New pharmacological properties of *Medicago sativa* and *Saponaria officinalis* saponin-rich fractions addressed to *Candida albicans*

Beata Sadowska,¹ Aleksandra Budzyńska,¹
Marzena Więckowska-Szakiel,¹ Małgorzata Paszkiewicz,¹ Anna Stochmal,²
Barbara Moniuszko-Szajwaj,² Mariusz Kowalczyk² and Barbara Różalska¹

¹Department of Infectious Biology, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland
²Department of Biochemistry, Institute of Soil Science and Plant Cultivation, State Research Institute, Czartoryskich 8, 24-100 Pulawy, Poland

The antifungal activity of the saponin-rich fractions (SFs) from *Medicago sativa* (aerial parts and roots) and *Saponaria officinalis* (used as a well-known source of plant saponins) against *Candida albicans* reference and clinical strains, their yeast-to-hyphal conversion, adhesion, and biofilm formation was investigated. Direct fungicidal/fungistatic properties of the tested phytochemicals used alone, as well as their synergy with azoles (probably resulting from yeast cell wall instability) were demonstrated. Here, to the best of our knowledge, we report for the first time the ability of saponin-rich extracts of *M. sativa* and *S. officinalis* to inhibit *C. albicans* germ tube formation, limit hyphal growth, reduce yeast adherence and biofilm formation, and eradicate mature (24 h) *Candida* biofilm. Moreover, *M. sativa* SFs (mainly obtained from aerial parts), in the range of concentrations which were active modulators of *Candida* virulence factors, exhibited low cytotoxicity against the mouse fibroblast line L929. These properties seem to be very promising in the context of using plant-derived SFs as potential novel antifungal therapeutics supporting classic drugs or as ingredients of disinfectants.

**INTRODUCTION**

*Candida albicans* is probably the best known and at the same time the most effective opportunistic fungal pathogen of humans and animals. It constitutes a commensal microflora in the gastrointestinal and genitourinary tracts of >70% of humans. However, in certain circumstances *C. albicans* could be pathogenic to critically ill, immunocompromised patients or even to healthy persons. It causes a diverse range of pathologies – from local cutaneous and mucosal infections (e.g. candidiasis of nail shafts and nails, oropharyngeal candidiasis, intestinal candidiasis) to life-threatening systemic infections, such as candidaemia, fungal pneumonia, meningitis or endocarditis (Cannon *et al.*, 2009; Kabir *et al.*, 2012; Kabir & Ahmad, 2013; Sardi *et al.*, 2013; Tlamčani & Er-rami, 2013). Endogenous candidaemia and the carrier state are the main mechanisms of *Candida* spp. spread and transmission, although exogenous infections are also possible (Mathé & van Dijck, 2013; Sardi *et al.*, 2013). The pathogenicity and high invasiveness of *C. albicans* arise from the broad range of its virulence factors. These include tissue-damaging hydrolytic enzymes: proteases (mainly secreted aspartic proteinases), phospholipases and haemolysins, but first of all the agents/abilities contributing to its strong adherence (e.g. two main *C. albicans* adhesions: agglutinin-like sequence and hyphal wall protein-1), biofilm formation and the transformation of morphological forms (yeast-to-hyphal). Also, the ability of *C. albicans* to survive in various anatomical body sites and to evade host defences seems to be essential (Chai *et al.*, 2009; Chandra *et al.*, 2001; Gropp *et al.*, 2009; Sardi *et al.*, 2013).

Invasive candidiasis usually results from tissue colonization by yeast and formation of biofilm – the structured multicellular microbial communities embedded in a self-produced extracellular polymeric substance (EPS) matrix containing β-glucan as the main component of the fungal EPS matrix. The reversible *C. albicans* conversion from yeast cells to either pseudohyphal or hyphal growth seems to be one of the most prominent factors contributing to tissue invasion and

**Abbreviations:** EPS, extracellular polymeric substance; GTF, germ tube form; Gs, saponin-rich fraction alone as a negative control; Gs, medium alone as a negative control; M, saponin-rich fraction of *Medicago sativa* aerial parts; Mr, saponin-rich fraction of *Medicago sativa* roots; MTT, 5-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; S, saponin-rich fraction of *Saponaria officinalis*; SF, saponin-rich fraction; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt.
resistance to phagocytosis. These forms also play a unique role in the process of C. albicans biofilm development. Their presence provides stability to the biofilm structure, and, in addition, the EPS matrix is bound to both yeast and hyphal cells (Kabir et al., 2012; Mathé & van Dijck, 2013; Sardi et al., 2013). In vitro research shows that yeast cells usually constitute the basal layer of the biofilm from which filamentous cells arise. However, biofilm morphology depends on many environmental factors (temperature, pH, serum availability), as well as on the concentration of quorum-sensing regulatory molecules such as farnesol, which exhibits an effect on mycelial growth (Sardi et al., 2013). Additionally, the possibility of more complex polymicrobial biofilm formation (consisting of both fungi and bacteria) should be assumed. C. albicans has been found to grow together with Staphylococcus aureus, Staphylococcus epidermidis and Enterococcus spp. in the course of systemic infections, with Gardnerella vaginalis during vaginal infections or with other fungal pathogens in skin infections (Dongari-Bagtzoglou, 2008; Mathé & van Dijck, 2013; Sardi et al., 2013). A number of factors contribute to the higher resistance of microbial biofilm to environmental stress conditions, including chemotherapeutics and host immune response, thus making biofilm an important aetiological agent of human disease. The reasons for this increased resistance must be sought primarily in the specific structure and unique physiology of this community, and the case of C. albicans biofilm in particular involves various morphological yeast forms and mixed bacterial–fungal biofilms, which are much more difficult to diagnose and treat (Bhattacharyya et al., 2013; Bink et al., 2011; Kabir et al., 2012; Mathé & van Dijck, 2013; Sardi et al., 2013).

