. The ^{1}H and ^{13}C spectroscopic data were consistent with literature values for this compound (Saxena et al., 1996). A molecular weight of 186 g/mol was obtained by electron impact mass spectrometry (EIMS). This compound (**1**) (Fig. 1) is of significant interest since it was previously reported to have antimicrobial, antihemorrhagic and vitamin K-like activities (Hausen and Schiedermair, 1988). It also displays anti-inflammatory and analgesic properties (Kosuge et al., 1985). Its modes of action are unknown. We determined antifungal Minimum Inhibitory Concentration (MIC) values for chimaphilin (**1**) against *S. cerevisiae*, *Malassezia globosa* and *Malassezia restricta*. These values were in the range of 0.05–0.55 mg/mL (Table 2) and were consistent with previous MIC estimates with eight fungal species including *S. cerevisiae* (Saxena et al., 1996). Together with this previous report, our results with *Malassezia* strains indicate that chimaphilin (**1**) has broad-spectrum antifungal activities, including activities against fungal pathogens of humans.

Good antioxidant activity by *C. umbellata* crude extract was observed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. The concentration of crude extract required to change the absorbance with respect to the control value by 50% (IC_{50}) was determined to be ~142.1 ± 3.6 ppm (Table 3). These results demonstrated that *C. umbellata* extract possesses an interesting antioxidant activity consistent with traditional uses such as in anti-inflammatory remedies and wound healing. We hypothesize that the antioxidant activity of *C. umbellata* extract is associated with the presence of flavonoids such as quercetin and isoquercetin in the plant (Duke, 1992). However, chimaphilin (**1**) may also play an antioxidant role, since other naphthaquinones such as plumbagin and shikonin have shown promising antioxidant activities (Tilak et al., 2004; Nishizawa et al., 2005).

The gene deletion array (GDA) analysis performed provides a preliminary chemical-genetic profile for the antifungal activity of chimaphilin (**1**). The 54 *S. cerevisiae* deletion mutants showing the greatest degree of susceptibility in this analysis were selected, and the cellular roles of

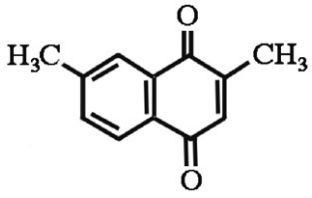

Fig. 1. Chimaphilin (**1**) molecular structure (mass 186.07 g/mol).

Table 2
MIC (mg/ml) of crude extract and chimaphilin extracted from *C. umbellata*

Strain	Crude extract	Chimaphilin
S. cerevisiae (S288c)	0.55	0.05 ± 0.01
M. globosa (CBS 9568)	ND[a]	0.39 ± 0.04
M. restricta (CBS 9569)	ND	0.55 ± 0.09

[a] ND = not determined.

Table 3
Antioxidant activity of *C. umbellata* crude extract

Extract	IC_{50} (ppm ± SE)	Slope	r_2	*P*-value
C. umbellata	142.1 ± 3.6	0.21	0.96	0.022
Quercetin (+control)	17.2 ± 0.1	2.75	0.99	<0.001
Ascorbate (+control)	22.1 ± 0.1	2.32	0.99	<0.001
Methanol (− control)	No effect			

the deleted genes were examined to determine possible cellular targets for chimaphilin (**1**) (Table 4). Exposure to chimaphilin (**1**) resulted in a minimum of a 70% reduction in colony size in each of the selected strains. The profile obtained reveals an interesting set of genes with diverse cellular functions falling into at least four general categories: stress response/multi-drug resistance, cell wall, mitochondrial and transcription functions. In the first category, the deletion of genes responsible for multi-drug resistance and genes that form part of the cell's normal stress response have been combined since deletion of these will likely produce an increased susceptibility to a wide variety of stresses, including drug treatments. Consequently, increased sensitivity to chimaphilin (**1**) caused by the deletion of these genes may be expected and therefore discarded from the analysis of the mode of activity of chimaphilin. Of the deletions with greater than 70% growth reductions, Pdr1p, Pdr11p, Stb5p, Tpo2p, and Vmr1p are all involved in multi-drug resistance. In addition, we place another gene connected to strong growth reduction, *PHO90*, into this group because of its membrane transporter activity and because it signals cell cycle arrest under low phosphate stress. The gene *UBI4* codes for ubiquitin, and makes up part of the cell's stress response, while Rpn10p is a sub-unit of the 19S regulatory particle of the 26S proteasome involved in ubiquitin-dependent protein degradation. Additional genes with deletion mutants showing strong chimaphilin (**1**) response include *HAL5*, *SOD1*, and *YVC1*, products of which are respectively involved in salt and pH tolerance, superoxide metabolism, and response to hyperosmotic shock.

