INFORMATION

Xerostomia is a medical term for the complaint of dry mouth due to a decrease in saliva secretion. Xerostomia can unbalance the oral microflora, mainly to the benefit of Candida albicans. The aim of the present study was to find a plant extract that could create an unfavourable environment for Candida, and would, therefore, be appropriate for use in a dry-mouth daily-care mouthwash. Water extract from the herbaceous plant Solidago virgaurea (Goldenrod) was selected due to its saponin content (plant detergents). Saponin concentrations reached 0.7 and 0.95 mg ml⁻¹ in S. virgaurea subsp. virgaurea and S. virgaurea subsp. alpestris extracts, respectively. C. albicans was grown in liquid medium and cells were counted by microscopic examination after 0, 4 and 24 h of incubation. Solidago extracts did not inhibit the growth of C. albicans (four strains), Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans, Streptococcus salivarius or Enterococcus faecalis. When inocula were incubated with Solidago extract for 4 and 24 h, we observed a decrease in Candida yeast–hyphal transition. Candida biofilms were then prepared in microtitre plates and treated with plant extracts at 0 h, to estimate biofilm formation, or at 18 h to estimate the effect of the saponin on pre-formed biofilms. Biofilm formation and pre-formed biofilms were both strongly inhibited. In conclusion, the S. virgaurea extract was efficient against two key virulence factors of C. albicans: the yeast–hyphal transition phase and biofilm formation.

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A supplementary figure is available with the online version of this paper.

Inhibition of Candida albicans yeast–hyphal transition and biofilm formation by Solidago virgaurea water extracts

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reactions. The host produces lactoferrin in several secretions including saliva. This glycoprotein displays both iron sequestering properties and direct action against Candida cell membranes. In response, Candida produces iron chelators (siderophores) and iron-transport proteins to obtain sufficient iron from the host to allow fungal growth (Nyilasi et al., 2005; Chen et al., 2010). Hyposalivation provokes a decrease in lactoferrin and other immune saliva components, causing an increased risk of oral candidiasis.

The treatment of xerostomia often includes treatment for xerostomia-induced candidiasis. Oral azoles and local polyenes are recommended to treat oral candidiasis, but only on a limited-time basis because of the risk of the Candida developing a resistance. Mouthwashes containing quaternary ammonium (chlorhexidine, hexetidine), vegetal apolar compounds (essential oils), sodium bicarbonate, alcohol and other antiseptic compounds can fight Candida proliferation, but they alter the bacterial part of the biofilm too and, therefore, carry a risk of worsening xerostomia symptoms. Some ranges of products, such as mouthwash, gel, toothpaste and lozenges, aim to fight the symptoms of dry mouth and mucosal inflammation by using ingredients such as lactoferrin and/or ovalbumin, lactoperoxidase, lysozyme, glucose oxidase and vitamins (Bioxtra, Biotène, Novasial, Orajel), but are not toxic when used orally because of their low digestive absorption rate (Milgat & Roberts, 1995; Matsuda, 1999). S. virgaurea polar extracts (aqueous, ethanolic or methanolic) contain noticeable levels of saponins and flavonoids (Tyszkievicz, 2008) but only ethanolic or methanolic Solidago extracts containing saponins that are purified and submitted to alkaline lysis have proven antifungal properties (Bader et al., 1995).

The aim of the present work was to screen the antibacterial and antifungal properties of this Solidago extract against four C. albicans strains and six bacterial strains (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans, Streptococcus salivarius, Enterococcus faecalis); to investigate the effect of S. virgaurea extract on C. albicans growth and yeast–hyphal transition; and to evaluate the effect of Solidago extract on C. albicans biofilm growth, at both early and intermediate stages.