Relatively few antimycotic groups have been identified, including polyenes, azaoles, echinocandins, allylamines and 5-flucytosine (Kabir & Ahmad, 2013; Pemán et al., 2009; Rajeshkumar & Sundararaman, 2012). Furthermore, the growing resistance of C. albicans and other Candida species to antifungal compounds and the participation of their biofilms in the development of pathological lesions have led to an urgent need to develop alternative therapy. Scientists and physicians have focused on naturally occurring substances, such as plant-derived compounds (e.g. polyphenols, essential oils, saponins) possessing direct biocidal/biostatic properties against fungi or inhibiting/impairing their virulence factor expression, adhesion and biofilm formation (Abid Ali Khan et al., 2012; Bhattacharyya et al., 2013; Naicker & Patel, 2013; Rajeshkumar & Sundararaman, 2012; Tsuuki et al., 2007). In this respect, one of the lesser known but promising agents seem to be saponins – surface-active phytochemicals present in most vegetables, beans and herbs. The best-known sources of saponins are peas, soybeans and some herbs with names indicating foaming properties, such as soapwort, sooproot, soapbark and soapberry. For many years saponins have been described as compounds influencing animals and humans via immunostimulatory, anti-inflammatory, hypcholesterolaemic, antioxidative and anticancerogenic activity. They also reduce the growth of some insects, protozoans and micro-organisms (Coleman et al., 2010; Francis et al., 2002; Sparg et al., 2004; Timbekova et al., 1996; Udgirkar et al., 2013; Weng et al., 2010).

Commercially available saponins are mainly extracted from Yucca schidigera and Quillaja saponaria. In the present study, saponin-rich fractions (SFs) extracted from the aerial parts and roots of Medicago sativa var. Radius and the roots of Saponaria officinalis L. were tested.

M. sativa, also called alfalfa or lucerne, is a plant from the pea family Fabaceae cultivated in many countries as an important forage crop. Alfalfa produces characteristic secondary metabolites, such as cumarins, isoflavones, naphthoquinones, alkaloids and saponins. Biological activities of Medicago spp. saponins include haemolytic activity (dependent on a type of aglycone), cytotoxic properties (including anticancer effect), nematocidal and insecticidal activity, and an antimicrobial effect mainly against Gram-positive bacteria (e.g. Staphylococcus aureus, Enterococcus faecalis), as well as against some fungi, including yeast from Saccharomyces spp., plant pathogens like Trichoderma viride, and some human pathogens from Blastomyces spp. and Candida spp. (Avato et al., 2006; Balestrazzi et al., 2011; Carelli et al., 2011; D’Addabbo et al., 2011; Tava & Avato, 2006). For comparison, S. officinalis, commonly named soapwort, as a well-known source of phytochemicals was included in our study. The biological activity of saponins obtained from S. officinalis (including anti-inflammatory, cytotoxic, haemolytic, anticancer, antifungal or hypcholesterolemic effects) has been widely described in the literature, which explains their use in traditional medicine, mainly as an expectorant during the course of upper respiratory tract infections or for the treatment of skin and rheumatic lesions (Böttger & Melzig, 2011; Czaban et al., 2013; Sparg et al., 2004; Weng et al., 2010).

Despite the broadly described biological activity of the saponins obtained from M. sativa and S. officinalis, their antifungal properties against C. albicans with regard to anti-biofilm activity and their synergistic effect with classic antimycotics have not been studied so far.

**METHODS**

**Plant material.** Seeds of M. sativa var. Radius were provided by the Plant Breeding and Acclimatization Institute, Radziów/Blonie, Poland. The seeds were planted in an experimental field of the Institute of Soil Science and Plant Cultivation, Pulawy, Poland. The aerial parts of the plants were harvested at the beginning of flowering and the roots were collected in the autumn, lyophilized and finely powdered. Dry, ground roots of S. officinalis L. were purchased from a commercial source (Herbapol). A voucher sample has been deposited at the Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation, State Research Institute, Pulawy, Poland.

**Extraction and purification of plant-derived SFs.** Powdered plant material was defatted with chloroform (M. sativa) or chloroform : hexane (1:1 v/v; S. officinalis) in a Soxhlet apparatus for 48 h. Defatted and dried material was extracted with 80% aqueous methanol twice under reflux for 1 h for M. sativa or three times at...
room temperature for 24 h for *S. officinalis*. The combined methanolic extracts were concentrated under reduced pressure and the residues were resuspended in water. The suspensions obtained were applied onto a 100 × 60 mm LiChroprep RP-18, 40–63 μm vacuum liquid chromatography column (Merck) preconditioned with water. Sugars and phenolics were removed from the column with water and 40% aqueous methanol, respectively. Saponins were then eluted with 85% (*M. sativa*) or 90% (*S. officinalis*) aqueous methanol. The solvent was evaporated *in vacuo* and the residues were lyophilized. The saponin mixture obtained from *S. officinalis* was suspended in water and extracted with ethyl acetate saturated with water. This separation extracted non-polar compounds, which went into the ethyl acetate layer, and yielded saponins.