The second deletion group, encompassing genes with functions associated with the cell wall or sporulation, is of special interest since these processes may be specific to fungi. Fungi are recognized as a sister group to animals and are more distantly related to plants. The development of antifungal compounds that do not harm either animal or plant hosts is difficult since all three eukaryotic groups have much in common biochemically. One of the defining characteristics of fungi, however, is the structure and composition of the cell wall. The development of compounds that target this cellular component may offer a high degree of specificity in the targeting of fungal pathogens. Eight genes in the profile appear to be related to cell wall function, suggesting that exposure to chimaphilin interferes with yeast cell wall biogenesis. These genes include *ECM8*, *ECM25*, *MNN10*, *MYO3*, and *SPR28*, which are involved in cell wall organization and assembly. Also included in this group are *SPR1* (encodes an exo-1,3-beta-glucanase) and *AUT7*, which are both linked to sporulation, and the endochitinase-encoding *CTS1*. Mutations in *MNN11*, while not directly connected in the literature to cell wall functions, increase sensitivity to Calcofluor White (CFW) when deleted. As CFW is a compound that binds to and interferes with cell wall function, a role in the cell wall's structure or assembly is predicted.

Since the above cell wall mutants could simply be more permeable to chimaphilin (**1**), and thus exhibit poor growth in our GDA experiments, we further tested whether chimaphilin (**1**) interferes with cell wall function through a physical stress assay using wild type yeast. Fig. 3A shows how the combination of chimaphilin (**1**) with 30 or 40 s of exposure to 65 °C reduced the number of viable cells by 10-fold compared to EtOH (carrier control). The increased sensitivity to heat shock supports the hypothesis that chimaphilin (**1**) interferes with fungal cell wall functions; a rapid thermal expansion of cells likely results in increased frequencies of death when cell wall integrity is compromised.

At least 10 of the deletions causing a strong reduction in growth are associated with mitochondrial function. These include genes involved in the mitochondrial ribosome (*RSM28*), NADPH regeneration (*GND1*), and the mitochondrial membrane (*COX18*). Also included are *PIB2*, *FUN14*, *MPA43*, and ORFs YNL144C and YKL187C, which are genes of unknown function but which appear to encode mitochondrial proteins. Also in this group are proteins Vmr1p, involved in multi-drug resistance, and Sod1p, involved in stress responses.

In addition, deletion of any one of 10 transcription factor genes resulted in significant susceptibility to chimaphilin (**1**). Two of these, *STB5* and *PDR1*, are genes that regulate multidrug resistance and may therefore compromise stress response. However, other deletions, including those for genes encoding the transcription factors Dep1p, Pgd1p, Opi1p, and Swi5p, and those for the transcriptional elongation factors Dst1p and Set2p, play quite different roles within the cell. The products of two additional transcription factors associated with susceptibility, Spt3p and Sgf73p, are both linked to SAGA, a multiprotein acetyltransferase complex involved in transcription regulation.

Strikingly, eight of the 10 transcription factors (including Pdr1p and Stb5p) are components of the core 14-protein interaction network identified within the profile (interaction information from SGD, Hong et al., 2006; see graphic abstract). This interaction network indicates that transcription processes in yeast are inhibited by

