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Antioxidant Activity, Polyphenols Content and Antimicrobial Activity of Several Native Pteridophytes of Romania

Liliana Cristina SOARE[1,2], Mariana FERDEŞ[2], Stefan STEFANOV[3], Zapriana DENKOVA[3] Radosveta NICOLOVA[3], Petko DENEV[4], Carmen BEJAN[5], Alina PĂUNESCU[6]

[1]*University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3 Mănăştur Street, Cluj-Napoca, Romania*

[2]*Politehnica University of Bucharest, Faculty of Medical Engeneering, Department of Bioengineering and Biotechnology, 313 Splaiul Independenţei, 060042, Bucharest; soleil_cri@yahoo.com*

[3]*University of Food Technology, Technical Faculty, 26 Maritza Boulevard, 4002 Plovdiv, Bulgaria*

[4]*Institute of Organic Chemistry with Centre of Phytochemistry-BAS, Laboratory of Biologicaly Active Substances, 95 Vasil Aprilov Boulevard, 4002 Plovdiv, Bulgaria*

[5]*National Institute of Research and Development for Bio-Technologies in Horticulture, Ştefăneşti, Argeş, Romania*

[6]*University of Piteşti, 1 Târgul din Vale Street, 110040 Piteşti, Argeş, Romania*

Abstract

The aim of this paper was to test the antioxidant activity, polyphenols content and antimicrobial activity of crude extracts obtained from leaves of pteridophyte species commonly found in Romania. The ORAC (Oxygen Radical Absorbance Capacity) of the investigated ferns varied between 421.90 µmol TE (Trolox equivalents/g FW (fresh weight) in *Dryopteris filix-mas* and 128.18 µmol TE/g FW in *D. affinis*. Methanolic extracts obtained from leaves of ferns have similar antioxidant activity to that of some medicinal plants. Polyphenols content in the leaves of ferns varies between 2340 mg Gallic acid equivalents (GAE)/100 g FW in *D. filix-mas* and 887 mg GAE/100 g FW in *D. affinis*. The correlation coefficient between ORAC and the total polyphenol content was R=0.985. This correlation suggests that phenolic compounds are major contributors to the antioxidant activity. The methanolic extract obtained from ferns inhibits the growth of Gram negative *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* NBJMCC1390, *Salmonella abony* and Gram positive *Staphyllococcus aureus* ATCC 25093 and *Enterococcus faecalis*. The highest antimicrobial activity was determined for the *Dryopteris* extract. The antimicrobial activity of methanolic extracts obtained from leaves of *D. filix-mas* and *D. affinis* is better than the *A. filix-femina* in the case of *Brevibacterium flavum* ATCC 14067, *Sarcina* sp., *Bacillus cereus* ATCC 1390, *Saccharomyces cerevisiae* and *Aspergillus niger*. The tested ferns could be used as cosmetic ingredients, as preservatives in food or in antimicrobial therapy.

Keywords: *Athyrium filix-femina, Dryopteris affinis, Dryopteris filix-mas,* leaves, microorganism, pathogen, saprophyte

Introduction

The Ayurvedic systems of medicine recommended the medicinal use of the pteridophytes. Ferns are also used in the Unani system of medicine (Uddin *et al*., 1998). In China, about 300 kinds of ferns were used as traditional medicinal herbs. In addition to their antioxidant activity, the ferns showed bioactivities such as antimicrobial, antiviral, antiinflammatory, antitussive, antitumor and anti-Human Immunodeficiency Virus (Chang *et al*., 2011). Efficient antioxidant properties of several ferns such as *Davallia*, *Hypolepis*, *Pteridium*, *Cytominum*, *Dryopteris*, *Polystichum*, *Dicranopteris*, *Lycopodium*, *Osmunda*, *Adiatum*, *Coniogramme*, *Polypodium*, *Pyrrosia*, *Pteris*, *Lygodium*, *Selaginella*, *Thelypteris*, *Athyrium*, *Matteuccia*, *Onoclea* and *Woodsia* were reported (Shin, 2010). Dryopteridaceae, Osmundaceae and Woodsiaceae exhibit powerful antioxidant activities. Some crude extracts obtained from ferns showed powerful antioxidant activities, more powerful than those of vitamin C. Analyzing antioxidant activities in many ferns will result in the development of health-care products for aging and chronic diseases due to their high bioactivities. The extracts obtained from pteridophytes have effective antimicrobial activities against Gram positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*), Gram negative bacteria (*Escherichia coli*, *Salmonella typhi*, *Pseudomonas aeruginosa*), as well as fungi. They could be developed into antibiotic sprays, packing material, toothpaste, hand wash, etc. for the protection of the human body and our living environment against undesirable microbials (Lee and Shin, 2011).