Before testing, crude *M. sativa* (aerial parts (M) and roots (Mr)) and *S. officinalis* (S) SFs were dissolved, respectively, in 50% ethanol (POCh) and Tissue Culture Water (Sigma) to make stock solutions, and then appropriate percentage of all peaks presented on the chromatograms corresponding to saponins were integrated and expressed as an concentration in these fractions was measured using a Gilson GX-281 HPLC column Waters HSS C18 column using a linear 1.8 μm Waters HSS C18 column using a linear 1.0 mm column Waters XBridge column. Samples were separated using a linear 50 min gradient from 20 to 50% acetonitrile in 0.1% formic acid with 0.3 ml min⁻¹ flow and the column temperature was held at 50°C. The chromatograms were examined with PAD detector set at 210 nm. The mass spectrometer was operated in the negative electrospray mode with the following ion source parameters: spray voltage 3.9 kV, capillary voltage 47 V, tube lens offset 60 V, capillary temperature 250°C. Full-scan spectra were acquired in the m/z range 150–2000. The automated MS/MS function was utilized at 35% normalized collision energy by molecular ion isolation with width of 1.0 and maximum acquisition time 250 ms. Data acquisition was conducted using the Xcalibur data system (version 1.3 SR1) (Thermo Fisher Scientific). The total saponins content in these fractions was measured using a Gilson GX-281 HPLC system equipped with an iSELECT II Detector (Gilson). Fractions were separated using a reverse-phase column and chromatographic parameters that were identical to those mentioned above. Peaks corresponding to saponins were integrated and expressed as an appropriate percentage of all peaks presented on the chromatograms.

Separation of *S. officinalis* SF was performed on a 100 × 1.0 mm internal diameter, 1.8 μm Waters HSS C18 column using a linear 16 min gradient from 20 to 55% of solvent B (acetonitrile containing 0.1% formic acid) in solvent A (doubly distilled water containing 0.1% formic acid) with a flow of 0.14 ml min⁻¹. The column was held at 50°C during separations and re-equilibration. The 2.5 μl sample was injected in the ‘partial loop needle overfill’ mode of a Waters Acquity autosampler. Column effluent was introduced into the ion source of the mass spectrometer, which operated in the negative ion mode with the following parameters of the ion source: cone voltage 20 V, capillary voltage 3 kV, extractor 3 V, RF lens 100 mV, source temperature 140°C, desolvation temperature 400°C, desolvation gas flow 1000 1 h⁻¹, cone gas flow 40 1 h⁻¹, collision cell entrance and exit were set to 50, collision energy was set to 2. Parameters of quadruples 1 and 3 were set to achieve unit-mass resolution: both LM and HM resolutions were set to 15 and ion energies were set to 0.8.

**Tested micro-organisms.** The reference strain *C. albicans* ATCC 10231 and five clinical *C. albicans* strains were used. Suspensions of the yeasts, at a cell density of 1–2 × 10⁶ cells ml⁻¹ in RPMI 1640 medium, were prepared from fresh (24 h) cultures grown at 35°C on Sabouraud Dextrose Agar (SDA; Difco).

**Evaluation of subMICs of SFs.** The MICs of the SFs against *C. albicans* strains were determined using an SDA dilution assay, with the tested products in the final concentration range of 125–1000 μg ml⁻¹ for *M. sativa* SFs and 250–3000 μg ml⁻¹ *S. officinalis* SFs. Each yeast suspension was spread on the surface of the SDA–SFs plates and the end point of the test system was determined after 48 h incubation at 35°C. The highest concentration of the given SFs resulting in the lack of inhibition of yeast growth, compared with the untreated control, was considered as its subMIC and was used for further experiments. All experiments were conducted in duplicate.

**Determination of antymycotic agent synergy with SFs.** Prepared inocula of *C. albicans* reference and clinical strains (OD₅₃₅ 0.5, nephelometer type Densimeter II) were spread with a sterile cotton swab on (i) control SDA or (ii) SDA plates containing SFs at a final concentration of 250 μg ml⁻¹ (subMIC) in duplicate. A standard disc diffusion test was performed (CLSI, 2009), using the following antymycotic agent set (per disc): amphotericin B (20 μg), miconazole (10 μg), clotrimazole (10 μg), ketoconazole (10 μg), nystatin (100 units), natamycin (10 μg), econazole (10 μg) and fluconazole (1 μg) (MASTRING-S; Mast Diagnostics). The plates were incubated at 37°C for 48 h and the growth inhibition zones were measured, including two inhibition zones for azoles (the complete growth inhibition zone and the zone with single small colonies resulting from the fungistatic activity of the azoles). The end points were determined according to the manufacturer’s instructions.

**Germ tube formation under the influence of SFs.** To determine serum-induced filamentation in liquid media and mycelium-like growth of *C. albicans* ATCC 10231, blastoconidia (4 × 10⁶ cells ml⁻¹) were incubated in RPMI 1640 medium containing 10% (v/v) FBS (RPMI-FBS) without (control) or with the addition of SFs at subMICs (final concentrations 125 and 250 μg ml⁻¹). At 1, 2, 3, 4, 24 and 48 h, the proportion of germ tube forms (GTFs), hyphae or other forms of cell morphology was evaluated in each culture by a microscopic examination of an aliquot of a culture using a light microscope (Zeiss Primo Star). Cells were considered as germinated if they had a germ tube at least twice the length of the cell. The results were expressed as GTFs per 100 cells (in five replications; total 500 cells counted) ±SD, and the percentage of GTFs after SF treatment in comparison with the control, i.e. GTFs formed by untreated *C. albicans* (considered as 100%), was calculated.

**Spider agar-invasive hyphal growth.** The test medium consisted of nutrient broth 1% (w/v), mannitol 1% (w/v), K₂HPO₄ 0.2% (w/v) and agar 1.35% (w/v) with the addition of SFs at subMICs (final concentrations 125, 250 and 500 μg ml⁻¹). *C. albicans* medium without SFs, containing ethanol (solvent) at appropriate concentrations (0.3–1.25%) served as the control. Aliquots (2 μl) of *C. albicans* ATCC 10231 suspension (5 × 10⁶ cells ml⁻¹) were spotted onto Spider agar plates in triplicates and the morphology of growing colonies (mycelium formation) was monitored daily for up to 7 days of incubation at 30°C. The presence of hyphal growth at the colony edges was determined using a stereomicroscope (PZO) under ×12 magnification and photographed using a digital camera.