Table 4
Deletion mutants identified as most sensitive to chimaphilin

Gene deletion (ORF)[a]		Gene function	GDA % growth reduction
Stress response and multi-drug resistance			
PDR1	(YGL013C)	Zinc-finger transcription factor; together with Pdr3p regulates the expression of a gene network involved in multiple drug resistance	75.4
PDR11	(YIL013C)	Membrane transporter of the ATP-binding cassette (ABC) superfamily	87.7
STB5	(YHR178W)	Protein with similarity to transcription factors	74.0
TPO2	(YGR138C)	Member of the multidrug-resistance 12-spanner (DHA12) family of the major facilitator superfamily (MFS-MDR)	79.3
*VMR1**	(YHL035C)	Member of the ATP-binding cassette (ABC) superfamily	90.1
*PHO90**	(YJL198W)	Protein with strong similarity to Pho87p and Ynr013p, member of the phosphate permease family of membrane transporters	74.2
UBI4	(YLL039C)	Mature ubiquitin is cleaved from polyubiquitin (Ubi4p) or from fusion with ribosomal proteins Rps31p, Rpl40Ap, or Rpl40Bp	78.9
RPN10	(YHR200W)	Non-ATPase component of the 26S proteasome complex	77.1
HAL5	(YJL165C)	Serine/threonine protein kinase involved in salt and pH tolerance	102.5
SOD1	(YJR104C)	Copper-zinc superoxide dismutase	96.4
*YVC1**	(YOR087W)	Protein of unknown function	96.1
Cell wall and/or sporulation			
ECM8	(YBR076W)	Protein possibly involved in cell wall structure or biosynthesis	78.2
ECM25	(YJL201W)	Protein possibly involved in cell wall structure or biosynthesis	94.8
MNN10	(YDR245W)	Subunit of the Anp1p-Hoc1p-Mnn10p-Mnn11p-Mnn9p mannosyltransferase complex, M-Pol II	99.4
MYO3	(YKL129C)	Myosin type I, may play a role in cellular growth or polarity that is partially redundant with the role of Myo5p; has an SH3 domain	94.5
SPR28	(YDR218C)	Septin-related protein expressed during sporulation	71.4
SPR1	(YOR190W)	Sporulation-specific exo-beta-1,3-glucanase	73.1
*AUT7/ATG8**	(YBL078C)	Protein that mediates attachment of autophagosomes to microtubules; in combination with Aut2p, also required for sporulation	76.1
CTS1	(YLR286C)	Endochitinase	79.3
MNN11	(YJL183W)	Subunit of the Anp1p-Hoc1p-Mnn10-Mnn11p-Mnn9p mannosyltransferase complex	72.9
Mitochondrial functions			
RSM28*	(YDR494W)	Protein of unknown function	80.7
GND1	(YHR183W)	6-Phosphogluconate dehydrogenase, decarboxylating, converts 6-phosphogluconate + NADP to ribulose-5-phosphate + NADPH + CO2	80.5
COX18	(YGR062C)	Protein required for activity of mitochondrial cytochrome oxidase	80.5
PIB2	(YGL023C)	Protein of unknown function, has similarity to Fab1p and Vps27p	92.0
FUN14*	(YAL008W)	Protein of unknown function	80.2
MPA43	(YNL249C)	Protein that leads to high levels of PDC1 (pyruvate decarboxylase) expression when overproduced	70.7
	(YNL144C)	Protein of unknown function	71.5
	(YKL187C)	Protein with similarity to 4-mycarosyl isovaleryl-CoA transferase	72.2
Transcription factors			
DEP1	(YAL013W)	Regulator of phospholipid metabolism	75.1
PGD1	(YGL025C)	Component of RNA polymerase II holoenzyme, involved in both positive and negative regulation of transcription	78.6
OPI1	(YHL020C)	Negative regulator of phospholipid biosynthesis pathway	80.7
SWI5	(YDR146C)	Transcription factor that controls cell cycle-specific transcription of HO, has three tandem C2H2-type zinc fingers	72.4
DST1	(YGL043W)	Transcription elongation factor S-II; DNA strand transfer protein catalyzing homologous DNA strand exchange, DNA metabolism	97.3
SET2	(YJL168C)	Protein involved in repression of basal transcription of GAL4	79.1
SPT3	(YDR392W)	Component of the nucleosomal histone acetyltransferase (Spt-Ada-Gcn5-Acetyltransferase or SAGA) complex, member of TBP (TATA-binding protein) class of SPT proteins	83.6
*SGF73**	(YGL066W)	Protein with similarity to *Dictyostelium discoideum* G-box-binding factor	85.1

(*continued on next page*)

Table 4 (*continued*)

Gene deletion (ORF)[a]		Gene function	GDA % growth reduction
Genes with additional functions			
RGA1	(YOR127W)	Rho-type GTPase-activating protein (GAP) for Cdc42p	85.7
SAC7	(YDR389W)	GTPase-activating protein for Rho1p	87.3
EFT2	(YDR385W)	Translation elongation factor EF-2, identical to Eft1p; contains diphthamide which is not essential for its activity	96.9
DPH2	(YKL191W)	Diptheria toxin resistance protein, required for diphthamide biosynthesis	70.9
XRS2	(YDR369C)	Protein required for DNA-repair and meiotic recombination and telomere maintenance	71.5
*ESC4/RRT107**	(YHR154W)	Protein involved in chromatin silencing, DNA metabolism	71.6
ZIP2	(YGL249W)	Protein involved in meiotic recombination, chromosome synapsis, and synaptonemal complex formation	76.4
ERG28	(YER044C)	Protein involved in synthesis of ergosterol	71.1
NPT1	(YOR209C)	Nicotinate phosphoribosyltransferase (NAPRTase), first step in the Preiss-Handler pathway leading to the synthesis of NAD	79.4
STE14	(YDR410C)	Farnesyl cysteine	82.1
WHI3	(YNL197C)	Protein involved in regulation of cell size; has 1 RNA recognition (RRM) domain	91.0
Genes with unknown function			
OCA5*	(YHL029C)	Protein of unknown function	88.7
*VPS63**	(YLR261C)	Protein of unknown function	97.3
	(YBR255W)	Protein of unknown function	83.6
	(YGR137W)	unknown/ Protein with similarity to tomato extensin PIR	77.9
	(YJL142C)	Protein of unknown function	76.2
	(YOL013W-A)	Protein of unknown function, has similarity to Ylr334p and Yol106p	75.4
	(YOL125W)	Protein of unknown function	74.2