The Romanian flora includes 70 pteridophytes and of these, only a few are used as medicinal plants (Ciocârlan, 2009). The aim of this paper was to test the polyphenols

contents, antioxidant and antimicrobial activity of crude extracts obtained from leaves of pteridophyte species commonly found in Romania to find new resources useful in therapy, cosmetics, food technology, etc.

Materials and methods

Plants

Leaf extracts were obtained from *Athyrium filix-femina* (L.) Roth (Lady Fern), *Dryopteris affinis* (Lowe) Fraser-Jenkins (Male Fern) and *Dryopteris filix-mas* (L.) Schott (Male Fern). The plants were colected in May 2011 from the Vâlsan Valley (Argeş county, Romania).

Extraction

Five grams of fresh leaves were washed with tap water and distilled water and then their surface was sterilized with 90% ethanol. Subsequently, the plant materials were immersed in 50 ml of methanol for alcoholic extracts. The methanolic macerates were kept for 24 h at room temperature. Macerates were squeezed through double-layered muslin cloth and filtered through filter paper. After filtration, aliquot was centrifuged at 6000 rpm for 20 min at room temperature. The supernatants were filtered through Whatman No.1 filter paper.

Oxygen Radical Absorbance Capacity (ORAC)

ORAC assay was measured according to the method of Ou *et al.* (2001) with some modifications. The method measures the antioxidant scavenging activity against peroxyl radical induced by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) at 37°C. Fluorescein (FL) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) as compared to the blank area in which no antioxidant is present. Solutions of AAPH, FL and Trolox were prepared in a phosphate buffer (75 mmol/l, pH 7.4). Samples were diluted in phosphate buffer as well. Reaction mixture (total volume 200 µl) contained FL-(170 µl, final concentration 5.36×10^{-8} mol/l), AAPH-(20 µl, final concentration 51.51 mmol/l), and sample (10 µl). FL solution and sample were incubated at 37°C for 20 min, and AAPH (dissolved in 37°C buffer) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, fluorescence readings were recorded at the end of every cycle after shaking. For the blank, 10 µl of phosphate buffer was used instead of a sample. Antioxidant activity was expressed in Trolox equivalents. Trolox solutions (6.25; 12.5; 25; 50 and 100 µmol/l) were used for defining the standard curve. One ORAC unit is assigned to the net protection area, provided by a Trolox solution with a concentration of 1 µmol/l. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area under the curve. The antioxidant activity was expressed in micromole Trolox equivalents per gram of fresh weight (FW).

Polyphenols content

The total polyphenols content was determined according to the method of Singleton and Rosi (1965) with Folin-Ciocalteu reagent. Gallic acid was employed as calibration standard; the results were expressed as Gallic acid equivalents (GAE) per g FW.

Antimicrobial assay

The antimicrobial activity of crude extracts was tested on 10 microorganism: five pathogen bacteria-*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* NBJMCC 1390, *Salmonella abony*, *Staphylococcus aureus* ATCC 25093 and *Enterococcus faecalis*, three saprophytic bacteria-*Brevibacterium flavum* ATCC 14067, *Sarcina* sp., *Bacillus cereus* ATCC 1390, one yeast-*Saccharomyces cerevisiae* and one mould-*Aspergillus niger*. The bacterial strains were cultivated on LBG Agar medium and the yeast and mould on Malt Agar medium. Plant extracts were tested in three concentrations: undiluted extract, 10^{-1} and 10^{-2}. The disc diffusion assay was used to determine the growth inhibition of microorganisms. Each paper disc was impregnated with 6 µl extract. The antimicrobial activity was expressed as the zone of inhibition (IZ) produced by the plant extract. For each extract was established the minimum inhibitory concentration (MIC) (ppm).

Results and discussion

Antioxidant activity

The antioxidant activity of fern species was evaluated with different methods, such as: DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay, ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] assay, FRAP (Ferric reducing ability of plasma) assay, ORAC (The oxygen radical absorbance capacity) assay, TRAP (The total radical trapping parameter) assay, DCFH-DA (Dichlorofluorescin-diacetate)-based assay, Cyclic voltammetry method, TOSC (total oxyradical scavenging capacity) assay, PCL (Photochemiluminescence) assay, Crocin CL test assay (crocin chemoluminescence assay), Chronocoulometric assay, CAA assay (Cellular antioxidant activity), Conjugated diene assay, Superoxide radical scavenging activity, Hydroxyl radical scavenging activity, Nitric oxide radical inhibition activity, Reducing power method, Phospho molybdenum method, Peroxynitrile radical scavenging activity, β-carotene linoleate method, Xanthine oxidase method, Cytochrome C test, Erythrocyte ghost system Microsomal lipid peroxidation or Thiobarbituric acid (TBA)