**Candida oxidative stress tolerance under the influence of SFs.** To test the oxidative stress tolerance, *C. albicans* ATCC 10231 was exposed (24 h) or not (control) to *M. sativa* SFs used at the concentration of 500 μg ml⁻¹ in agar medium. Then, 1 ml prepared *Candida* cell suspension (1 × 10⁶ cells ml⁻¹) pre-exposed to SFs or control was transferred to Eppendorf tubes and treated for 1 h at 35°C with hydrogen peroxide at concentrations of 10, 25 or 50 mM (chosen after the preliminary studies). Finally, fungal suspensions...
were centrifuged (1200 r.p.m., 10 min), diluted (from $10^5$ to $10^3$ cells ml$^{-1}$) and spotted (5 µl) onto YPG (yeast peptone glucose) agar plates. The morphology of Candida spot growth (diameter and number of microcolonies) was monitored for 48 h at 30 °C and compared with the growth of the control yeasts not treated with SFs and then with hydrogen peroxide.

C. albicans adhesion, biofilm formation on abiotic surfaces and biofilm eradication under the influence of SFs. A suspension of C. albicans ATCC 10231 (OD$_{550}$ 0.3, nephelometer type Densimeter II) in RPMI medium supplemented with 2 % (v/v) glucose (RPMI/Glc), prepared from a fresh overnight culture on SDA, was added (100 µl) to the wells of a 96-well tissue culture polystyrene microplate (Nunc) to estimate fungal adhesion and biofilm formation. Then, 100 µl SFs alone at a final concentration of 500 µg ml$^{-1}$ or SFs in combination with caspofungin at final concentrations of 500 and 0.25 µg ml$^{-1}$, respectively, were added. C. albicans suspension was cultured in the absence (control) or constant presence of SFs/SFs with caspofungin (five replications for each) for 2 h to estimate adhesion or 24 h to assess biofilm formation, at 37 °C. Then, non-attached fungal cells were removed and metabolic activity of the remaining cells was tested based on an XTT [2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt; Sigma] reduction assay. XTT solution (0.5 mg ml$^{-1}$), containing 10 µM menadione (Sigma), was put into wells (100 µl). The microplates were incubated for 2 h at 37 °C in the dark and the absorbance of the wells (490 nm) was measured. The results are presented as the percentages of adherent cells or biofilm biomass calculated from the mean absorbance values ± SD of the control (considered as 100%) and test wells.

The same starting yeast suspension was diluted twofold and added (200 µl) to the wells of a 96-well tissue culture polystyrene microplate for 24 h at 37 °C to investigate the influence of SFs on preformed (24 h) C. albicans biofilm (biofilm eradication). Then, non-attached fungal cells with the medium were removed and SFs alone (at a final concentration of 500 µg ml$^{-1}$) or SFs together with caspofungin (at final concentrations of 500 and 2 µg ml$^{-1}$, respectively) were added to fungal biofilm to a total volume of 200 µl. After 24 h incubation of C. albicans biofilm at 37 °C with or without (control) SFs/SFs with caspofungin (five replications for each), the degree of biofilm survival (%) was assessed as described above using an XTT reduction assay.

Cytotoxicity activity of SFs. L929 cells (ATCC CCL-1, NCTC clone 929) were grown in RPMI 1640 medium with L-glutamine and sodium bicarbonate (Sigma) supplemented with 10 % (v/v) heat-inactivated FBS (Cytogen) and 1 % (v/v) penicillin/streptomycin (Sigma) at 37 °C in a humidified incubator with 5 % CO$_2$ atmosphere for 3 days to obtain a confluent cell layer. Cells were detached with 0.25 % trypsin, and 100 µl cell suspension at a density of 1 × 10$^6$ cells ml$^{-1}$ was seeded into a 96-well tissue culture plate (Nunc) for 24 h at 37 °C and 5 % CO$_2$. Then, the culture medium was replaced with 200 µl medium containing the SFs in the concentration range 3.9–500 µg ml$^{-1}$ and exposed for 0.5 or 24 h. At the same time, the following controls were set up: cells with culture medium as growth positive control, cells with 1.25 % of ethanol as a solvent control, the SFs alone as a negative control for the samples (Ks) and medium alone as a negative control for the cells growth (Km). The cytotoxicity of the saponins was evaluated by the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay. At the end of each test period, the cells were centrifuged (1400 r.p.m., 10 min) and washed with culture medium. Finally, the supernatants were removed, and 100 µl fresh culture medium and 50 µl MTT in PBS at 1.5 mg ml$^{-1}$ were added to the cells followed by 2 h incubation in the dark. Subsequently, the medium and MTT were aspirated from the wells, and 75 µl 20 % (v/v) SDS (Sigma) in a mixture of dimethylformamide with water (1:1 ratio) was added to the wells for 18 h at room temperature in the dark to dissolve the insoluble purple formazan product into a coloured solution. The absorbance was read at a wavelength of 550 nm using a microplate reader (Victor2; Wallac). The results obtained for the samples and the controls were used to calculate the percentage of cell viability: cell viability (%) = ([A$_{550}$ sample–A$_{550}$ Ks]/(A$_{550}$ growth positive control–A$_{550}$ Km) × 100). A dose–response curve was derived from eight concentrations in the test range 3.9–500 µg ml$^{-1}$ using four wells per concentration to determine the mean of each point.