[a] Source of gene function information is YPD (Hodges et al., 1999) and SGD (Hong et al., 2006) except where indicated by * = SGD only.

chimaphilin (**1**) but could also be an artifact associated with these particular mutants. We therefore utilized a β-galactosidase reporter gene assay to monitor transcriptional activity in yeast grown with or without exposure to subinhibitory concentrations of chimaphilin (**1**). As seen in Fig. 3B, the addition of chimaphilin (**1**) to yeast cells resulted in a reduction of β-galactosidase activity in a dose dependent manner. Compared to the carrier solvent controls, significant reductions in β-galactosidase activities were observed when chimaphilin (**1**) was at 7.5 μg/mL ($P = 0.01$, paired-*t*-test) and 15 μg/mL ($P = <0.001$). Since these chimaphilin (**1**) concentrations did not inhibit yeast growth in our assays (data not shown), these results are consistent with the hypothesis that chimaphilin (**1**) interferes with transcription processes. However, from these experiments we cannot rule out the possibility that chimaphilin (**1**) interferes with protein synthesis. This later possibility is unlikely given the predominance of transcription factors in the core interaction network derived from our GDA data.

Once the genes associated with general stress response, cell wall, mitochondrial, and transcription functions are removed, there remain 18 genes in the profile. These have minor associations with other genes in the profile. For example, both Rga1p and Sac7p are GTPase-activating proteins, and both deletions show similar (between 85% and 90%) reductions in colony size. Protein Eft2p, a translational elongation factor, contains diphthamide, the production of which requires Dph2p. It is possible, therefore, that the reduction in growth seen with the *DPH2* deletion is caused by a reduction in Eft2p activity. *XRS2*, *ESC4* and *ZIP2* encode products involved in DNA damage repair or in recombination. Additional genes of known function whose deletion confers high chimaphilin susceptibility include *ERG28*, *NPT1*, *STE14* and *WHI3*. Finally, the list of vulnerable mutants includes those for *OCA5*, *VPS63*, YBR255W, YGR137W, YJL142C, YOL013W-a, and YOL125W, all genes of unknown function. Each of these deleted genes appears to have a role important in maintaining cellular integrity during exposure to chimaphilin (**1**).

2.1. Concluding remarks

The diversity of cellular processes affected by chimaphilin (**1**) that is evident in our GDA analysis suggests that the compound has multiple targets within the yeast cell. This is expected of an effective antifungal since multiple mutations in genes associated with diverse pathways would be necessary to gain resistance to the compound. Further assays are required to verify the inhibitory targets of chimaphilin (**1**). However, results from the two secondary assays performed support the notion that chimaphilin (**1**) interferes with cell wall and transcription processes, and provides confirmation of the veracity of the GDA approach as a whole. Significantly, activities that interfere with specifically fungal processes, such cell wall functions, are of special interest and clearly warrant further study.

3. Experimental

3.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were obtained on a Varian INOVA 500 spectrometer (Palo Alto, CA) with standard pulse sequences operating at 500 MHz in ^{1}H NMR and 125 MHz in ^{13}C NMR. $CDCl_3$ was used as solvent and TMS as internal reference. EIMS data were obtained on a Kratos concept II H mass spectrometer (Manchester, UK). UV spectra were performed in MeOH on a DU 640 Beckman–Coulter UV/visible spectrophotometer (Mississauga, ON).

3.2. Plant material and extract preparation

C. umbellata dried leaf and stem material was purchased from Richters Herb Specialists (Goodwood, ON). A voucher has been retained at the University of Ottawa herbarium (voucher #19901). About 100 g of dried plant material was extracted two times in 1 L of EtOH–H_2O (4:1, v/v) at room temperature overnight with shaking, filtered through Whatman #1 paper (Maidstone, UK) in a Buchner funnel and partitioned against hexane (1:2, v/v) to obtain two fractions. H_2O was added to the EtOH residue (1:1.5, v/v) and the mixture was washed with EtOAc (1:2, v/v); the resulting two fractions were separated and the organic extraction was dried by addition of Na_2SO_4. The three fractions were rotoevaporated at 45 °C to near dryness, then freeze dried for 48 h to achieve complete dryness.

3.3. Isolation and analysis of antifungal compound

Each fraction was tested using 2 mg/disc for antifungal activity by disc diffusion assays with *S. cerevisiae*. Significant antifungal activity was observed with the hexane fraction only, and this fraction was therefore further separated. Thin layer chromatography (TLC, 20×20 cm, 0.25 mm thickness, 60 F_{254} EMD Chemicals, Darmstadt, Germany) with hexane/EtOAc (3:1) as the mobile phase was used to resolve 14 bands under UV light. Each band was collected separately, dissolved in 10 mL of MeOH, filtered and dried (System AES2010, Savant Instruments, Holbrook, NY) and then weighed. Disc diffusion bioassays with *S. cerevisiae* (0.60 mg/disc) were again performed with each fraction to determine the antifungal activities.