**METHODS**

**Plant material.** Plant materials of the two taxa were collected during flowering. S. virgaurea L. subsp. virgaurea was collected in the Tinée valley, Col Saint Martin, la Colermine, France (44.067° N 7.217° E), on 23 August 2011 at an altitude of 1,500 m. S. virgaurea subsp. alpestris (Waldst. & kit.) Greml was collected in the Tinée valley, Piste Roubine, Isola 2000, France (44.186° N 7.157936° E), on 12 August 2011 at an altitude of 2,130 m. Voucher specimens have been deposited in the Botanic Garden of the City of Nice (France) (voucher numbers C-3173 and C-3174, respectively).

Plant water extract was obtained from 6 g dried aerial plant parts by placing them in 100 ml of distilled sterile water, warming for 2 h at 30 °C and subjecting them to ultrasound twice for 15 min. The decoction was then filtered. Large particles were precipitated by addition of 30 mg edible milk calcium caseinate (Protilight IP4, Armor Protéines) and 15 ml M. citrate buffer (pH 4) ml⁻¹ of decoction. After 60 min of incubation at 20 °C and filtration, the decoction was concentrated at 60 °C in a rotary evaporator.

In assays using blood agar plates, isotonic Solidago extracts were obtained by addition of sodium chloride 0.9%. Powder of escin (Sigma–Aldrich) in solution, a mixture of saponins obtained from Aesculus hippocastanum (Horse-chestnut), was used as control.

Saponins cannot be quantified with a spectrophotometer (Oleszek & Bialy, 2006). In order to estimate the saponins content in each culture of Candida albicans, we used a simple screening protocol using the radial inhibition of growth and yeast–hyphal transition; and to evaluate the effect of Solidago extract on C. albicans biofilm growth, at both early and intermediate stages.

**Microbial strains and culture conditions.** We used three reference strains recommended by the Association Française de Normalisation (AFNOR) to test antibacterial compounds: E. coli ATCC 25922, Staph. aureus ATCC 25923 and P. aeruginosa ATCC 27853. We also tested an oral wild-type strain of E. faecalis obtained from the Bacteriology Laboratory at Nice University Hospital (courtesy Dr F. Girard-Pipau). These strains were grown aerobically at 37 °C.
overnight, on Mueller–Hinton agar (bioMérieux). Two other oral strains were also tested: *Strep. salivarius* ATCC 7073 and a wild-type strain of *Strep. mutans* (courtesy of Dr F. Girard-Pipau). Streptococci were grown on 5% sheep’s blood agar for 5 days at 37 °C, both in an anaerobic chamber and under microaerophilic conditions in a 5% CO2 chamber.

Four *C. albicans* strains were used: *C. albicans* ATCC 10231 (AFNOR) and three wild-type oral strains named IM001, IM003 and IM007, isolated at Nice University Hospital Laboratory (Courtesy Dr Martine Gari-Toussaint). In order to increase the yeast–hyphal transition, *C. albicans* ATCC 10231 was pre-cultivated anaerobically on 5% sheep’s blood agar (bioMérieux) for 48 h at 37 °C. Then all *C. albicans* strains were cultivated aerobically on Sabouraud Chloramphenicol agar (bioMérieux) for 48 h at 37 °C.

**Microbial growth.** Microbial growth inhibition was investigated by the diffusion method with 100 μl of bacterial (106 c.f.u.) or fungal (105 c.f.u.) inocula smeared onto agar plates, and 40 μl of *Solidago* extract deposited into pits of 5 mm diameter. Diameter of growth inhibition was measured after 24 h and after 3 days of incubation.

**Effect of plant extracts on Candida yeast–hyphal transition.** To estimate the effect of *S. virgataea* water extracts on yeast–hyphal transition, *C. albicans* was cultivated in YEP liquid medium, containing yeast extract (11 mg ml⁻¹), Sigma–Aldrich), peptone (22 mg ml⁻¹), Oxoid) and 2% w/v N-acetyl-D-glucosamine (GlcNAc) (Calbiochem), under aerobic conditions at 37 °C for 24 h. A *C. albicans* inoculum of 105 cells ml⁻¹ was incubated with *S. virgataea* water extract at a final saponin concentration of 0.25 mg ml⁻¹. Yeast and hyphae were counted using a KOVA Glastic slide by observation under an optic microscope after 0, 4 and 24 h of incubation. The control consisted of *C. albicans* incubated with YEP GlcNAc using water acidified with citrate buffer pH 4 instead of plant water extract. The numbers of hyphae and germ tubes were determined and the percentage inhibition of hyphae and germ tube formation was calculated by comparison with control. All assays were performed in triplicate and three independent experiments were performed.