Haemolytic activity of SFs. To determine haemolytic activity, Tryptic Soy Agar (TSA; BTL) containing 5 % human erythrocytes was used. Aliquots of 5 µl of previously prepared SFs at concentrations of 125, 250 and 500 µg ml$^{-1}$ were applied to TSA as the spots to yield a circular inoculation site of ~5 mm in diameter and then incubated at 37 °C for 24 h. After incubation, a transparent zone around the spots was considered as positive haemolytic activity. Purified saponin from Quillaja saponaria (Quil A; Superfios Specialty Chemicals) prepared in the same concentration range was used as a standard. Triton X-100 (1 % v/v in PBS; Sigma) was used as a positive control.

Statistical analysis. Most of the results are expressed as mean ± SD. When applicable, statistical differences were evaluated using the program STATISTICA 6.0 (Stat Soft). P<0.05 was considered significant.

RESULTS

Content of the saponins in SFs

SFs of M. sativa var. Radius aerial parts (M) and roots (Mr) contained 97.29 and 99.70 % of the saponins, respectively. The saponin content of S. officinalis root extract (S) was 862.97 mg g$^{-1}$ (86.29 %; total amount of dry matter).

Antifungal activity of SFs and their synergy with antimycotics

M. sativa (M and Mr) and S. officinalis (S) SFs were assessed for antifungal activity in vitro. Initially, the effect of these products alone was evaluated against C. albicans ATCC 10231, and then the same reference strain and five C. albicans clinical strains were used to test synergistic activity of SFs with known antimycotics. The MICs of M and Mr against C. albicans ATCC 10231 were set to 1000 µg ml$^{-1}$. The growth of C. albicans was not inhibited by the S SF at relevant concentrations (the MIC exceeded its highest value used, i.e. 3000 µg ml$^{-1}$; data not shown).

The synergy of antifungal activity of classic antimycotics with plant-derived SFs was observed only for azoles, whilst it was weak or had no effect for amphotericin B, nystatin, natamycin and flucytosine. M. sativa root extracts seem to be the most promising to potentiate azole activity against C. albicans ATCC 10231. The best effect was described for ketoconazole, for which in the presence of Mr the complete growth inhibition zone reached 23.0 ± 0.8 mm, whereas in the control (activity of ketoconazole alone) such zones did not exist and there was only a 20.0 ± 0.0 mm zone with single small colonies, indicating weak fungistatic activity of ketoconazole alone. The synergy of Mr with clotrimazole
was manifested by the enlargement of the complete growth inhibition zone from 20.0 ± 0.0 mm in the control to 26.5 ± 0.6 mm in the presence of the SF. In the case of miconazole and econazole, extension of the complete growth inhibition zone was observed from 15.0 ± 0.0 mm in the control to 22.0 ± 0.0 mm and from 11.0 ± 0.0 mm in the control to 21.5 ± 0.6 mm, respectively. However, it is worth noting that 20.0 ± 0.0 mm zones with single small colonies were also seen on the control plates for these two azoles.

Similar *M. sativa* roots SF synergy with ketoconazole and econazole was also observed against three *C. albicans* clinical strains (nos 10, 17 and 22). However, extension of the complete growth inhibition zone caused by clotrimazole in the presence of Mr (from 20.0 ± 0.0 mm in the control to 24.0 ± 0.0 mm) was demonstrated only for *C. albicans* clinical strain no 10, whilst miconazole did not exhibit synergy with Mr. Representative results are presented in Fig. 1. No synergistic effect of *M. sativa* aerial parts and *S. officinalis* SFs with antifungal agents against *C. albicans* was observed. Only a slight broadening of the complete growth inhibition zone caused by natamycin action against *C. albicans* ATCC 10231 in the presence of *S. officinalis* SF (from 12.0 ± 0.0 mm in the control to 14.0 ± 0.0 mm) was seen.

**Influence of SFs on germ tube formation and hyphal growth**

All tested saponin-rich plant extracts inhibited germ tube formation by *C. albicans* cells, clearly visible for the first 3 h of the incubation of yeast suspension with or without (control) SFs. *S. officinalis* SF demonstrated the strongest activity, resulting in a decrease in the percentage of germinated cells by 50.4, 39.2 and 38.7 % when used at 250 µg ml⁻¹, and by 61.5, 53.5 and 48.1 %, when used at 500 µg ml⁻¹ after 1, 2 and 3 h of incubation, respectively. The percentages were calculated in comparison with the control (considered as 100 %). *M. sativa* aerial (M) and root (Mr) SFs exhibited slightly lower but still significant activity against germ tube formation, reaching 47.7, 36.3 and 10.5 % decrease in germinated *Candida* cells after 1, 2 and 3 h incubation with M at 250 µg ml⁻¹, and 39.0, 21.2 and 41.1 % after yeast incubation with Mr at 250 µg ml⁻¹. Interestingly, reducing the concentration of these fractions to 125 µg ml⁻¹ resulted in stronger inhibition of germ tube formation, achieving values of 57.8, 54.4 and 39.2 % for M, and 65.6, 46.9 and 27.5 % for Mr, respectively. Large aggregates of *Candida* cells, which were observed on microscope slides prepared from 4 h of incubation, made it impossible to assess the exact number of cells forming germ tubes. However, the different appearance of the control cells forming hyphae and cells treated with SFs forming aggregates of typical blastospores was still evident (Fig. 2). The demonstrated changes in germ tube formation correlate with the efficiency of Spider agar medium invasion by *C. albicans* in the presence of SFs. We observed strong, dose-dependent inhibition of the hyphal growth of yeast (mycelium formation) by SFs used at 125–500 µg ml⁻¹. As the effect of M and Mr was similar, images of the representative *C. albicans* colonies only after Mr and S are presented in Fig. 3. The mean diameters (mm ± SD) of the dense growth area forming the centre of the colony alone (value a) and the area also including the hyphal growth zone on the edge of the colony (value b) were calculated based on the dimensions of a few representative colonies.