To increase the yield of the active compound, 1.26 g of dried hexane fraction was dissolved in hexane and fractionated by silica gel CC (Davisil 62–230 mesh column, 38×2.5 cm column). The fractions were eluted using a stepwise gradient of increasing polarity consisting of 100 mL each of *n*-hexane, *n*-hexane/EtOAc (98:2 to 2:98), EtOAc. Twelve fractions were obtained from the combination of the 200 samples collected and were examined by TLC with hexane/EtOAc (3:1) as mobile phase. Column fractions that had compounds with similar Rf values were combined. To further purify the material, the antifungal fraction 3 (120 mg) was separated on a silica gel column (Davisil) prepared with *n*-hexane. Fractions were eluted using a stepwise gradient consisting of 50 ml each of *n*-hexane, *n*-hexane/CH_2Cl_2 (9:1 to 1:9), CH_2Cl_2. The single antifungal fraction from this second column yielded yellow needles (48 mg). Thus compound (**1**) (Fig. 1) was identified on the comparison of its ^{1}H NMR and ^{13}C NMR spectroscopic data to literature values.

3.4. Antifungal activity

3.4.1. Fungal strains

S. cerevisiae (S288c, Ontario Cancer Institute, Toronto, ON, Canada), *M. globosa* and *M. restricta* (Centraalbureau voor Schimmelcultures strains CBS 9568 and CBS 9569; Guého et al., 1996) were used in antifungal activity assays. *M. globosa* and *M. restricta* are commensal fungi that appear to be associated with the causation of dandruff (Gemmer et al., 2002) as well as atopic and seborrheic dermatitis and pityriasis versicolor (Crespo et al., 2000; Sugita et al., 2004; Batra et al., 2005).

3.4.2. Inoculum preparation

S. cerevisiae was cultured on YPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose [Difco, Detroit, MI]) or Sabouraud's dextrose medium (Difco) at 30 °C. *M. globosa* and *M. restricta* were cultured on Sabouraud's medium modified by addition of 2% olive oil and 1% glycerol at 30 °C, or Christensen's broth (Nakamura et al., 2000). All transfers of fungal strains were done under a biological containment hood (Bioclone 2, Microzone, Ottawa, ON).

3.4.3. Antifungal assays

The antifungal activity of crude extracts was tested using *S. cerevisiae* disc diffusion assays performed in triplicate. *S. cerevisiae* was inoculated into Sabouraud's broth medium and grown to an optical density of 600 nm (OD_{600}) of ~2.0 and diluted 1:200. Aliquots (100 μL) of the diluted broth cultures were spread over the surface of Sabouraud's agar plates. Paper discs (7.5 mm diameter) were impregnated with crude extract (2 mg) dissolved in 20 μL of solvent, allowed to air-dry and placed on the agar plates. Berberine (E. Merck, batch #119H0687, Darmstadt, Germany) was used as an antifungal positive control and the solvents (hexane, EtOAc, MeOH or EtOH–H_2O (6:4, v/v)), applied to the paper discs and allowed to air-dry, were used as negative controls. Each fraction was tested under two conditions: following UV-light irradiation (10 W/m^2 for 2 h using three 20 W black-light blue tubes, 320–400 nm range) and without UV irradiation (dark treatment). For the latter, plates were wrapped in aluminum foil. All treatments were subsequently incubated in the dark for 24 and 48 h at 30 °C, at which times growth inhibition zones were measured. Similarly, the antifungal activities of the 14 thin layer chromatography bands were tested using *S. cerevisiae* disc diffusion assays, performed in dupli-

cate, with the paper discs impregnated separately with 0.60 mg/disc of each fraction dissolved in MeOH (20 μL).

Minimum Inhibitory Concentration (MIC) assays were done in sterile 96-well plates using the method described by Ficker et al. (2003). *S. cerevisiae* was grown overnight in YPD broth, adjusted to OD_{600} ~0.8 and diluted 1:500 (to obtain ~10^3 CFU/ml). *M. globosa* and *M. restricta* were adjusted to OD_{545} ~ 1.0 and diluted 1:50 in Christensen's broth. Chimaphilin (**1**) was prepared at 3.126 mg/ml in EtOH–H_2O (15:85, v/v) and volumes of 20–90 μL of this solution were mixed with inoculum (~100 cells/well), brought up to 150 μL/well with medium and distributed into the last column of a microtiter plate. A three-fold-dilution was performed along the rows so that a concentration range of ~1.8 to 7.0×10^{-6} mg/mL was covered. The last column served as a drug-free control. The microtiter plates were incubated in the dark for 24 and 48 h at 30 °C and MICs were recorded as the concentration at which there was ⩾80% reduction in growth in comparison to wells with no inhibitor present. Growth reduction was determined by eye and by spectrophotometric optical density readings (OD_{600}, Spectra Max 340PC, Molecular Devices, Sunnyvale, CA).

