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Original Research

Antifungal Activity of *Juglans regia* (L.) Leaf Extracts Against *Candida albicans* Isolates

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Abstract

The objective of performed study was aimed at evaluating the antifungal potential of four extract fractions (methanolic, ethyl acetate, alkaloid, and hydrolyzed methanolic) derived from *Juglans regia* (L.) leaves against pathogenic *Candida albicans* strains. Furthermore, total phenolics and alkaloid content as well as the antioxidative potential of examined extract fractions were determined. Tested yeasts comprised 140 isolates from diverse biological specimens (oropharyngeal, rectal and vulvovaginal swabs, skin lesions, sputum, urine, and faeces), and one reference strain (*C. albicans* ATCC 90029). Methanolic extract from walnut leaves characterized by the highest anticandidal activity, the alkaloid fraction possessed a slightly lower antifungal efficacy, while ethyl acetate and hydrolyzed methanolic preparates inhibited the growth rate of examined fungal pathogens in the lowest degree. Additionally, it has been elucidated that all tested strains were susceptible for nystatin and amphotericin B, and only one yeast strain was resistant to flucytosine. On the contrary, the group of azole antimycotics were characterized by reduced effectiveness against the candidal isolates.

Keywords: antifungal activity, antimycotic susceptibility testing, *Candida albicans*, *Juglans regia*, total phenolics content, alkaloids, antioxidative potential

Introduction

Candida albicans is a pleomorphic diploid yeast existing as part of normal microbial communities of skin surface, mucous membrane, and gastrointestinal tract in the vast majority of healthy humans [1]. Switching the ecological strategy of these fungi from commensalism to parasitism depends on the enhanced susceptibility of the host, rapid transcriptional reprogramming, and profound phenotypic modifications in the fungal cells in response to environmental stimuli [2]. Pathogenic *C. albicans* strains are involved in multifarious infections from mild cutaneous or mucosal disorders, through urinary tract infections to severe and life-threatening systemic invasions with candidaemia [3]. In the past decade, there has been reported an increasing number of nosocomial *Candida* spp. infections [3-5]. Unfortunately, available options of the antifungal therapy are very limited in opposition to a wide battery of antibiotics used in eradication of bacterial infections. Secondarily, prolonged antifungal monotherapy or a combination of regiments is linked with a significant risk of hematologic, hepatic, and/or renal toxicity [6]. The plant kingdom may provide a wide spectrum of highly active antimycotic compounds exhibiting dissimilar modes of action in comparison with the agents broadly used in medicine [7-9]. In addition, synergistic interactions between the chemical constituents in plant extracts may significantly improve its anticandidal activity [7, 10].

It has been signally reported that aqueous extracts from the fruit of several varieties of the walnut (*Juglans regia* L.,

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Juglandaceae family) possessed antimycotic efficacy towards the reference yeast strain (*C. albicans* CECT 1394) [9]. Furthermore, Noumi and co-workers also reported that methanolic, ethyl acetate, and diluted acetone extracts of *J. regia* bark sampled in Tunisia were characterized by the antifungal properties against many *Candida* spp. strains isolated from the oral cavity of humans [11].

In the context of previous reports regarding quantitative-qualitative analyses of selected secondary metabolites (e.g. phenolic acids, flavonoids, and naphtoquinones) in the leaves of the walnut foliar tissues, this plant species may serve as a plentiful source of biologically active constituents [12-16]. Therefore, there is a need to elucidate whether leaf extracts obtained from the walnut may possess the antifungal potential against pathogenic *C. albicans* isolates.

The purpose of the performed studies was to assess *in vitro* antifungal activity of the four extracts (methanolic, ethyl acetate, alkaloid, and hydrolyzed methanolic fraction) derived from *J. regia* leaves against the candidal isolates differing in resistance patterns to the antimycotic drugs. Moreover, contents of total phenolics and alkaloids, and antioxidative capacity of the investigated walnut preparates were determined. Additionally, the conducted bioassays were focused on evaluating the susceptibility patterns of pathogenic strains of *C. albicans* isolated from various biological specimens (oropharyngeal, rectal and vulvovaginal swabs, skin lesions, sputum, urine, and faeces) toward 10 antimycotic drugs (nystatin, amphotericin B, flucytosine, voriconazole, itraconazole, fluconazole, ketoconazole, clotrimazole, miconazole, and econazole).

Material and Methods

Plant Material

Mature leaves of the walnut (*Juglans regia* L.) cv. 'Albi' were harvested in June 2010 from plants grown in a small cottage orchard near Siedlce, Poland (52º11'N, 22º17'E). No phytosanitary procedures were applied. Only healthy green leaves without any visible damage were sampled.

Preparation of Extracts

Groups of chemical substances tested in microbiological experiments were extracted and fractionated according to the procedures described by Chrzanowski et al. [12] and Chrzanowski [17].

In order to obtain the methanolic fraction (MF), 20 g of leaf dry weight (d.w.) was vigorously vortexed with 0.5 dm^3 80% methanol (v/v) for 6 h. The extract was filtered two times through Whatman No. 1 under reduced pressure. The filtrate was evaporated at 40ºC using a vacuum rotary evaporator (Heidolph Hei-VAP Precision), and the dry residue was stored until antimycotic activity determination.