**Effect of plant extracts on the formation of *C. albicans* biofilms.** *C. albicans* biofilms were grown on commercially available pre-sterilized, polystyrene, conical bottomed 96-well microtitre plates (Corning). Biofilms were formed by pipetting standardized cell suspensions into wells; 100 μl of a suspension containing 106 cells ml⁻¹ in RPMI 1640 buffered with MOPS (Nett et al., 2011; Ramage et al., 2001). In order to determine whether *Solidago* water extracts had an effect on biofilm formation, 50 μl of SVV or SVA extracts were added immediately into the microplate wells after the *Candida* suspensions to a final concentration of 0.25 mg ml⁻¹. *C. albicans* incubated with acidified water instead of *Solidago* extract and biofilm-free wells were included to serve as positive and negative controls, respectively. Plates were incubated for 18 h at 37 °C on an orbital shaker at 200 r.p.m. After biofilm formation, the medium was aspirated. Non-adherent cells were removed by thoroughly washing the biofilms twice with PBS (pH 7.2). A semiquantitative measure of biofilm formation was calculated using a 2,3-bis-(2-methoxy-4-nitro-sulfo-phenyl)-2H-tetrazolium-5 carboxanilide (XTT) reduction assay (Hawser et al., 1998; Nett et al., 2011). Briefly, 100 μl volumes of water were added to each of the prewashed biofilms and into the control well. Then 50 μl volumes of the XTT reaction mixture (activation reagent and XTT reagent) prepared according to manufacturer’s recommendations (Cell Proliferation kit XTT, AppliChem) were added. Plates were incubated in the dark for 2 h at 37 °C. A colorimetric change resulting from XTT reduction and representing a direct correlation of metabolic activity of the biofilm was measured in a microtitre plate reader (ELX800, Biotek Instruments) at 490 nm. An inhibitory percentage was calculated by the following formula: [(control – treatment)/control] × 100. Assays were performed in triplicate and three independent experiments were performed.

**Effect of plant extracts on pre-formed *C. albicans* biofilms.** *Candida* biofilms were obtained as described before, but 100 μl SVV or SVA extracts (final concentration 0.75 mg ml⁻¹) or water acidified (control) were added into wells after 18 h of incubation. Microtitre plates where incubated for 2 hours on an orbital shaker at 200 r.p.m. Biofilms were then washed and measured as described before.

**Scanning electron microscopy.** For SEM, *C. albicans* biofilms were grown on polymethylmethacrylate (PMMA) (8 mm diameter) (Pesci-Bardon et al., 2006). Biofilms were formed by dispensing standardized cell suspensions (1 ml of a suspension containing 106 cells ml⁻¹ in RPMI 1640) onto resin discs within six-well cell culture plates (Corning) and incubating at 37 °C. Discs were removed 18 h later and washed with 0.1 M phosphate buffer (2 × 3 min). Biofilms were treated for 2 h with SVV, SVA or acidified water (control) as described before. Discs were then washed in 0.1 M phosphate buffer (2 × 3 min). Discs were placed in fixative (4%, v/v, formaldehyde in PBS) overnight. Samples were dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min, 100% for 20 min) and, finally, air-dried in a desiccator. Samples were observed at ×100, ×500 and ×1500 magnification in a scanning electron microscope (ISM-5310LV, JEOL) in low vacuum mode at 25 and 30 kV. Images were processed for display using SemAfor software (JEOL AB).

