ANTICANDIDAL EFFECT OF THE EXTRACTS FROM *MELILOTUS OFFICINALIS* AND *ARISTOLOCHIA CLEMATITIS*

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ABSTRACT
The aim of the present study was to assess the anticandidal effects of ethanolic and aqueous extracts of *Melilotus officinalis* (sweet clover) and *Aristolochia clematitis* (birthwort) against selected isolates of 9 *Candida* species. The aerial parts of the plants were collected in Romania. Anticandidal activities of aqueous and ethanolic extracts were tested by microtiter broth dilution method against isolates of *C. inconspicua*, *C. pulcherrima*, *C. guilliermondii*, *C. albicans*, *C. krusei*, *C. lusitaniae*, *C. glabrata*, C. *parapsilosis*, *C. methapsilosis* and *C. ortopsilosis*. Aqueous and ethanolic extracts of sweet clover showed broad spectrum anticandidal activity and inhibited the growth of most tested yeast strains. From the species tested, the isolates of *C. guilliermondii* and *C. parapsilosis* were the most sensitive. Extracts of birthwort showed no growth reduction effect on some *Candida* species: growth of *C. krusei*, *C. lusitaniae* and *C. glabrata* was even enhanced by these extracts, and aqueous extract had also a growth supporting effect on *C. albicans*.

Keywords: *Candida*, *Melilotus officinalis*, *Aristolochia clematitis*, antifungal activity

INTRODUCTION

The need for new and useful natural compounds with beneficial effect on human health is growing, and a lot of publications appear about the evidence-based use of medicinal plants supporting the treatment of various diseases. The antimicrobial activity of natural compounds is in the focus of interest also because of the growing microbial resistance against frequently used antibiotics. The flora of Central Europe has large potential for plants which can be used for these purposes. That is why we aimed to perform antimicrobial screening tests with two well-known medicinal plants: sweet clover and birthwort.

Melilotus officinalis (L.) Pallas, belongs to the family *Fabaceae*. It is an annual to biennial erect or decumbent plant, 30-250 cm tall. The flowering stems or just the flowers of the plant are used in traditional medicine to treat inflammation and infection in the throat and gastrointestinal system (ANWER ET AL., 2008). It was documented that sweet clover is used to reduce spasm; its coumarinic extract have effects on lymphedema and its polysaccharides have immunocorrecting, anti-anemia and adaptogenic effects (ZHAO ET AL., 2007). The constituents include melilotin and other coumarin glycosides, an essential oil, and flavonoid pigments making *M. officinalis* aromatic (MARTINO ET AL., 2006).

Aristolochia species are widely used in traditional medicine and have a number of physiologically active compounds of different classes, with aristolochic acid derivatives as the main compounds (BENZAKOUR ET AL., 2011; WU ET AL., 2004). Ingestion of herbal remedies containing aristolochic acid is associated with the development of a syndrome of chronic renal failure, designated aristolochic acid nephropathy (POZDZIK ET AL., 2010). Although, the toxicity of aristolochic acid has been well studied (MENGS AND STOTZEM, 1993), *Aristolochia* species are still used as alternative medicines. Aristolochic acid from the roots of *A. bracteata* showed good antibacterial effect (ANGALAPARAMESWARI ET AL.,

2012) whereas methanolic extracts of the leaves showed good antibacterial and moderate antifungal activity (DEEPA ET AL., 2012).

In this study we investigated the antifungal effect of aqueous and ethanolic birthwort extracts against *Candida* species. *C. albicans* and other *Candida* species are now recognized as major agents of hospital acquired infection worldwide. Recent data from the US National Nosocomial Infections Surveillance system rank these organisms as the fourth most common cause of bloodstream infection. Mortality rates are high and treatment is costly. Other *Candida* spp. are also frequently identified as agents of nosocomial pneumonias and urinary tract infections (LUPETTI ET AL., 2002).

Biofilm formation plays an important role in the pathogenicity of *C. albicans*. One defining characteristic of a biofilm is presence of a matrix of extracellular polymeric material in which the microorganisms are embedded. Probably the most significant feature of microbial biofilms is their notorious resistance to a variety of antimicrobial agents, including antibiotics, antiseptics and industrial biocides. Natural antimicrobials are thought to overcome resistance problems since they have more than one active ingredients acting by different mechanisms.

MATERIALS AND METHODS

Plants

Melilotus officinalis (L.) Pallas and *Aristolochia clematitis* L. were collected in Romania and were identified in the University Botanical Garden of Szeged.