Plant material (50 g of leaf d.w.) was vigorously vortexed with 1 dm^3 80% methanol for 6 h. The extract was filtered through Whatman No. 1 paper under reduced pressure. Next, the filtrate was collected, and the residual pellet was secondly vortexed with 0.5 dm^3 80% methanol (v/v) at ambient temperature for 3 h, and filtered as mentioned above. The mixtures were pooled and evaporated at 40ºC using the rotary evaporator to obtain the final 60% methanol concentration, and the solution was defatted with petroleum ether.

The purified methanolic extract was divided into two portions. The first part of the volume was subjected to acidic hydrolysis, whereas the second one was acidified to pH 2.0 with 6 M hydrochloric acid and shaken with ethyl acetate. The extraction was performed three times, using one volume of ethyl acetate to 1.5 volumes of the methanolic extract. Subsequently, the pooled organic layers were evaporated to dryness with the use of a vacuum evaporator at 40ºC. The dry residue represented the ethyl acetate fraction without hydrolysis (EAF) that was tested in further stages of the study. In order to perform the hydrolysis, methanolic extract was acidified to pH 2 using concentrated hydrochloric acid, and the process was carried out for 6 h at 80ºC under a water-cooled condenser.

After completion of hydrolysis, the extract was acidified to pH 2 (using 6 M HCl) and shaken with ethyl acetate (three portions of 150 cm^3 each). The combined organic phases were evaporated to dryness to obtain the hydrolyzed methanolic fraction (HMF). Separation of the alkaloid fraction (AF) was made from 50 g of leaf d.w. by extraction with 1 dm^3 0.1 M hydrochloric acid for 3 h at 80ºC. The mixture was filtered through Whatman No.1 paper, and the residue was treated again with 0.1 M HCl for 1 h at the same temperature. The combined filtrates were alkalized to pH 9.5 with 5 M NaOH, and shaken three times with diethyl ether.

The organic layers were pooled and evaporated to dryness at 40ºC. The isolated fractions of walnut leaves were stored in dry form at -20ºC until analysis. Before assessing the antimycotic activity of the walnut fractions, each tested preparate was dissolved in 50 cm^3 96% ethanol (molecular biology grade) to yield the crude extract. Next, several concentrations (0.01, 0.05, 0.10, 0.25, 0.5, 1.0, 1.5, and 2.0 $mg\cdot cm^{-3}$) of the fractions were prepared from the relevant stock solutions and subsequently used in the microbiological assays.

Chemical Analyses

Total phenolics content of the methanolic, ethyl acetate, and hydrolyzed methanolic fraction from the walnut leaves were measured using Folin-Ciocalteu's spectrophotometric method, according to the procedure described by Singleton and Rossi [18]. The phenolics content was expressed as a percentage of dry weight residue of the respective extract fraction.

The alkaloid content in AF fraction of *J. regia* leaves was determined by the spectrophotometric method with the use of Dragendorff's reagent [17]. Extract was acidified with 1 M HCl to pH 2.0-2.5, and 2 cm^3 of Dragendorff's reagent was added. The precipitate was washed with ethanol and centrifuged (6,000 × g for 15 min). Next, 2.0 cm^3 1% solution of sodium sulphide (Na_2S) was added to the residue. The brown-black pellet was dissolved in

2.0 cm^3 of concentrated nitric acid (V) and diluted with 10 cm^3 of deionized water. A portion (1 cm^3) of the mixture was combined with 5 cm^3 3% solution of thiourea. The absorbance value was measured at 435 nm, and the alkaloid content was presented as a percentage of dry weight of the extracted residue. Quinine was used as a standard, and the calibration curve was prepared in the concentration range 10-75 $\mu g \cdot cm^{-3}$.

Antioxidative capacity of the investigated walnut preparates against DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals was estimated with the protocol of Brand-Williams et al. [19]. The reaction mix contained 380 mm^3 0.04% methanolic solution of $DPPH^\bullet$ and 20 mm^3 of the respective *J. regia* extract (2 $mg \cdot cm^{-3}$). Subsequently, the preparates were vigorously vortexed for 1 min, and incubated at ambient temperature for 15 min. After this procedure, absorbance values of the samples were measured at 517 nm. The antioxidant potential of the tested extracts was expressed as a percentage (%) inhibition of $DPPH^\bullet$ radicals.

Microorganisms Tested

The investigated human pathogenic *C. albicans* strains (N=140) were isolated from a broad range of biological samples (oropharyngeal, rectal and vulvovaginal swabs, skin lesions, sputum, urine, and faeces). The yeast strains were provided by the Department of Medical Microbiology at Warsaw Medical University and Damian Medical Centre in Warsaw (both in Poland). Furthermore, *C. albicans* ATCC 90029 was used as a reference strain.

Species Identification and Antimycotic Susceptibility Assay

Species level confirmation of the candidal isolates and their *in vitro* susceptibility profiles to 10 standardized antifungal agents: nystatin (NY), amphotericin B (AMB), flucytosine (5-fluorocytosine, AFY), voriconazole (VO), itraconazole (ITC), fluconazole (FLU), ketoconazole (KCA), clotrimazole (CLO), miconazole (MCL), and econazole (ECN) were verified by using the Integral System YEASTS Plus (Liofilchem, Italy), following the manufacturer's protocol. Additionally, the antifungal effects of these drugs toward the investigated *C. albicans* strains were determined using the agar-based disk diffusion method according to the protocol M44-A2 of the Clinical and Laboratory Standards Institute (CLSI) [20]. Fluconazole and voriconazole disks were purchased from Becton Dickinson & Co. (USA), itraconazole disks were provided from Abtek Biologicals LTD (Liverpool, United Kingdom), and the other antimycotic disks were produced and supplied by Liofilchem (Italy).