**Statistical analysis.** Statistical analyses were performed using StatView software version 5.0 (1998, SAS Institute). For multigroup comparisons, the Kruskal–Wallis test was used to determine if any group exhibited statistically significant different percentages of inhibition of biofilm formation or of yeast–hyphal transition. If the Kruskal–Wallis test demonstrated >1 of the groups to be statistically different, a post hoc analysis using the Mann–Whitney U test correction was used to adjust the significance value (P) for the number of comparisons.

**RESULTS**

The saponin concentration was estimated by an indirect method of quantification. It reached 0.7 ± 0.15 mg ml⁻¹ in the SVV extract and 0.95 ± 0.15 mg ml⁻¹ in the SVA extract.

**Microbial growth**

The SVV and SVA plant extracts did not inhibit bacterial (*E. coli*, *Staph. aureus*, *P. aeruginosa*, *Strep. mutans*, *Strep. salivarius*, *E. faecalis*) or fungal (*C. albicans*, four strains) growth by the diffusion method on agar plates (data not shown).

**Effect of plant extracts on *C. albicans* yeast–hyphal transition**

*C. albicans* growth in liquid medium was not inhibited by the addition of plant extracts (data not shown). However, these extracts did inhibit yeast–hyphal transition (Table 1). Shorter forms of germ-tubes were also observed.
**Table 1. Impact of *S. virgaurea* extracts on *C. albicans* hyphal formation**

At 4 and 24 h, the number of hyphae and germ tubes was determined and the percentage was calculated by comparison with the total. Values are means ± SD. P-values represent significant difference between *Solidago* treatments and controls (no treatment) using Kruskal–Wallis and Mann–Whitney tests.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hyphae at 4 h (%) Control</th>
<th>Hyphae at 4 h (%) SVA</th>
<th>Hyphae at 24 h (%) Control</th>
<th>Hyphae at 24 h (%) SVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 10231</td>
<td>51.5 ± 13.0</td>
<td>1.6 ± 4.6*</td>
<td>7.7 ± 6.6†</td>
<td></td>
</tr>
<tr>
<td>IM001</td>
<td>25.2 ± 8.6</td>
<td>14.3 ± 5.7‡</td>
<td>10.7 ± 6.3§</td>
<td></td>
</tr>
<tr>
<td>IM003</td>
<td>18.7 ± 8.0</td>
<td>1.1 ± 1.1*</td>
<td>0.1 ± 1.6*</td>
<td></td>
</tr>
<tr>
<td>IM007</td>
<td>16.9 ± 6.7</td>
<td>2.7 ± 2.0*</td>
<td>4.3 ± 2.9*</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.0001.
†P<0.05.
‡P<0.005.
§P<0.0005.

**Effect of plant extracts on the formation of *C. albicans* biofilms**

*Solidago* extracts strongly inhibited *C. albicans* biofilm formation.

All *C. albicans* strains produced a significant biofilm (OD<sub>490</sub>; ATCC 10231, 2.06 ± 0.47; IM001, 3.09 ± 0.55; IM003, 2.22 ± 0.62 and IM007, 3.38 ± 0.17). Compared to control cultures without *Solidago* extracts, SVV and SVA extracts significantly reduced biofilm formation for all the strains tested (*P<0.0005*). The percentages of reduction in biofilm formation observed were: ATCC 10231, 98.41 ± 1.97%; IM001, 99.18 ± 2.45%; IM003, 97.32 ± 5.94%; and IM007, 96.49 ± 3.73%, for SVV, and ATCC 10231, 95.86 ± 4.88%; IM001, 96.00 ± 2.22%; IM003, 99.46 ± 5.94%; and IM007, 95.14 ± 4.11%, for SVA (Fig. 1). Using the non-parametric test of Mann and Whitney, there were no statistically significant differences between SVA and SVV treatments.