**Oxidative stress cell wall stability**

*C. albicans* ATCC 10231 cells exposed to SFs (500 µg ml⁻¹) exhibited lower oxidative stress tolerance after treatment with various doses of hydrogen peroxide (10, 25 and 50 mM) in comparison with control cells. The SF from *M. sativa* aerial parts (M) seemed to be slightly more effective than saponins obtained from the roots (Mr) in the process of fungal cell wall destabilization, which resulted in sensitization of yeasts to hydrogen peroxide. As seen from Fig. 4, *C. albicans* cells pre-exposed to M or Mr and then treated with 50 mM hydrogen peroxide did not grow, in contrast to the control cells. Their growth after treatment with 25 mM hydrogen peroxide was also inhibited, but the effect was slightly stronger for *C. albicans* pre-exposed to M (Fig. 4b).
SF activity against *C. albicans* adhesion, biofilm formation and eradication

All tested SFs used at a final concentration of 500 μg ml⁻¹ influenced *C. albicans* adhesion leading to its inhibition by 5.1 ± 16.1 (P=0.54), 16.9 ± 8.6 (P=0.0373) and 29.7 ± 4.8 % (P=0.0122) for M, Mr and S SFs, respectively. Strong synergy in the anti-adherent activity between *M. sativa* SFs and caspofungin (0.25 μg ml⁻¹) was also demonstrated. The significant (P=0.0199) limitation of yeast adhesion reached 36.7 ± 13.4 % for aerial (M) SF mixed with caspofungin and 57.5 ± 8.4 % for root (Mr) SF with caspofungin, whilst for caspofungin alone it did not exceed 10 % (P=0.391) (Fig. 5a). In the constant presence of *M. sativa* SFs, *C. albicans* biofilm formation was significantly (P=0.0122) inhibited by 19.8 ± 1.4 % and 36.3 ± 7.8 %, respectively, for M and Mr. The SF from *S. officinalis* extract used at the same concentration (500 μg ml⁻¹) had no effect on yeast biofilm formation, similarly to caspofungin used alone at 0.25 μg ml⁻¹. However, we observed strong synergy in the anti-biofilm activity of all tested SFs (500 μg ml⁻¹) and caspofungin (0.25 μg ml⁻¹) applied together (Fig. 5b). The most potent in this respect was the mixture of *S. officinalis* SF and caspofungin, which almost completely inhibited *C. albicans* biofilm formation (biofilm biomass decreased by 95.5 ± 0.2 %, P=0.0122). *M. sativa* SFs used in combination with caspofungin were also potent biofilm inhibitors, causing 29.9 ± 6.2 (P=0.0122) and 74.9 ± 3.1 % (P=0.0122) reduction in fungal biofilm formation for M and Mr, respectively. Moreover, when applied to already-formed 24 h *C. albicans* biofilm at subMIC (500 μg ml⁻¹) these SFs reduced its viability. The percentage of mature fungal biofilm eradication was significant (P=0.0122) and reached 16.9 ± 3.4, 20.6 ± 6.9 and 18.9 ± 4.4 %, respectively, for M, Mr and S. More importantly, the observed effect of *Candida* biofilm eradication was improved by the simultaneous presence of caspofungin used at 2 μg ml⁻¹, which corresponded to 4×MIC. Mature yeast biofilm biomass was strongly reduced (P=0.0122) by 43.3 ± 1.4, 51.9 ± 2.0 and 47.4 ± 1.7 % by M, Mr and S, respectively, used in combination with caspofungin. Caspofungin used alone at the same concentration showed no effect on *C. albicans* 24 h-old biofilm (eradication at the level 0.99 ± 5.6 %, P=0.834).

SF influence on mouse fibroblasts

The results of an MTT test with L929 fibroblasts demonstrated that the SF from aerial parts of *M. sativa* (M) at 3.9–250.0 μg ml⁻¹ and ethanol 0.0098–1.25 % did not reduce vital cell numbers (P>0.05) in comparison with control cells (Fig. 6). Cytotoxicity of *M. sativa* root (Mr) SF increased in a dose- and time-dependent manner, but it
was still much lower than the cytotoxicity of reference saponins obtained from *S. officinalis* (Fig. 6). The LD$_{50}$ values, determined for L929 fibroblasts after 0.5 and 24 h exposure to the SFs, reached 500 and 500 mg ml$^{-1}$ for M, and 187.5 and 15.6 mg ml$^{-1}$ for Mr, respectively. The highest cytotoxicity (90.94 ± 0.00 % of dead cells after 0.5 h exposure to SF and 92.4 ± 0.01 % of dead cells after 24 h exposure to SF) was observed for *S. officinalis* SF (S) used at 500 mg ml$^{-1}$. It should be noted that S was also equally cytotoxic at the lower concentration tested (3.9 mg ml$^{-1}$) used for 24 h (94.61 ± 0.02 % of dead cells). The high cytotoxicity of *S. officinalis* saponin-rich extract correlated with its haemolytic activity shown on TSA plates with 5 % human erythrocytes (haemolytic zones: 3.0 ± 0.67 mm for S at 250 mg ml$^{-1}$, 3.94 ± 0.5 mm for S at 500 mg ml$^{-1}$, 9.33 ± 0.94 mm for Triton X-100). No haemolytic activity was demonstrated for the other SFs and their solvents.

**Fig. 3.** Impact of the SFs from *M. sativa* roots (Mr) and *S. officinalis* (S) used at 125, 250 and 500 mg ml$^{-1}$ on *C. albicans* ATCC 10231 hyphal growth on Spider agar medium. The presence or lack of hyphae was determined using a stereomicroscope (×12 magnification). The mean diameters (mm ± SD) of the dense growth area forming the centre of the colony alone (value a) and the area also including the hyphal growth zone on the edge of the colony (value b) were calculated based on the dimensions of a few representative colonies.