Evaluation of the Antifungal Activity of Walnut Leaf Extracts

All tested yeast isolates (N=140) and one reference strain (*C. albicans* ATCC 90029) were used to assess the antimycotic activity of methanolic, ethyl acetate, alkaloid, and hydrolyzed methanolic fractions derived from the walnut leaves. The anticandidal potential of investigated extracts was screened using the disk diffusion method of Noumi et al. with minor modifications [11]. It used the Mueller-Hinton agar (Merck, Poland) supplemented with 2% glucose and 0.5 $\mu g \cdot cm^{-3}$ methylene blue (Sigma-Aldrich, Poland). Sterile 6 mm disks (bioMérieux, Poland) were impregnated with the relevant extract fractions obtained from *J. regia* leaves and placed on the agar plates (120 mm diameter, 4 mm depth) previously inoculated with the yeast cells suspended in sterile 0.85% NaCl solution and adjusted to a 0.5 McFarland turbidity standard (approx. 10^6 CFU cm^{-3}). Individual *C. albicans* strains were tested on separate Petri dishes (n=3), and after 24 h incubation at 35ºC, diameters of the growth inhibition zone (in mm) were measured. Furthermore, negative controls (blank disks saturated with the extrahent) were included in all experiments.

Determination of Growth Rate of the Reference Candidal Strain in the Presence of Examined *J. regia* Extract Fractions

The bioassay comprised one reference *C. albicans* strain (ATCC 90029). The effect of four leaf walnut fractions (MF, EAF, HMF, and AF) on growth intensity of the investigated strain was evaluated. Inoculum preparation and assessment of the yeast growth rate was conducted according to Hoot et al. method [21], with slight modifications. The examined *C. albicans* strain was cultured at 37ºC in liquid yeast peptone dextrose (YPD) broth containing 2.0 $mg \cdot cm^{-3}$ of the relevant extract fraction derived from the walnut leaves. Optical density (OD_{600}) of the yeast suspensions (aliquots of 200 mm^3) was measured after 4 and 8 h post inoculation (hpi) in 96-well sterile, flat-bottom, and transparent microplates (Greiner Bio-One GmbH, Austria) using an Epoch UV-Vis spectrophotometer (BioTek, USA). Negative controls (inoculated YPD medium untreated with the walnut extracts) were also included.

Statistical Analysis

The experiments were performed in three independent replications (n=3) and the obtained results were presented as the mean values ± standard deviation (SD). The effect of yeast strain and concentration of *J. regia* extract fractions on the growth inhibition zone of candidal isolates, as well as the influence of tested preparates and exposure time on the growth intensity (OD_{600}) of *C. albicans* ATCC 90029, were analyzed using a factorial analysis of variance (ANOVA). Significance of differences between the mean values was evaluated using Tukey's *post-hoc* test at $P < 0.05$. All calculations were carried out using STATISTICA 10.0 software (StatSoft, Poland).

Results and Discussion

The performed antifungal biotests revealed that all examined isolates (N=140) from a variety of specimens

Table 1. *In vitro* susceptibility profiles of *C. albicans* strains (N=140) against the examined antimicotic drugs.

Antifungal agents	Dose per disk	No. of *C. albicans* isolates tested (%)		
		S	SDD	R
Nystatin (NY)	100 IU	140 (100)	0 (0.0)	0 (0.0)
Amphotericin B (AMB)	20 µg	140 (100)	0 (0.0)	0 (0.0)
Flucytosine (AFY)	1 µg	139 (99.3)	0 (0.0)	1 (0.7)
Voriconazole (VO)	1 µg	135 (96.5)	3 (2.1)	2 (1.4)
Itraconazole (ITC)	8 µg	130 (92.9)	4 (2.8)	6 (4.3)
Fluconazole (FLU)	25 µg	125 (89.3)	6 (4.3)	9 (6.4)
Ketoconazole (KCA)	10 µg	118 (84.3)	15 (10.5)	7 (5.0)
Clotrimazole (CLO)	10 µg	107 (76.4)	28 (20.0)	5 (3.6)
Miconazole (MCL)	10 µg	104 (74.3)	34 (24.3)	2 (1.4)
Econazole (ECN)	10 µg	103 (73.6)	30 (21.4)	7 (5.0)

Disk diffusion method was performed in three separate replicates (n=3), according to the protocol M44-A2 of the Clinical and Laboratory Standards Institute, CLSI [20]. The Mueller-Hinton agar (MH-GMB) plates were inoculated with the yeast suspension and incubated at 35ºC for 24 h.
S – susceptible; SDD – susceptible dose-dependent; R – resistant.