**Effect of plant extracts on pre-formed *C. albicans* biofilms**

*Solidago* extracts significantly decreased *C. albicans* pre-formed biofilms. Exposure of pre-formed 18 h biofilms to SVV and SVA extracts for 2 h resulted in significant reduction in viability compared to control biofilms for all the strains tested (*P<0.0005*). The percentages of reduction observed were: ATCC 10231, 77.85 ± 13.35%; IM001, 91.16 ± 4.82%; IM003, 79.21 ± 8.28%; and IM007, 90.85 ± 7.19%, for SVV, and ATCC 10231, 92.37 ± 7.79%; IM001, 82.17 ± 9.53%; IM003, 76.26 ± 10.71%; and IM007: 91.91 ± 4.84%, for SVA (Fig. 2). Using the non-parametric test of Mann and Whitney, there were statistically significant differences between SVA and SVV treatments. SVA was more efficient than SVV against *C. albicans* ATCC 10231 (*P=0.0243*) and SVA was more efficient than SVA against *C. albicans* IM001 group (*P=0.038*). However, there were no statistically significant differences between SVV and SVA against *C. albicans* IM003 and IM007 (*P>0.05*).

**DISCUSSION**

In the present study, we investigated a new strategy to relieve dry mouth symptoms and focused on the creation of an unfavourable environment for *C. albicans*. For this purpose we investigated *Solidago* water extracts containing saponins on *C. albicans* biofilm formation. Cells were incubated for 18 h, in the presence or absence of *S. virgaurea* water extracts (final concentration 0.25 mg ml<sup>-1</sup>), and assayed for XTT reduction activity. A reference strain, *C. albicans* ATCC 10231, and three wild-type strains, IM001, IM003 and IM007, were tested. Kruskal–Wallis and Mann–Whitney tests were used to compare *Solidago* treatments and controls (no treatment), *P<0.005.*

**Fig. 1.** Effect of *Solidago virgaurea* water extracts containing saponins on *C. albicans* biofilm formation. Cells were incubated for 18 h, in the presence or absence of *S. virgaurea* water extracts (final concentration 0.25 mg ml<sup>-1</sup>), and assayed for XTT reduction activity. A reference strain, *C. albicans* ATCC 10231, and three wild-type strains, IM001, IM003 and IM007, were tested. Kruskal–Wallis and Mann–Whitney tests were used to compare *Solidago* treatments and controls (no treatment), *P<0.005.*
saponins, which are plant detergents with iron-chelator properties. Saponins could deprive *Candida* from the iron available in serum exudates and saliva. However, in *vivo*, the precise role of plant iron chelators in mediating conditions affecting the oral mucosa and microflora may be controversial. Hameed et al. (2008) performed iron deprivation experiments by using a synthetic chelator (bathophenanthrolene disulfonic acid), which promoted hyphal development without affecting the growth of *Candida* cells, or their ability to form biofilms on catheters.

The present plant extraction process allowed the preservation of a natural edible aroma (saponins/liquorice), dye (flavonoids/yellow) and maybe other compounds of interest such as anti-inflammatory acid phenols (salicylic acid), or chacones (flavonoids) with intrinsic yeast–hyphal transition and biofilm inhibitory properties (Messier et al., 2011).

In order to develop a dry mouth-specific mouthwash and to preserve oral mucosa hydration, we first aimed to preserve oral bacteria in the healthy microflora, which are natural competitors of *C. albicans*. For this reason, we first verified that *Solidago* extracts did not inhibit bacterial growth. This absence of antibacterial activity could be explained because saponins can interfere with sterols, namely phytosterols (plants), cholesterol (mammalian cells and human viruses) and ergosterol (fungi), while most of bacteria are lacking in membrane sterols (Alvarez et al., 2007).