**Fig. 4.** Oxidative stress tolerance of *C. albicans* ATCC 10231 pre-cultured for 24 h on (a) control SDA or on SDA with (b) *M. sativa* aerial (M) SF or (c) *M. sativa* roots (Mr) SF at 500 μg ml$^{-1}$. The yeasts were further incubated with different concentrations of hydrogen peroxide (0, 10.0, 25.0 or 50.0 mM), diluted (10$^5$–10$^3$ cells ml$^{-1}$) and spotted (5 μl) on YPG plates. The growth intensity was evaluated after 48 h incubation at 30 °C.
DISCUSSION

The progress of medicine, including invasive medical procedures, usually affecting the human body by the application of biomaterials (e.g. vascular and urinary catheters, joint and bone prostheses, cardiac valves, pacemakers, bypass devices), is probably the main reason for the increasing number of immunocompromised patients. In this group, infections involving \textit{C. albicans} and other pathogenic fungi appear with high prevalence. Meanwhile, the availability of well-established pharmaceutics is greatly reduced due to the rapidly growing number of multidrug-resistant strains. The current paradigm in the discovery of novel promising antimicrobial therapeutics postulates their targeting to microbial virulence factors/abilities rather than direct biocidal activity (Bink \textit{et al.}, 2011; Chevalier \textit{et al.}, 2012; Gauwerky \textit{et al.}, 2009; Naicker \& Patel, 2013; Rajeshkumar \& Sundararaman, 2012). The antifungal potential of tested SFs from plant extracts seems to fulfil these needs.

The direct antifungal activity of examined phytocompounds should be considered as moderate (for \textit{M. sativa} aerial part and root SFs) or even weak (for \textit{S. officinalis} SF). Similar to the results obtained by Avato \textit{et al.} (2006), the MICs of tested SFs against \textit{C. albicans} were >500 µg ml$^{-1}$. However, the demonstrated synergy between the SFs (mainly obtained from the roots of \textit{M. sativa}) and the azoles (drugs commonly used to treat fungal infections) is worth noting. In our research, even complete restoration of \textit{C. albicans} sensitivity on, for example, ketoconazole in the presence of SFs was observed. Azoles interfere with sterol biosynthesis by inhibiting 14z-lanosterol demethylase, which leads to the reduction in the ergosterol content in the membranes, finally causing fungal growth repression (Cannon \textit{et al.}, 2009). Many strains of \textit{Candida} spp. particularly clinical isolates and the species naturally less sensitive to azoles, such as \textit{Candida glabrata} or \textit{Candida krusei}, are resistant to azoles by overexpression of plasma membrane efflux pumps. \textit{C. albicans} may possess...
CaCdr1/2p belonging to ATP-binding cassette pumps with a broad spectrum of substrates and CaMdr1p belonging to the major facilitator superfamily transporters (Cannon et al., 2009). Therefore, we could consider the possibility of blocking or modifying these pumps by saponins as a potential mechanism of their synergistic effect. However, at the same time we observed slight synergism between SFs and natamycin. The complete C. albicans ATCC 10231 growth inhibition zone caused by natamycin in the presence of S. officinalis SF was extended from 12.0 ± 0.0 mm in the control to 14.0 ± 0.0 mm. It points to other, probably simpler mechanisms of saponin synergy with antymycotics. Natamycin belongs to the polyenes – antifungals with the rare occurrence of resistance caused mainly by a reduction in the amount of ergosterol in the plasma membrane (te Welscher et al., 2008). Therefore, we believe that the mechanism of demonstrated synergy is based on unspecific yeast cell membrane damage, which leads to its increased permeability (also for antifungal drugs), rather than blocking or modification of the efflux pumps. These assumptions were confirmed by the results of our research concerning the influence of the SFs on the effect of oxidative stress. Tested SFs decreased C. albicans tolerance to hydrogen peroxide, as we had previously demonstrated for essential oils (Budzyńska et al., 2013). C. albicans shows great natural resistance to oxidative stress resulting mainly from the activity of numerous anti-oxidative enzymes (e.g. catalase, superoxide dismutases, glutathione oxidase) (Missall et al., 2004). Based on our results, it can be suggested that the SFs had a direct effect on Candida cell wall stability rather than on the activity of the enzymes mentioned above. Such physical alteration might influence the correct positioning and anchoring of cell-walllocalized proteins responsible for counteracting the oxidative stress (e.g. superoxide dismutases). Importantly, C. albicans activates an oxidative stress response, among others, after exposure to human blood or phagocytosis, which promotes pathogen invasion and survival inside the host cells, including phagocytes (Enjalbert et al., 2007; Missall et al., 2004). In summary, the parallel use of antifungals and saponin-rich plant extracts (SFs) in the course of yeast infections can support classic therapy, decrease the risk of invasive mycosis, reduce the applied doses of antymycotics and limit the possibility of resistant strain generation. However, it should also be taken into consideration that the presence of subinhibitory concentrations of antymycotics can lead to overexpression of other fungal virulence factors, as described by Wu et al. (2000) for C. albicans under fluconazole subMIC concentrations.