were sensitive to both nystatin and amphotericin B (Table 1). Furthermore, 139 *C. albicans* strains were susceptible to flucytosine (99.3%). Additionally, more than 90% of the candidal strains were highly sensitive to voriconazole and itraconazole, whereas dose-dependent susceptibility to these antimycotics was identified in 3 (2.1%) and 4 (2.8%) isolates. A slightly lower number of the yeasts was sensitive for fluconazole (125/140; 89.3%) and ketoconazole (118/140; 84.3%). It is important to note that the group of tested fungal strains was markedly less sensitive to three azole antifungals: clotrimazole, miconazole, and econazole (76.4, 74.3 and 73.6%, accordingly). Moreover, the status of dose-dependent susceptibility to these agents occurred in 20.0-24.3% of the investigated microorganisms (28-34 yeast strains), depending on the antimycotic tested. The highest percent of *C. albicans* isolates were resistant to fluconazole (6.4%), moderate resistance levels were demonstrated with respect to ketoconazole (5.0%), econazole (5.0%), itraconazole (4.3%), and clotrimazole (3.6%), whereas the lowest percentage of the examined strains were resistant to miconazole (1.4%) and voriconazole (1.4%).

It has been identified that *C. albicans* is a predominant fungal pathogen responsible for multifarious forms of primary and re-emerging human candidiasis [22]. Therefore, identification and careful monitoring of environmental circulation of multi-drug resistant yeast strains seems to be an important issue in public health management. The conducted investigations revealed an excellent potency of nystatin and amphotericin B against the examined pathogenic yeast strains. These observations are coherent with findings reported by many other authors [22-24]. In contrast, Mishra et al. demonstrated an unusually high proportion of nystatin-resistant candidal isolates (20/57; 35.1%) from catheterized patients [25]. Nystatin and amphotericin B belong to polyene macrolide antibiotics and the mechanism of action involves their binding to ergosterol molecules within the yeast plasma membrane, which secondarily leads to uncontrolled leakage of ions and cell death [26]. However, therapeutic applications of these antifungals are very limited because of their high toxicity and haemolytic activity. In the present study, only one candidal strain (0.7%) was resistant to flucytosine (5'-fluorocytosine). Similarly, Dorocka-Bobkowska and Konopka established that 1.4% of the yeast strains isolated from patients with a diagnosed denture stomatitis were resistant to this antifungal agent [24]. High anticandidal efficiency of this drug has been evidenced by many authors from different countries [3, 6, 27].

Flucytosine is a fluorinated pyrimidine analogue transformed in the yeast organisms to 5-fluorouracil and other metabolites that profoundly repress the biosynthesis of nucleic acids and proteins. However, monotherapy with this antifungal is not recommended because of possible prompt selection of resistant yeast strains [6, 27]. Tested azole antifungals characterized with significantly lower efficacy toward the investigated yeast isolates in comparison with flucytosine. The proportion of resistant yeast strains against tested azole drugs slightly differed and ranged from 6.4% (fluconazole) to 1.4% (voriconazole and miconazole). Additionally, this should be stressed to a considerable degree (above 20%) of dose-dependent susceptibility of examined candidal isolates to three azole agents: clotrimazole, miconazole, and econazole. In contrast to polyene antimycotics, many researchers have documented an increasing tendency in the emergence of azole resistance in clinical candidal strains [3, 22, 24, 25]. Rathor et al. suggest that susceptibility levels to members of this antifungal class varied significantly in dependence on the geographic

Table 2. *In vitro* anticandidal activity of methanolic and ethyl acetate extract fractions derived from *J. regia* leaves.

Yeast strains tested (*n*)	Extract concentration (mg·cm^{-3})							
	0.01	0.05	0.10	0.25	0.5	1.0	1.5	2.0
Methanolic fraction								
S (103)	–	–	8.5±0.1^{j}	9.2±0.1^{j}	14.6±0.8^{g}	18.3±0.9^{d}	20.8±1.1^{c}	21.2±1.2^{c}
RI (20)	–	–	9.0±0.3^{j}	9.5±0.3^{ij}	15.8±0.5^{ef}	16.5±0.7^{e}	21.5±1.3^{c}	23.4±1.6^{a}
RII (12)	–	–	–	8.4±0.1^{j}	15.2±0.9^{fg}	16.0±0.8^{ef}	20.7±1.0^{c}	22.8±1.3^{ab}
RIII (5)	–	–	–	11.0±0.5^{hi}	14.6±0.3^{fg}	17.2±0.9^{de}	18.4±0.9^{d}	20.5±1.1^{c}
Ref	–	–	8.5±0.1^{j}	12.5±0.6^{h}	18.3±0.9^{d}	20.4±1.0^{c}	21.0±1.0^{c}	21.6±1.2^{bc}
Ethyl acetate fraction								
S (103)	–	–	–	–	–	–	10.2±0.5^{e}	16.8±0.9^{a}
RI (20)	–	–	–	–	–	–	9.6±0.4^{ef}	14.5±0.6^{b}
RII (12)	–	–	–	–	–	–	10.0±0.5^{e}	12.4±0.6^{c}
RIII (5)	–	–	–	–	–	–	9.5±0.4^{ef}	13.0±0.7^{c}
Ref	–	–	–	–	–	9.0±0.3^{f}	11.4±0.5^{d}	15.2±0.8^{b}