assay (Mubashir and Shah, 2011). The ORAC method is preferred for the measurement of the antioxidant activity of foods and biological samples. Ninfali *et al*. (2005) performed a comprehensive evaluation of different foods and spices using this method, Wojcikowski *et al.* (2007) investigated the ORAC antioxidant activity of 55 medicinal plants and Kratchanova *et al.* (2010) evaluated the ORAC antioxidant activity of 25 medicinal plants of Bulgaria. Tab. 1 shows the ORAC antioxidant activity of the investigated ferns. The antioxidant activity varied between 421.90 μmol TE/g FW in *Dryopteris filix-mas* and 128.18 μmol TE/g FW in *D. affinis*. The antioxidant activity of plants varies by species and within the same species differences were found depending on the solvent extraction, the physical condition of the plant material (fresh or dried) or environmental factors (Kratchanova *et al*., 2010).

Methanolic extracts obtained from the leaves of three fern species have a similar antioxidant activity to that of some medicinal plants. Thus, close values to those obtained for *D. filix-mas* have been reported in extracts obtained from *Calendula officinalis* flowers (Marigold) (407 μmol TE/g DW), *Ocimum basilicum* leaves (Basil) (402 μmol TE/g DW) and *Matricaria chamomilla* (Chamomile) flowers (469 μmol TE/g DW) (Kratchanova *et al.,* 2010). A similar antioxidant activity to that obtained in *Athyrium filix-femina* (186.53 μmol TE/g FW) was reported for aqueous extracts obtained from aerial parts of *Taraxacum officinale* (Dandelion) (193 TE/g DW) and from leaves of *Laurus nobilis* (Laurel) (170 μmol TE/g DW). The antioxidant activity determined in *D. affinis* (128.18 μmol TE/g FW) is close to that reported for *Cichorium inthybus* (Chicory) (aerial parts, water extraction) (132 μmol TE/g DW).

Polyphenols content

The polyphenols content in the leaves of ferns varies between 887 mg GAE/100 g FW in *D. affinis* and 2340 mg GAE/100 g FW in *D. filix-mas* (Tab. 2). Zheng and Wang (2001) reported excellent correlation for medicinal plants and culinary herbs when antioxidant activity (determined using the oxygen radical absorbance assay, ORAC assay) was compared with the polyphenols content. A linear correlation between antioxidant activity and the content of polyphenols have been reported by Katalinic *et al*. (2006), Kiselova *et al*. (2006), Kratchanova *et al*. (2010), Maizura *et al*. (2011) and Vasco *et al.* (2008). Other authors found no such correlation (Souri *et al*., 2008).

In this study, the correlation coefficient between ORAC and the total polyphenols content was R=0.985. This correlation suggests that phenolic compounds are major contributors to the antioxidant activity. Also, Rice-Evans *et al*. (1997), Shan *et al*. (2005), Wong *et al*. (2006) and Wu *et al*. (2006) reported that phenolic compounds in spices and herbs significantly contributed to their antioxidant properties. Cai *et al*. (2004) showed a linear correlation between antioxidant activity and the total phenolic content in the 112 traditional Chinese medicinal plants associated with anticancer properties. Chang *et al*. (2007) showed a good correlation between antioxidant capacity and the total polyphenols content in six ferns used in the Chinese system of medicine. Kratchanova *et al*. (2010) reported a correlation coefficient R=0.950 in the case of the water extract of 25 Bulgarian medicinal plants and R=875 in the case of acetone extract. A good correlation was demonstrated between the antioxidant activity and the total phenolic compounds in the Labiatae and Asteraceae from Poland (Wojdyło *et al*., 2007). A positive correlation coefficient between the total phenolic content and DPPH assay of plants extracts obtained from kesum (*Polygonum minus*), ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) was reported by Maizura *et al*. (2011).

Antimicrobial activity

The methanolic extract obtained from ferns inhibits the growth of Gram negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella abony* and Gram positive *Staphyllococcus aureus* and *Enterococcus faecalis* (Tab. 3). The highest antimicrobial activity was determined in *Dryopteris filix-mas* extract. 60 ppm is the MIC for *E. coli*, *Salmonella abony* and *Enterococcus faecalis*. The same MIC was obtained in *D. affinis* extract in the case of *Salmonella abony* and *Enterococcus faecalis*. *A. filix-femina* extract has the lowest antimicrobial activity. The highest IZ was determined in the *D. affinis* extract against *Enterococcus fecalis*, and the lowest in the *A. filix-femina* extract against *Staphyllococcus aureus* and *Enterococcus faecalis*. The antimicrobial activity of methanolic extracts obtained from leaves of *D. filix-mas* and *D. affinis* is better than those of *A. filix-femina* in the case of *Brevibacterium flavum*, *Sarcina* sp., *Bacillus cereus*, *Saccharomyces cerevisiae* and *Aspergillus niger* (Tab. 4). Microorganism growth was inhibited by ferns extracts; the IZ was larger than 8 mm in *Dryopteris* species extract. Parihar *et al*. (2010) shows that leaves extract of *Athyrium pectinatum* inhibited the growth of *Salmonella arizonae*, but did not inhibit the growth of *E. coli*, *Salmonella typhi*