3.4.4. Cell wall integrity assay

Two millilitres of a 10^4 cells/mL culture of S288C yeast in mid log phase was transferred to a culture tube and either chimaphilin (**1**) (3.0 μg/mL) or an equivalent volume of carrier solvent (EtOH) without compound was added. The cultures were incubated overnight with gentle shaking at 30 °C and the cell density was adjusted to 10^7 cells/mL. Cell wall disruption was done by heat-shock treatments in a 65 °C water bath for 0, 30 and 40 s. Following heat-shock, cell viability was estimated by drop-plate assays as described in Chen et al. (2003) using a 10-fold dilution series.

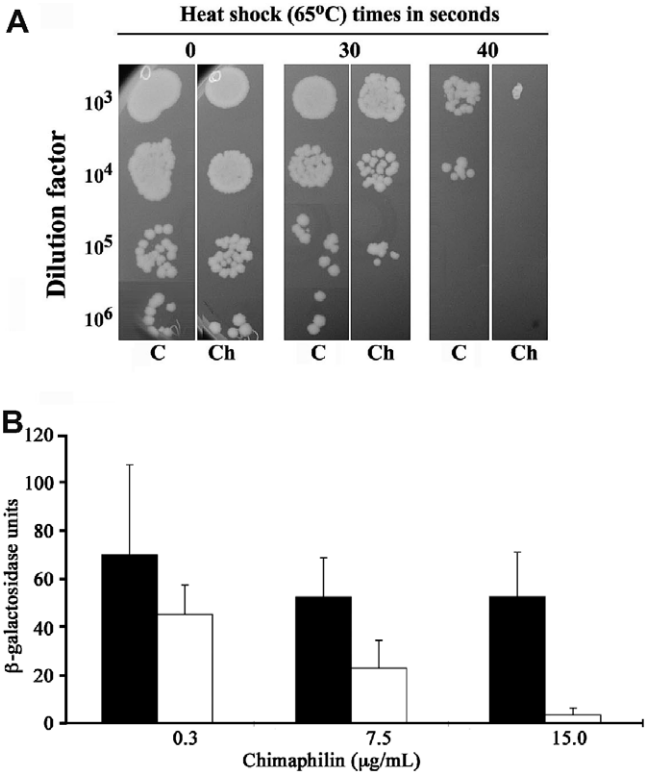

Fig. 3. Secondary assays support GDA-based indications that chimaphilin (**1**) inhibits cell wall and transcription functions in yeast. Panel A: Drop-plate assays of S288C treated with carrier control (C) or 3.0 μg/mL of chimaphilin (**1**) (Ch) followed by heat shock at 65 °C for 0, 30 or 40 s. Chimaphilin (**1**) treatments resulted in a 10-fold greater sensitivity to heat shock, which is predicted to stress cell wall integrity. Panel B: Decreases in β-galactosidase reporter gene expression in *S. cerevisiae* with increasing concentrations of sub-inhibitory levels of chimaphilin (**1**). β-Galactosidase activity ($n = 5$, ±SD) in yeast was significantly reduced at 7.5 and 15 μg/mL of chimaphilin (**1**) (open bars) relative to controls (carrier solvent, closed bars) supporting GDA-based results that chimaphilin (**1**) interferes with transcription processes.

3.4.5. β-Galactosidase expression assay

For this assay we used strain YBR115CΔ that contains the β-galactosidase reporter gene on plasmid pUKC815. Mid-log phase cells were grown in SC-ura medium with subinhibitory amounts of chimaphilin, or with the carrier control (EtOH), for 4 h at 30 °C. Yeast cells were harvested by centrifugation, resuspended in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 0.27% β-mercaptoethanol, pH 7.0) and kept on ice. Initial cell density was measured at OD_{600} and β-galactosidase activity was measured as described in Miller (1972).

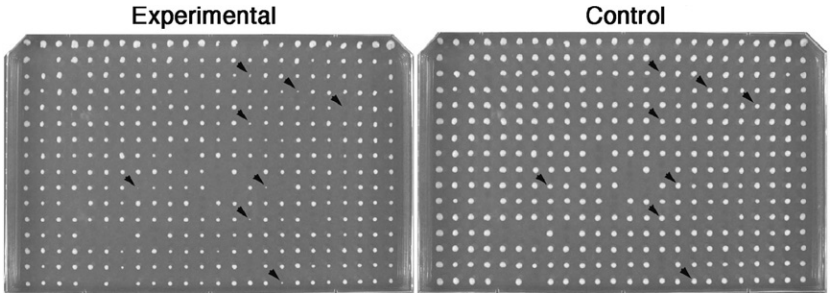

Fig. 2. Examples of deletion mutants significantly inhibited (arrows) when exposed to chimaphilin (**1**) (experimental) in comparison to no-chimaphilin (control) in growth medium.