(*n*) – number of yeast isolates tested; S – *C. albicans* strains susceptible for all tested antimycotic drugs; RI, RII, and RIII – groups of the yeast isolates resistant against 1, 2, and 3 (or more) antifungal drugs, respectively; Ref – reference strain (*C. albicans* ATCC 90029). All experiments were performed in triplicates. The anticandidal activity was expressed as a diameter of the inhibition zone (IZ) in millimeters (mean ± SD). Strong antifungal activity, IZ 20-25 mm; moderate antifungal activity, IZ 14-19 mm; low antifungal activity, IZ 8-13 mm; "–" – no zone of inhibition. Letters (superscript) indicate significant differences between average values of the inhibition zone, presented separately for each extract fraction ($P < 0.05$; Tukey's test for different *N*).

distribution of *C. albicans* strains, the nosocomial mode of transmission, and types of clinical specimens [3]. It has been postulated that a long-term and/or uncontrolled administration of azole agents, the main group of antimycotics used in treatment of chronic or recurrent candidiasis, leads to rapid selection and widespread use of resistant *Candida* spp. strains [23, 28]. Fluconazole is one of the most commonly used antifungal drugs, which is probably the main cause of the increased resistance of the candidal strains to this chemical compound. The mode of action of azole antibiotics is involved with a disturbed ergosterol biosynthesis within the plasma membrane of the yeasts [4, 8].

Results regarding evaluation of the antifungal potential of several extracts obtained from walnut leaves toward the investigated *C. albicans* strains are presented in Tables 2-3. The lowest concentrations (0.01 and 0.05 mg·cm^{-3}) of all tested extract fractions did not evoke any growth inhibition of the studied fungal strains. Similarly, slightly higher concentrations (0.10 mg·cm^{-3}) of the extracts did not exhibit any antifungal activity, with the exception of the reference strain and two groups of isolates (S – susceptible for all antimycotic drugs, and RI – resistant to one of the tested antimycotics) that were slightly sensitive to the methanolic fraction (MF).

Application of increased extract content (0.25 mg·cm^{-3}) resulted in low antifungal activity of the MF fraction toward the investigated yeast strains, whereas the alkaloid fraction (AF) was only effective against the group of *C. albicans* strains susceptible for all examined antifungal agents. Further increasing the extract concentrations (0.5 and 1.0 mg·cm^{-3}) led to a gradual increment in the anticandidal potential of MF, HMF, and AF preparates. Conversely, the ethyl acetate fraction (EAF) at these concentrations were characterized by a lack of antifungal activity against the pathogenic yeast isolates and low anticandidal activity toward the reference strain. The tested fungal strains were found to be more sensitive to higher concentrations (1.5 mg·cm^{-3}) of the examined walnut leaf preparates. The growth inhibition zones (IZ) of the candidal strains ranged from 9.5 to 21.5 mm (in diameters), depending on the respective extract fractions and the analysed group of yeasts. It should be emphasized that the highest tested concentration (2.0 mg·cm^{-3}) of MF extract derived from *J. regia* leaves characterized with strong antifungal activity against the tested *C. albicans* strains (20.5-23.4 and 18.6-22.0 mm of growth inhibition zones, respectively). The alkaloid extract possessed a moderate antimycotic efficiency (IZ 16.0-18.5 mm), whereas the lowest antifungal activity was demonstrated in the case of HMF and EAF extracts (growth inhibition zones of 12.4-16.8 and 12.5-18.0 mm, accordingly). The performed statistical analyses proved the significant effects of examined indicators (strain of yeast, concentration of the relevant extract fractions) and interactions between these variables on growth inhibition zones of the tested fungal pathogens (Table 4).

The conducted studies revealed that tested extract fractions (MF, EAF, HMF and AF) obtained from *J. regia*

Table 3. *In vitro* anticandidal activity of the hydrolyzed methanolic and alkaloid extract fractions derived from *J. regia* leaves.

Yeast strains tested (*n*)	Extract concentration (mg·cm^{-3})							
	0.01	0.05	0.10	0.25	0.5	1.0	1.5	2.0
Hydrolyzed methanolic fraction								
S (103)	–	–	–	–	8.4±0.1^{j}	15.2±0.5^{de}	15.8±0.5^{c}	16.5±0.6^{b}
RI (20)	–	–	–	–	–	8.6±0.1^{ij}	14.9±0.4^{de}	16.2±0.5^{b}
RII (12)	–	–	–	–	9.0±0.2^{i}	10.0±0.4^{h}	15.4±0.5^{cd}	18.0±0.7^{a}
RIII (5)	–	–	–	–	–	11.4±0.5^{g}	12.0±0.3^{fg}	12.5±0.4^{f}
Ref	–	–	–	–	–	9.2±0.2^{i}	14.5±0.4^{e}	5.4±0.5^{cde}
Alkaloid fraction								
S (103)	–	–	–	8.5±0.1^{j}	9.0±0.2^{i}	9.5±0.2^{h}	15.5±0.5^{cd}	16.0±0.5^{c}
RI (20)	–	–	–	–	8.5±0.1^{j}	10.0±0.4^{h}	16.2±0.7^{c}	18.5±0.8^{a}
RII (12)	–	–	–	–	–	11.5±0.5^{g}	15.8±0.5^{cd}	17.0±0.6^{b}
RIII (5)	–	–	–	–	9.5±0.3^{hi}	10.2±0.4^{h}	14.0±0.4^{f}	16.2±0.5^{c}
Ref	–	–	–	–	10.0±0.4^{h}	14.5±0.5^{ef}	15.2±0.5^{de}	18.0±0.9^{a}