Tab. 1. ORAC antioxidant activity (mean ± S.D. in μmol TE/g FW) of the investigated ferns

	Sample	ORAC
1	*Athyrium filix-femina*	186.53 ± 1.52
2	*Dryopteris affinis*	128.18 ± 7.37
3	*Dryopteris filix-mas*	421.90 ± 15.14

Tab. 2. Polyphenols content (mean ± S.D. in mg GAE/100 g FW) of ferns leaves

	Sample	Total polyphenols
1	*Athyrium filix-femina*	915.53±25.16
2	*Dryopteris affinis*	887±108.16
3	*Dryopteris filix-mas*	2340±85.44

Tab. 3. Antimicrobial activity of methanolic extract against some pathogenic bacteria

Sample	*Escherichia coli* ATCC 25922 2×10^{10} cfu/cm^3		*Pseudomonas aeruginosa* NBJMCC 1390 1×10^{13} cfu/cm^3		*Salmonella abony* 1.6×10^{10} cfu/cm^3		*Staphylococcus aureus* ATCC 25093 1×10^{13} cfu/cm^3		*Enterococcus faecalis* 1.7×10^{12} cfu/cm^3	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
Athyrium filix- femina	8.00±1.41	>600	8.50±0.70	>600	8.50±0.70	>60	7.00±0.0	>600	7.00±0.0	>600
Dryopteris affinis	8.00±0.0	>600	9.00±1.41	>600	9.00±1.41	>60	10.00±1.41	600	10.50±0,70	>60
Dryopteris filix- mas	10.00±0.0	60	8.00±0.0	>600	9.00±0.0	60	10.00±0.0	600	9.00±1.41	60

IZ-inhibition zone (mm); MIC-minimum inhibitory concentration (ppm); cfu-colony forming units

Tab. 4. Antimicrobial activity of methanolic extract against some saprophytic microorganisms

Sample	*Brevibacterium flavum* ATCC 14067, $3.6\cdot10^{13}$ cfu/cm^3		*Sarcina* sp., $1.7\cdot10^{10}$ cfu/cm3		*Bacillus cereus* ATCC 1390 $1.4\cdot10^{11}$ cfu/cm^3		*Saccharomyces cerevisiae,* $3.9\cdot10^{7}$ cfu/cm^3		*Aspergillus niger* $1\cdot10^{5}$ cfu/cm^3	
	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC	IZ	MIC
Athyrium filix- femina	8.00±0.0	>600	0	0	9.00±0.0	600	10.00±0.0	600	8.00±0.0	>600
Dryopteris affinis	9.50±0.70	600	9.00±0.0	>600	10.50±0.70	600	10.50±0.70	600	9.00±1.41	600
Dryopteris filix- mas	9.00±1.41	600	8.00±0.0	>600	10.50±0.70	600	12.00±0.0	>60	8.00±0.0	>600

IZ-inhibition zone (mm); MIC-minimum inhibitory concentration (ppm); cfu-colony forming units

and *Staphyllococcus aureus*. Different researches showed that the strong antibiotic activities of different ferns from *Dryopteris* genus. *D. crassirhizoma* and *D. filix-mas* can be used against Methicillin resistant *S. aureus* (Lee *et al*., 2009), and *D. cochleata* against gram positive and negative bacteria and fungi (Banerjee and Sen, 1980). The aqueous extract obtained from leaves of *D. cochleata* was effective against *E. coli* and *Salmonella typhi*, while the alcoholic extract was effective against *Agrobacterium tumefaciens*, *E. coli* and *Salmonella typhi* (Parihar *et al*., 2010). *D. crassirhizoma* is patented as an anti-tooth decay substance because of its high activity against *Streptococcus* in Korea.

The extracts obtained from some ferns could be utilized as effective natural cosmetic ingredients for the treatment or prevention of acne (Lee and Shin, 2011); some *Dryopteris* spp. exhibit strong antimicrobial activities against *Propionibacterium acnes*, known as a main factor of acne (pimple) (Kim *et al*., 2006).

Conclusions

The methanolic extracts obtained from leaves of *Athyrium filix-femina*, *Dryopteris affinis* and *D. filix-mas* ferns have shown a good antioxidant activity. A positive correlation was obtained between the antioxidant activity and the total phenolic compounds. These plants could be a good source of natural antioxidants. The best antimicrobial activity was demostrated by the *D. filix-mas* extract. The tested ferns could be used as cosmetic ingredients, as preservatives in food or in antimicrobial therapy.

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