3.4.6. Gene deletion array (GDA) analysis

A set of haploid *S. cerevisiae* strains representing ~4700 gene deletions maintained at Carleton University was used. These mutants had been generated in the background strain BY4741, a derivative of strain S288C, as described in Tong et al. (2001). We use standard conventions for designating yeast genes (e.g. *ERG28*) and proteins (e.g. Erg28p). Chimaphilin-associated growth inhibition was monitored in each mutant by methods similar to those described by Parsons et al. (2004). YPD agar plates without (control), and with 0.05 mg/mL chimaphilin (**1**) (experimental), were inoculated by hand-pinning sets of ~384 mutant strains per plate using a floating pin replicator. Plates were then incubated for 1–2 days at 30 °C, and photographed with a Hewlett Packard PhotoSmart 735 digital camera. Colony areas were determined by digital analysis (Memarian et al., 2007) and saved to a Microsoft Excel spread sheet. For both experimental and control plates, the size of each colony was compared to the average size for all colonies on the plate (Fig. 2). We identified as potentially interesting mutants those colonies that were, (a) significantly smaller than the average colony on the experimental plate, and (b) not significantly different in size from the average colony on the control plate. After normalizing for mutation-associated colony size differences and overall growth inhibition due to the presence of chimaphilin (**1**), a set of the 54 (~1% of total mutants examined) most inhibited mutants was identified and then verified as highly susceptible by visual comparison. Gene functions of highly susceptible mutants were extracted from the Saccharomyces Genome Database (SGD, Hong et al., 2006) and The Yeast Proteome Database (YPD, Hodges et al., 1999).

3.5. Antioxidant activity

Antioxidant activity was tested by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (McCune and Johns, 2002). A solution of 100 μM DPPH dissolved in methanol was freshly prepared and the absorbance was monitored until it reached 1.030–1.035. DPPH solution (1.5 mL) was mixed with 0.25 mL of methanol as a negative control, or with treatments that included quercetin and ascorbate (29 μM) as positive controls, or the EtOH–H_2O (4:1, v/v) extracts of *C. umbellata* at concentrations of 25, 50, 100, 200 and 400 ppm in methanol. After 30 min, absorbance at 517 nm (A_{517}) was measured with a Beckman model DU-640 spectrophotometer, for comparisons between treatment and control absorbance values using the following formula:

$$\%\ \text{inhibition} = (\text{negative control } A_{517} - \text{treatment } A_{517}) \times 100/\text{negative control } A_{517}$$

The IC_{50} (concentration required to bring about a 50% change in absorbance in relation to the control value) was determined using the inhibition of the rate of DPPH reduction versus plant extract concentration.

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References

Arnason, T., Hebda, R.J., Johns, T., 1981. Use of plants for food and medicine by native peoples of eastern Canada. Can. J. Bot. 59, 2189–2325.

Batra, R., Boekhout, T., Guého, E., Cabañes, F.J., Dawson Jr., T.L., Gupta, A.K., 2005. *Malassezia baillon*, emerging clinical yeasts. FEMS Yeast Res. 5, 1101–1113.

Chen, C.Y., Nace, G.W., Irwin, P.L., 2003. A 6 × 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. J. Microbiol. Method 55, 475–479.

Chevalier, A., 1996. The Encyclopedia of Medicinal Plants. Dorling Kindersley Publishing, London.

Crespo Erchiga, V., Ojeda Martos, A., Vera Casaño, A., Crespo Erchiga, A., Sanchez Fajardo, F., 2000. *Malassezia globosa* as the causative agent of pytiriasis versicolor. Br. J. Dermatol. 143, 799–803.

Duarte, Rocha A., Braga, de Oliveira A., Dias, de Souza Filho J., Lombardi, J.A., Castro, Braga F., 2004. Antifungal constituents of *Clytostoma ramentaceum* and *Monsoa hirsuta*. Phytother. Res. 18, 463–467.

Duke, J.A., 1992. Handbook of Phytochemical Constituents of GRAS Herbs and Other Economic Plants. CRC Press, Boca Raton, FL.

Ficker, C.E., Arnason, J.T., Vindas, P.S., Alvarez, L.P., Akpagana, K., Gbéassor, M., De Souza, C., Smith, M.L., 2003. Inhibition of human pathogenic fungi by ethnobotanically selected plant extracts. Mycoses 46, 29–37.

Foster, S., Duke, J.A., 1990. A Field Guide to Medicinal Plants. Houghton Mifflin Co., Eastern and Central N. America.

Gemmer, C.M., DeAngelis, Y.M., Theelen, B., Boekhout, T., Dawson, T.L., 2002. Fast, noninvasive method for molecular detection and differentiation of *Malassezia* yeast species on human skin and application of the method to dandruff microbiology. J. Clin. Microbiol. 40, 3350–3357.