Interestingly, the tested SFs seem to be very promising in the context of their effect targeted against fungal invasion and biofilm formation. All the more so as only a small number of drugs can be used to treat invasive fungal infections and biofilm-associated infections of implanted biomaterials. As described above, C. albicans hyphal growth and the possibility of morphological form transformation (yeast-to-hyphal) play a pivotal role in tissue invasion and biofilm stability. Therefore, the ability of test SFs to inhibit Candida germ tube formation, corresponding to the limitation of hyphal growth on Spider agar medium demonstrated here, is extremely important. This activity could prevent the development of invasive mycosis and fungal biofilm formation in the early stages of infection. Moreover, we showed the significant influence of SFs on the reduction of C. albicans adherence and biofilm formation, as well as on the eradication of mature (24 h) Candida biofilm. It is important to note that bactericidal and fungicidal (mainly against some plant fungi and a limited number of human pathogens) activity of M. sativa saponins, based on an assessment of MIC, have been reported previously (Avato et al., 2006; Balestrazzi et al., 2011; Carelli et al., 2011; D’Addabbo et al., 2011; Sung & Lee, 2008; Tava & Avato, 2006). However, data on antifungal activity of M. sativa and S. officinalis SFs in the context of yeast adhesion and biofilm formation, to the best of our knowledge, are reported here for the first time. In our research, we also demonstrated strong synergy between SFs and caspofungin in terms of their ability to inhibit C. albicans adhesion and biofilm development, and even to eliminate mature fungal biofilm. The effect of caspofungin used alone at MIC/2 (against yeast adhesion and biofilm formation) or 4 × MIC (against biofilm elimination) was very limited. We noticed only insignificant inhibition of C. albicans adhesion (P=0.3913) and no effect of caspofungin alone on fungal biofilm. However, caspofungin and amphotericin B have been shown to possess efficacy against yeast biofilm, but only when used in much higher concentrations. It was confirmed for different Candida spp. that the drug concentrations required for reducing fungal metabolic activity by half had to be five to eight times higher in biofilms compared with planktonic cells, leading to at least a 30-fold increase in MIC (Mathe & van Dijck, 2013). Soustre et al. (2004) showed that the same subMIC of caspofungin as that used in our research (MIC/2) decreased C. albicans adhesion, although mainly in the case of strains susceptible to fluconazole. The authors explained this phenomenon by an original mechanism of caspofungin action. Caspofungin belongs to echinocandins – cyclic lipopeptides interfering with cell wall biosynthesis by the inhibition of 1,3-β-glucan synthase (Cannon et al., 2009; Rajeshkumar & Sundararaman, 2012; Soustre et al., 2004). Therefore, its influence on the yeast cell wall may lead to a disturbance in the expression of some cellular adhesins.

Considering the biological activity of saponins, the composition of a crude saponin mixture seems to be important, but not fully understood. Even the extraction method can lead to different constitutions and thus various biological properties. According to the research performed by Timbekova et al. (1996), the medicagenic acid glucopyranoside as well as hederagenin glycosides and their derivatives (containing medicagenic acid or hederagenin as aglycones) are the main components of M. sativa extracts. However, the antimicrobial activity of crude saponin
mixtures obtained from *M. sativa* aerial parts, roots and their individual components was still very diverse, and depended on both the type of extract/compound and the tested micro-organism. For example, the growth of *C. albicans*, *Candida utilis*, *Bacillus mesentericus* and *Pseudomonas lachrymans* was not inhibited by the tested phytocompounds, whereas whole-root extract and the glucopyranoside of medicagenic acid limited the growth of *Candida tropicalis*. All tested preparations also fully suppressed the development of *Agrobacterium tumefaciens* and *Corynebacterium michiganense* (Timbekova et al., 1996). In other studies, *Saccharomyces cerevisiae* appeared to be the most susceptible among all tested fungi (including *C. albicans*, *C. tropicalis* and *Candida laurentii*) to saponins, mainly to sapogenin mixtures and medicagenic acid (Avato et al., 2006). Moreover, in many cases the whole-plant-derived extract (especially enriched in interesting phytocompounds) exhibits higher biological activity than its individual components, which may result from their synergistic effect (Liu, 2003; Seeram et al., 2005). It can be assumed that a similar phenomenon occurred in our *M. sativa* and *S. officinalis* SFs, in which the number of individual saponins reached 24.

In the light of the literature data on the negative effects of some components of plant extracts on eukaryotic cell viability and functions, cytotoxicity tests on mouse fibroblast line L929 were performed for all saponin-rich extracts. L929 fibroblast cells are ISO approved (according to EN ISO 10993-5) and the most common cell type that could be the target of the chemicals released from materials (e.g. dressings, drugs) having contact with damaged tissue. L929 fibroblasts were investigated with an MTT assay – a good indicator of cell viability. Our results showed that the SF from aerial parts of *M. sativa* was the least cytotoxic, even when used at 250 µg ml⁻¹, which was a potent inhibitor of *C. albicans* germ tube formation and hyphal growth. This is encouraging for the future application of this kind of preparation directly to eukaryotic tissues (e.g. in the form of surface-active ointments, lotions or dressings). The strong cytotoxicity of *S. officinalis* saponin-rich extract (even at the lowest concentration tested of 3.9 µg ml⁻¹), correlating with its haemolytic activity, excludes it from potential direct therapeutic use in humans. However, its ability (alone or in combination with caspofungin) to eradicate *C. albicans* biofilm, as well as to inhibit germ tube, hyphal growth and fungal biofilm formation, seems attractive in the context of the use of the SF from *S. officinalis* as an ingredient of antifungal disinfectants.

In conclusion, our results indicate an important antifungal potential of the saponins obtained from aerial parts and roots of *M. sativa* and roots of *S. officinalis*, used alone or in synergy with antimycotics. The ability of saponin-rich extracts to adversely affect such virulence factors of *C. albicans* as germ tube formation, hyphal growth, adhesion and biofilm formation/eradication was demonstrated. These attributes of *Candida* cells play a role as new potential drug targets and thus the properties of saponins seem to be very promising in the context of their possible medical applications. We suggest the use of plant-derived saponins as novel therapeutics supporting classic drugs in the course of fungal infections or as an ingredient of antifungal disinfectants. Further studies, in particular clinical investigations, are needed.

**ACKNOWLEDGEMENTS**

The study was partially supported by funds of the National Science Centre (grant 2012/05/N/NZ7/01216).

**REFERENCES**


http://jmm.sgmjournals.org