Anti-*Candida* properties are required in a dry mouth-specific mouthwash (ten Cate et al., 2009; Humphrey & Williamson, 2001); however, in clinical practice, a full eradication of *C. albicans* from oral ecosystems is not a therapeutic objective (ten Cate et al., 2009). In response to the host environment, *C. albicans* yeast–hyphal transition is important for virulence, and Saville et al. (2003) demonstrated that mutants that are locked in yeast or hyphal forms were not as virulent. It has also been shown that filamentation is not a compulsory prerequisite during the formation of a biofilm (Blankenship & Mitchell, 2006; Douglas, 2003). Thus, the second aim of this work was to inhibit the *C. albicans* yeast–hyphal transition using *Solidago* extracts, and in experiments, such an effect was
observed. This could be explained by the difference in total lipid content of hyphal forms, which is higher than that of the yeast and rather immature forms (C16:0 versus C18:0 fatty acids) due to an increased biosynthesis of lipids in hyphal forms (Bahn et al., 2007). Additional studies will now be necessary in order to investigate whether Solidago saponins can reduce Candida pathogenesis linked to hyphal forms, focusing on membrane polarization and production of extracellular proteolytic enzymes (SAP), as well as the synthesis of adhesins promoting adherence to epithelial cells, denture resin, Candida cells and other oral bacteria (Hwp1, Als, Alal) (Hope et al., 2008; Alvarez et al., 2007; Martin & Konopka, 2004). Potential interactions between saponins and Candida membrane-associated lipomannans should also be investigated, as well as resistance-inducing factors (Masuoka, 2004).

Candida hyphae can co-aggregate to form a three-dimensional scaffolding colonized by oral bacteria, leading to macroscopic deposits on oral surfaces (Villar et al., 2007). Although in vitro mutant cells fixed in either hyphal or yeast form could develop into biofilms (Douglas, 2003), these biofilms were rudimentary and were not as stable as wild-type biofilms (Blankenship & Mitchell, 2006). The third aim of this work was to investigate whether Solidago extracts could prevent C. albicans biofilm growth, at both early and intermediate stages of development. This was confirmed by growth onto two different supports, microtitre plates and PMMA resin discs mimicking prosthetic dentures. SEM results suggested that saponin extracts displayed anti-adherent activity but the XTT method is only able to demonstrate that there is less metabolic activity after both treatments (Nett et al., 2011). However, the anti-biofilm effects of saponins observed in the present study are in line with the results of Coleman et al. (2010) who showed that, similarly, plant derived saponins disrupted C. albicans biofilms. Several authors demonstrated that, unlike brand antiseptic mouthwashes, fluconazole and polyenes (nystatin and amphotericin B) were inefficient against pre-formed Candida biofilms in vitro (Ramage et al., 2011; Lamfon et al., 2004). The S. virgaurea extracts used in this study displayed anti-biofilm properties similar to those of antiseptic mouthwashes (used at the exposure time recommended by the manufacturers) containing quaternary ammonium compounds [chlorhexidine gluconate (Cordosyl), GlaxoSmithKline; hexetidine-triclosan (Oraldene), Warner-Lambert] or essential oils in combination (Listerine, Pfizer). However, unlike antiseptic mouthwashes, these Solidago extracts did not exhibit any fungicidal or bactericidal properties. In vivo, inhibition of hyphal forms by a saponin-containing mouthwash should also reduce macroscopic biofilms too. This property could be reinforced by the intrinsic detergent properties of saponins against lipid molecules, particularly serum lipoprotein exudates (Enjalbert & Whiteway, 2005). Currently, serum is commonly added to Candida culture medium to promote the yeast–hyphal transition. Besides, saponins have both haemolytic and iron chelator properties, and iron is necessary for Candida growth (Matsuda, 1999). Thus, saponins could interfere with blood exudates from inflamed oral mucosa and indirectly reduce Candida proliferation by sequestering iron.

In conclusion, we showed that S. virgaurea water extracts had no antibacterial or antifungal activity sensu stricto. However they displayed inhibitory effect on the C. albicans yeast–hyphal transition, strongly inhibited biofilm formation and decreased pre-formed biofilms. Some Candida strains were more inhibited by SVV and others by SVA, so it could be beneficial to mix these two plant extracts. Chemical analysis is in progress, in order to better understand how saponins can inhibit the Candida yeast–hyphal transition.

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