(*n*) – number of yeast isolates tested; S – *C. albicans* strains susceptible for all tested antimycotic drugs; RI, RII, and RIII – groups of the yeast isolates resistant against 1, 2, and 3 (or more) antifungal drugs, respectively; Ref – reference strain (*C. albicans* ATCC 90029). All experiments were performed in triplicates. The anticandidal activity was expressed as a diameter of the inhibition zone (IZ) in millimeters (mean±SD). Strong antifungal activity, IZ 20-25 mm; moderate antifungal activity, IZ 14-19 mm; low antifungal activity, IZ 8-13 mm; "–" – no zone of inhibition. Letters (superscript) indicate significant differences between average values of the inhibition zone, presented separately for each extract fraction ($P < 0.05$; Tukey's test for different *N*).

Table 4. Results of the factorial ANOVA calculated for the group of candidal strains (G), extract concentration (EC), and interaction between these indicators.

Extract fraction	Tested effects		
	Group of candidal strains (G)	Extract concentration (EC)	Interaction (G×EC)
MF	$F_{4, 1120} = 144.7$ ($P < 0.0001$)	$F_{7, 1120} = 7219.5$ ($P < 0.0001$)	$F_{28, 1120} = 89.5$ ($P < 0.0001$)
EAF	$F_{4, 1120} = 215.5$ ($P < 0.0001$)	$F_{7, 1120} = 12419.3$ ($P < 0.0001$)	$F_{28, 1120} = 181.7$ ($P < 0.0001$)
HMF	$F_{4, 1120} = 2083.2$ ($P < 0.0001$)	$F_{7, 1120} = 21965.8$ ($P < 0.0001$)	$F_{28, 1120} = 740.0$ ($P < 0.0001$)
AF	$F_{4, 1120} = 960.3$ ($P < 0.0001$)	$F_{7, 1120} = 27973.4$ ($P < 0.0001$)	$F_{28, 1120} = 1264.5$ ($P < 0.0001$)

Extract preparates derived from the walnut leaves: MF – methanolic fraction, EAF – ethyl acetate fraction, HMF – hydrolyzed methanolic fraction, AF – alkaloid fraction.

leaves markedly suppressed the growth rate of *C. albicans* ATCC 90029 strain when compared to the non-treated (control) samples. It has been elucidated that methanolic fraction possessed the strongest antifungal efficacy against the reference strain (Fig. 1). Moreover, a slightly lower anticandidal potential was demonstrated in the case of the alkaloid fraction, whereas hydrolyzed methanolic and ethyl acetate extracts inhibited the yeast growth in the lowest degree in relation to the extract-free control. Furthermore, the antifungal efficacy of all investigated extract fractions after 8 hours post inoculation (8 hpi) was higher when compared to shorter exposure time (4 hpi). The significant influence of the extract fractions ($F_{4, 20} = 130.6$; $P < 0.0001$), exposure time ($F_{1, 20} = 545.5$; $P < 0.0001$), and interactions between these indicators ($F_{4, 20} = 42.4$; $P < 0.0001$) on growth intensity of the reference candidal strain has been confirmed.

The obtained results indicated that the highest contents of phenolic compounds occurred in EAF and HMF fractions, while MF preparate possessed a significantly lower amount of the examined group of walnut leaf constituents. Moreover, it has been elucidated that alkaloids constituted approx. 60% of dry weight of the alkaloid extract residual (Table 5). It has also been found that the EAF fraction of walnut leaves characterized by the highest level of antioxidative activity toward DPPH radicals (70.8% inhibition of DPPH$^{\bullet}$) and MF and HMF preparates were moderately active (63.57 and 56.97%, respectively), whereas the alka-

Table 5. Levels of tested parameters (total phenolic and alkaloid contents) in walnut leaf extract fractions.

Extract fraction	Dry weight of the residue (mg)	Total phenolic content [a)]	Alkaloid content [a)]
MF	1,619	46.8±0.3	-
EAF	1,595	80.3±0.5	-
HMF	608	76.2±1.8	-
AF	703	-	57.2±0.2

Extract preparates derived from the walnut leaves: MF – methanolic fraction, EAF – ethyl acetate fraction, HMF – hydrolyzed methanolic fraction, AF – alkaloid fraction;
[a)] – total phenolics and alkaloid contents are expressed as percent of dry weight of the respective extract residue; "–" – not measured.

loid extract possessed the lowest antiradical potential (15.87%) (Fig. 2). Additionally, the statistical analyses revealed significant differences in DPPH radical scavenging activity of the tested *J. regia* preparates ($F_{3,8}$ = 2098.3; $P < 0.0001$).