Microorganisms

C. inconspicua CBS 619, *C. pulcherrima* CBS 566, *C. guilliermondii* ATCC 10231, *C. albicans* CBS 573, *C. krusei* CBS 6936, *C. lusitaniae* CBS 138, *C. glabrata* CBS 604, C. *parapsilosis* SZMC 8092, *C. methapsilosis* SZMC 8116 and *C. ortopsilosis* strains were used in the experiments. Yeast strains were obtained form the American Type Culture Collection (ATCC, Manassas, VA, USA), from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), and from the Szeged Microbial Collection (SZMC, Szeged, Hungary). Inocula from 72 hours old yeast cultures and YEPD medium (1% (w/v) yeast extract, 2% peptone, 2% glucose, 2% agar) were used for the tests. Inocula (10^5 cells mL^{-1}) were prepared in RPMI 1640 medium (Sigma-Aldrich) (KRISCH ET AL., 2009).

Preparation of plant samples

The aerial parts of the plants were naturally air dried in shade for 14 days. Once dried, they were milled with an electric grinder into a fine powder which was stored at 4 °C until use.

Preparation of extracts

To 10 g powdered plant material 100-100 ml distilled water or 96% (v/v) ethanol was added, and the solution was agitated for 24 hours at room temperature in the dark. The extracts were filtered and concentrated by lyophilization or by vacuum drying at 50 ^oC. After the dehydration process, dry plant extracts were dissolved in 30% (v/v) ethanol to a final concentration of 50 mg mL^{-1} and sterilized by filtration through a 0.45 µm membrane filter (Millipore). The extracts were stored in labeled sterile bottles at -20 °C.

Determination of anti-candidal activity by broth micro dilution assay

In vitro antifungal activities were evaluated by 96-well microtiter plates assay in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), buffered with 0.165 M morpholino-propanesulfonic acid (pH 7) according to National Committee for Clinical Laboratory Standards (CLSI RECOMMENDATION, 2002). In the wells, 100 µl sterile-filtered plant extracts were mixed with 100 µl cell suspension (10^5 cell/ml). Each test plate contained an uninoculated control, a positive growth control, a medium sterile control and a drug sterile

control. The assay was performed in triplicates and absorbance was read at 620 nm after incubation without shaking at 37 °C for 24 hours. Measured absorbance values were converted into percent growth, taking the control as 100%.

RESULTS AND DISCUSSION

Overall, sweet clover aqueous and ethanolic extracts showed broad spectrum anti-candidal activity, and inhibited the growth of all tested strains (*Fig. 1* and *Fig. 2*). From the species tested, *C. guilliermondii* and *C. parapsilosis* were the most sensitive, while the growth of *C. albicans*, *C. krusei*, *C. lusitaniae*, *C. glabrata* were less affected.

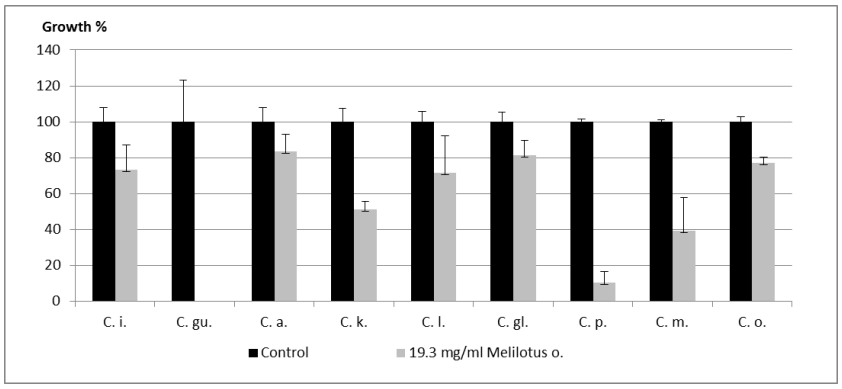

Figure 1. Fungal growth (%) in the presence of *M. officinalis* alcoholic extracts
Control was taken as 100%. C.i., C.gu., C.a., C.k., C.l., C.gl., C.p., C.m., and C.o., are *C. inconspicua*, *C. guilliermondii*, *C. albicans*, C. *krusei*, C. *lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. methapsilosis*, and *C. ortopsilosis*, respectively.