Guého, E., Midgley, G., Guillot, J., 1996. The genus *Malassezia* with the description of four new species. Antonie van Leeuwenhoek 69, 337–355.

Hausen, B.M., Schiedermair, I., 1988. The sensitizing capacity of chimaphilin, a naturally-occurring quinone. Contact Derm. 19, 180–183.

Hodges, P.E., McKee, A.H., Davis, B.P., Payne, W.E., Garrels, J.I., 1999. The yeast proteome database (YPD): a model for the organization and presentation of genome-wide functional data. Nucleic Acids Res. 27, 69–73.

Hong, E.L., Balakrishnan, R., Christie, K.R., Costanzo, M.C., Dwight, S.S., Engel, S.R., Fisk, D.G., Hirschman, J.E., Livstone, M.S., Nash, R., Park, J., Oughtred, R., Skrzypek, M., Starr, B., Theesfeld, C.L., Andrada, R., Binkley, G., Dong, Q., Lane, C., Hitz, B., Miyasato, S., Schroeder, M., Sethuraman, A., Weng S., Dolinski, K., Botstein, D., Cherry, J.M., 2006. Saccharomyces Genome Database. http://www.yeastgenome.org/ (accessed August 2006).

Jones, N.P., Arnason, J.T., Abou-Zaid, M., Akpagana, K., Sanchez-Vindas, P., Smith, M.L., 2000. Antifungal activity of extracts from medicinal plants used by First Nations Peoples of eastern Canada. J. Ethnopharmacol. 73, 191–198.

Kosuge, T., Yokota, M., Sugiyama, K., Mure, T., Yamazawa, H., Yamamoto, T., 1985. Studies on bioactive substances in crude drugs used for arthritic diseases in traditional Chinese medicine. III. Isolation and identification of anti-inflammatory and analgesic prin-

ciples from the whole herb of *Pyrola rotundifolia* L.. Chem. Pharm. Bull. 33, 5355–5357.

Loeffler, J., Stevens, D.A., 2003. Antifungal drug resistance. Clin. Infect. Dis. 36 (Suppl. 1), S31–S41.

Marles, R.J., Clavelle, C., Monteleone, L., Tays, N., Burns, D., 2000. Aboriginal plant use in Canada's northwest boreal forest. Nat. Resources Canada. UBC Press.

McCune, L.M., Johns, T., 2002. Antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the indigenous peoples of the north of American boreal forest. J. Ethnopharmacol. 82, 197–205.

Memarian, N., Jessulat, M., Alirezaie, J., Mir-Rashed, N., Xu, J., Zareie, M., Smith, M., Golshani, A., 2007. Colony size measurement of the yeast gene deletion strains for functional genomics. BMC Bioinform. 8, 117.

Miller, J., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, NY, pp. 352–355.

Nakamura, Y., Kano, R., Murai, T., Watanabe, S., Hasegawa, A., 2000. Susceptibility testing of *Malassezia* species using the urea broth microdilution method. Antimicrob. Agents Chemother. 44, 2185–2186.

Nishizawa, M., Kohono, M., Nishimura, M., Kitagawa, A., Niwano, Y., 2005. Presence of peroxyradicals in cigarette smoke and the scavening effect of shikonin, a naphthoquinone pigment. Chem. Pharm. Bull. 53, 796–799.

Parsons, A.B., Brost, R.L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G.W., Kane, P.M., Hughes, T.R., Boone, C., 2004. Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. Nat. Biotechnol. 22, 62–69.

Saxena, G., Farmer, S.W., Hancock, R.E., Towers, G.H., 1996. Chlorochimaphilin: a new antibiotic from *Moneses uniflora*. J. Nat. Prod. 59, 62–65.

Sugita, T., Tajima, M., Amaya, M., Tsuboi, R., Nishikawa, A., 2004. Genotype analysis of *Malassezia restricta* as the major cutaneous flora in patients with atopic dermatitis and healthy subjects. Microbiol. Immunol. 48, 755–759.

Tilak, J.C., Adhikari, S., Devasagayam, T.P., 2004. Antioxidant properties of *Plumbago zeylanica*, an Indian medicinal plant and its active ingredient, plumbagin. Redox Rep. 9, 219–227.

Tong, A.H., Evangelista, M., Parsons, A.B., Xu, H., Bader, G.D., Pagé, N., Robinson, M., Raghibizadeh, S., Hogue, C.W., Bussey, H., Andrews, B., Tyers, M., Boone, C., 2001. Systematic genetic analysis with ordered arrays of yeast deletion mutants. Science 294, 2364–2368.

Wainwright, M., 1998. Photodynamic antimicrobial chemotherapy (PACT). J. Antim. Chem. 42, 13–28.