The English walnut (*J. regia*), also known as common or Persian walnut, is a commonly cultivated crop tree in Europe and Asia [29]. This light-demanding woody species grows in moist soil and reaches a height up to 35 m and 2 m of trunk diameter. The walnut leaves are commonly used in traditional Chinese and Iranian medicine, which is attributed to their unique phytochemical composition [30]. Mohammadi and co-workers uncovered that ethanolic extracts derived from *J. regia* leaves possessed hypoglycemic and hipolipidemic effects in type 2 diabetic rats [31]. Moreover, Salimi et al. elucidated the antiproliferative activity of the *J. regia* chloroform fraction against several human cancer cell lines [32]. Our previous report evidenced the presence of eleven phenolic acids (caffeic, chlorogenic, *trans*-cinnamic, *o*- and *p*-coumaric, ferulic, gallic, *p*-hydroxybenzoic, syringic, tannic, and vanillic) in methanolic extracts obtained from the leaves of *J. regia* cv. 'Albi' [12]. Moreover, it was found that foliar tissues of this walnut cultivar characterized with the highest amounts of *p*-hydroxybenzoic, vanillic, tannic, and *p*-coumaric acids, whereas *o*-coumaric and gallic acids occurred in the lowest concentrations. Salimi et al. also demonstrated that methanolic extracts derived from the walnut leaves contained high amounts of total phenols, flavonoids, and tannins [32]. Moreover, Cosmulescu et al. [33] revealed that mature leaves of five cultivars of *J. regia* ('Germisara', 'Jupanesti', 'Franquette', 'Vilna', and 'Valcor') grown in Romania markedly differed in the content of juglone (5.4-22.8 mg/100 g fresh weight). According to many researcher groups, walnut leaf extracts provide a significant source of secondary metabolites exhibiting a strong antioxidant efficacy [14, 32-34]. Seasonal dynamics of phenolic compounds in walnut leaves is associated with a profound increase in the content of these constituents in June and July, with a subsequent decline in August [14]. Based on the phytochemical analyses, *J. regia* leaves should be collected before this downward trend. Furthermore, strong correlation between the foliar concentration of polyphenols and the specific walnut genotype has been evidenced. Nour et al. uncovered a significant intervarietal variability in the content of phenolic compounds in walnut leaves [34]. Comparative analyses of flavonoids in foliar tissues of nine *J. regia* cultivars revealed high concentrations of catechin hydrate, myricetin, and rutin, and the low content of epicatechin and quercetin aglycones. Additionally, Amaral et al. demonstrated significant differences in the concentrations of individual phenolic substances in the walnut leaves

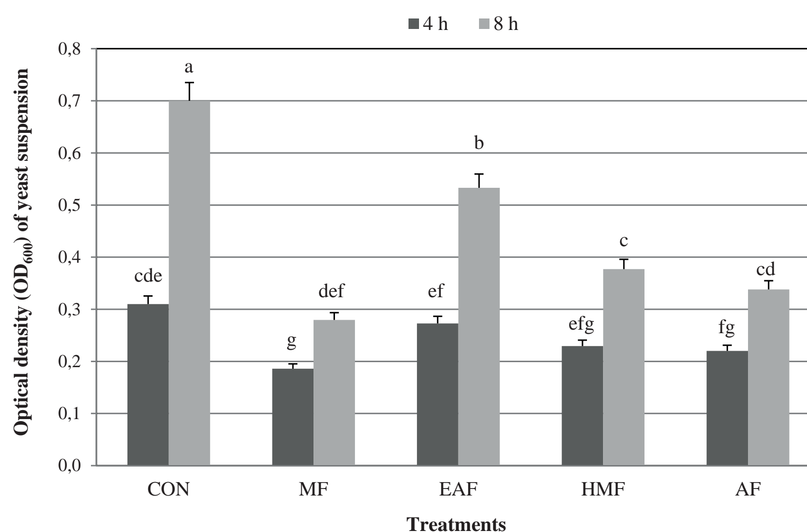

Fig. 1. The effect of tested extract fractions obtained from walnut leaves on the growth intensity of *C. albicans* ATCC 90029.
The growth rate of the reference strain is expressed as optical density of yeast suspension (OD_{600}). CON – control (yeast suspension in YPD medium untreated with the walnut extract); MF, EAF, HMF and AF – yeast suspensions in YPD broth containing 2.0 mg·cm^{-3} of the relevant extract fraction derived from walnut leaves. The measurements of OD_{600} were performed after 4 and 8 hours post inoculation. All data are presented as mean values (±SD). Different letters above the bars indicate significant differences between average values of optical density ($P < 0.05$; Tukey's test).

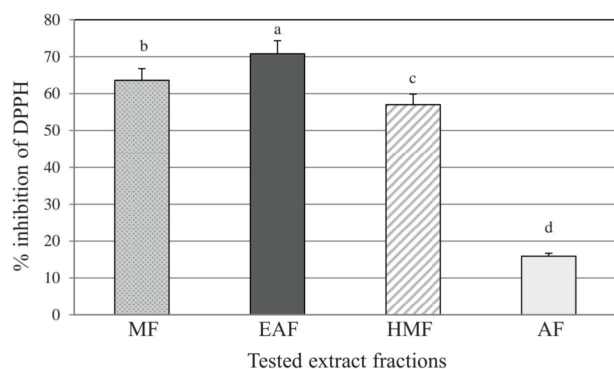

Fig. 2. The antioxidative potential of investigated extract fractions obtained from *J. regia* leaves.
Antioxidative potential is expressed as percent inhibition of DPPH radicals. All data are presented as mean values (±SD). Different letters above the bars indicate significant differences between average values of antioxidative potential ($P < 0.05$; Tukey's test).

between three consecutive crop seasons (2002-04), indicating the circumstantial effect of climatic factors on levels of the studied allelocompounds [35].