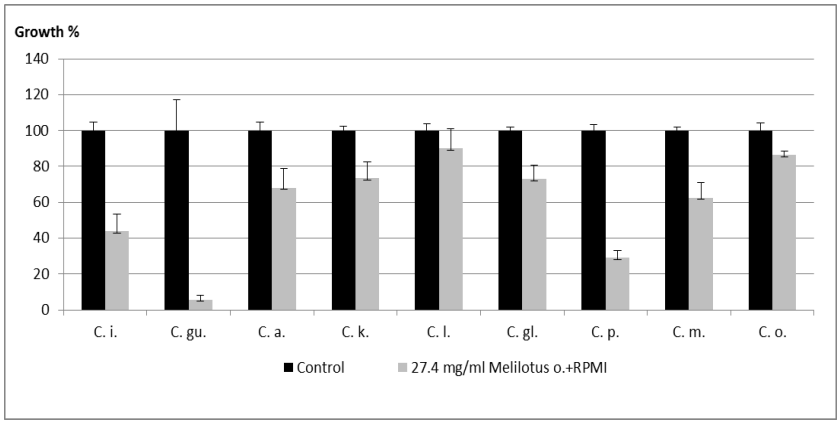

Figure 2. Fungal growth (%) in the presence of *M. officinalis* aqueous extracts.
Control was taken as 100%. C.i., C.gu., C.a., C.k., C.l., C.gl., C.p., C.m., and C.o., are *C. inconspicua*, *C. guilliermondii*, *C. albicans*, C. *krusei*, C. *lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. methapsilosis*, and *C. ortopsilosis*, respectively.

Surprisingly, extracts of *A. clematitis* showed no growth reduction effect on some *Candida* species and the growth of *C. krusei*, *C. lusitaniae*, and *C. glabrata* was even supported by

both types of extracts. The aqueous extract had also growth supporting effect on *C. albicans* (*Fig. 3* and *Fig. 4*). Reasons of this phenomenom are unknown and need further investigation. It is worth to mention that the same strains were the less sensitive ones also to sweet clover extracts.

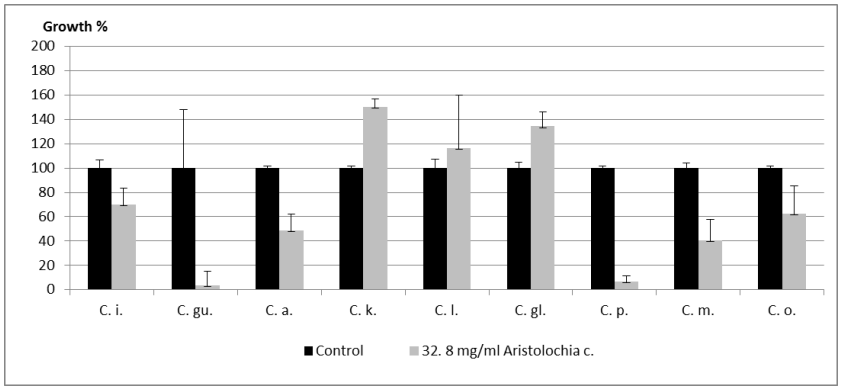

Figure 3. Fungal growth (%) in the presence of *A. clematitis* alcoholic extracts. Control was taken as 100%. C.i., C.gu., C.a., C.k., C.l., C.gl., C.p., C.m., and C.o., are *C. inconspicua*, *C. guilliermondii*, *C. albicans*, C. *krusei*, C. *lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. methapsilosis*, and *C. ortopsilosis*, respectively.

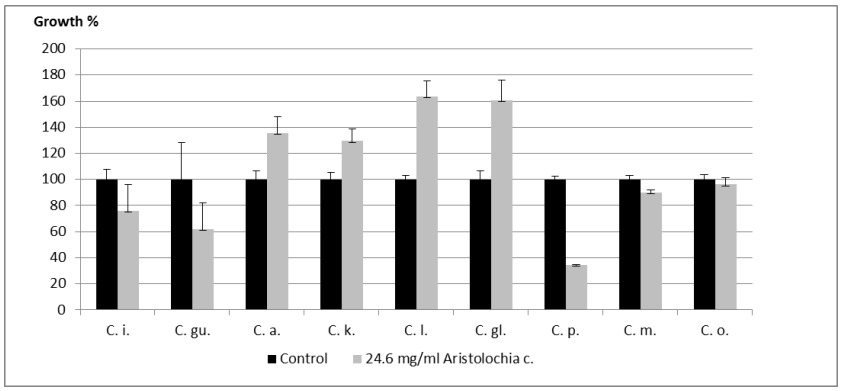

Figure 4. Fungal growth (%) in the presence of *A. clematitis* aqueous extracts. Control was taken as 100%. C.i., C.gu., C.a., C.k., C.l., C.gl., C.p., C.m., and C.o., are *C. inconspicua*, *C. guilliermondii*, *C. albicans*, C. *krusei*, C. *lusitaniae*, *C. glabrata*, *C. parapsilosis*, *C. methapsilosis*, and *C. ortopsilosis*, respectively.

These results suggest, that *M. officinalis* (sweet clover) extracts are useful as antimycotic agents against wide range of *Candida* species. Further studies, to clarify the role of different constituents of sweet clover extracts in this anti-candidal effect, and their activity against other fungal pathogens are in progress.

ACKNOWLEDGEMENTS

This study was supported in part by the contracts supported by the Ministry of Rural Development, Hungary (VM: SF/533/2012 and 1002908849).

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