To the best of our knowledge, it is the first report comparing the antifungal effects of four extract fractions of walnut leaves against pathogenic strains of *C. albicans*. A dose-dependent inhibitory impact of the investigated foliar preparates on the growth of candidal strains has been evidenced. Noumi et al. revealed that ethyl acetate extracts of the walnut bark exhibited a low antimycotic activity towards clinical isolates of *C. albicans* [30]. Importantly, Pereira et al. proved that aqueous extracts from the *J. regia* fruits of five varieties ('Franquette,' 'Lara,' 'Margot,' 'Mayotte,' and 'Parisienne') grown in Portugal possessed an antifungal potential against *C. albicans* CECT 1394 [9]. Extracts derived from tissues of 'Lara' and 'Franquette' walnut plants exhibited the strongest antifungal activity against *C. albicans*, 'Mayotte,' and 'Parisienne' varieties, demonstrating the high activity, whereas preparates from the 'Marbot' cultivar were characterized by a slight anticandidal potential. Several reports regarding the antimycotic potential of extracts from various organs of many plant species against the reference and clinical yeast isolates have been published [8, 36-39].

Höfling et al. revealed that methanolic extracts obtained from several plants (*Mentha piperita* L., *Syzygium cumini* (L.) Skeels, *Tabebuia avellanedae* Lorentz ex Griseb., *Rosmarinus officinalis* L., and *Punica granatum* L.) showed antimycotic activity against the yeast reference strain [37]. Oguro et al. established that the antifungal defensin AFP1 isolated from *Brassica juncea* (L.) Czern. induced oxidative stress with subsequent membrane permeabilization, leading to circumstantial growth inhibition of *C. albicans* cells [38]. Interestingly, the combination of root and leaf ethanolic extracts from *Hypericum havvae* Güner displayed a markedly higher antifungal activity toward the reference strain *C. albicans* ATCC 10231 and many other species among *Candida* genus than the effects caused by using single preparates derived from these organs [36]. In the context of these findings, it is highly probable that a synergistic mode of action of a wide array of allelocompounds extracted from diverse plant systems may profoundly enhance the anticandidal efficacy against the opportunistic yeast pathogens. Furthermore, Pozzatti and colleagues elucidated fungistatic and fungicidal activity of cinnamon, ginger, Mexican oregano, oregano, and thyme essential oils towards the clinical isolates of *C. albicans* [39]. It was found that fluconazole-resistant candidal strains were also less sensitive to cinnamon essential oil in comparison with fluconazole-susceptible ones [8]. Pinto et al. documented that essential oil from aerial parts of *Ferulago capillaris* (Link ex Spreng.) Cout. possessed antifungal activity against pathogenic *C. albicans* strains, and suppressed germ tube formation at sub-inhibitory concentrations [40].

It should be stressed that Amber et al. evidenced a significant antimycotic potential of *Ocimum sanctum* L. essential oil (OSEO) against pathogenic isolates of *C. albicans* and its synergistic effect with azole antifungals: fluconazole and ketoconazole [7]. Implementation of complex therapy strategies involving both natural and synthetic antifungal compounds may lead to a more rapid and effective eradication of *Candida*-related mycoses in humans. Messier and Grenier revealed the synergistic antifungal effect of nystatin and two allelocompounds isolated from *G. glabra*, glabridin, and licochalcone A [41]. Interestingly, Budzyńska et al. elucidated that saponin-rich fractions (SAPFs) of *Trifolium alexandrinum* L., *T. incarnatum* L., and *T. resupinatum* cv. *resupinatum* Gib & Belli significantly reduced the invasive capacity and germ tube formation of *C. albicans*. Moreover, SAPFs exhibited synergistic interactions with azole antimycotic drugs against the reference yeast strain [42].

In summary, the present study evidenced the antifungal activity of several extract fractions of *J. regia* leaves against a wide range of pathogenic *C. albicans* strains characterized by different susceptibility levels to standardized antimycotics. The obtained results provide a basis for further experiments focused on identification of highly bioactive constituents in the walnut preparates exhibiting the anticandidal potential, assessment of cytotoxicity profiles to human cell lines, and deciphering their possible interactions with the antifungal drugs used in treatment of candidiasis.

Conclusions

It has been found that all the investigated yeast clinical isolates were sensitive to nystatin and amphotericin B, and only one strain of *C. albicans* showed resistance to flucytosine. Additionally, azole antimycotics were significantly less effective against the examined yeast pathogens. The methanolic fraction derived from *J. regia* leaves exhibited strong antifungal activity and the alkaloid fraction displayed moderate anticandidal potential, whereas hydrolyzed methanolic and ethyl acetate preparates possessed low antimycotic effects against examined *C. albicans* strains.

Furthermore, we documented the dose-dependent anticandidal effect of tested walnut fractions. It has also been elucidated that EAF and HMF fractions contained the highest levels of total phenolics. Moreover, ethyl acetate preparate characterized with the greatest antioxidative capacity, HMF and MF fractions possessed intermediate activity, and AF fraction showed the lowest antiradical potential.

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