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Article

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Essential Oil Constituents and Antioxidant Activity of *Asplenium* Ferns

Saoussen Hammami[1,*], Ali Snène[1], Ridha El Mokni[2,3], Khaled Faidi[1], Danilo Falconieri[4], Hatem Dhaouadi[1], Alessandra Piras[5], Zine Mighri[1], and Silvia Porcedda[5]

[1]Research Unit Applied Chemistry and Environment 13ES63, Faculty of Sciences of Monastir, Monastir University, Monastir 5000, Tunisia, [2]Laboratory of Botany and Plant Ecology, Faculty of Sciences, University of Bizerta, Jarzouna, Bizerta 7021, Tunisia, [3]Laboratory of Pharmaceutic Botany, Monastir Faculty of Pharmacy, Monastir University, BP. 80, Avenue Avicenne, Monastir 5000, Tunisia, [4]Industrial Technical Institute "Michele Giua", Via Montecassino 09134, Cagliari, Italy, and [5]Department of Chemical and Geological Science, University of Cagliari, Cittadella Universitaria di Monserrato, S.P. Monserrato-Sestu km 0,700, Monserrato, CA, 09042, Italy

*Author to whom correspondence should be addressed. Email: h_saoussen@yahoo.fr

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Abstract

Two fern species *Asplenium adiantum-nigrum* L. and *Asplenium trichomanes* L. collected from the Kroumiria region (Northwest of Tunisia) were individually submitted to hydrodistillation in a Clevenger type apparatus. Volatile organic compounds were identified by GC–MS and GC–FID. Thus, 35 compounds were identified in *A. adiantum-nigrum* essential oil accounting for 77.5% of the whole constituents dominated by palmitic acid (34.5%); however, only 29 volatiles were identified in *A. trichomanes* showing a high amount of phytol, an odorous diterpene alcohol, representing 14.4% of the total oil contents. The total phenolic content and the antioxidant effects of crude extracts from both pteridophytes were determined using Folin–Ciocalteu and 2,2′-diphenyl-1-picrylhydrazyl free radical-scavenging assays, respectively. *A. adiantum-nigrum* ethyl acetate extract is shown to be lower in total phenolic contents (49.3 mg gallic acid equivalent/g) than similar extract from *A. trichomanes* (55.4 mg GAE/g).

Introduction

Pteridophytes are represented by about 305 genera comprising more than 10,000 species all over the world (1). Ferns, constituting the major class of pteridophytes, are reported for their medicinal uses to treat ascaris infections, bleeding, trauma, burning diarrhea and cold (2). Previous studies mentioned their various bioactivities such as antioxidant, antitumor, anti-HIV, antimicrobial, anti-inflammatory and antiviral effects (3). According to the French society of perfumes, ferns can be considered of an important use into flavoring and fragrances (4). *Aspleniaceae* is one of the largest families of leptosporangiate ferns included in the *polypodiales* order. The genus *Asplenium* (*Aspleniaceae*, *Pteridophyta*) is a large and complex one comprising more than 650 species (5) from which 10 grow naturally in Tunisia (6). The most abundant in Kroumiria are *Asplenium trichomanes* (the maidenhair spleenwort) and *Asplenium adiantum-nigrum* (the black spleenwort).

Within the objective to search for natural antioxidants and biologically active compounds, the present work deals with a phytochemical investigation of two ferns belonging to *Asplenium* genus. The *A. adiantum-nigrum* is a leptosporangiate fern with 15–40 cm tall and from 2 to 4 pinnate, ascending rhizome and dark brown scales narrowly triangular and hair-like in upper half. Fronds have a conspicuously swollen base with scattered black hair-like scales. It is widely distributed along the Mediterranean basin in regions characterized by a relatively mild winter (7). In Tunisia, the fern is widespread particularly in the northwest, growing within cracks of shady rocks under rainy forest areas of Aïn Draham, Béni Mtir and El Feïdja regions. The *A. trichomanes* is a 5–30 cm tall fern with dark brown to black fronds.

In Tunisia, it grows naturally in several forest areas including those of Aïn Draham, Béni Mtir and El Feïdja regions (7). Some phytochemical investigations performed on the crude extracts of both *Asplenium* species growing in various countries, led to the isolation of phenolic derivatives, triterpenoids and flavonoid glycosides including 3,6,8-tri-C-xylosylapigenin, scutellstrein 6-*O*-glucoside, genkwanin 4′-*O*-glucosylrhamnoside, acacetin 7-*O*-glucosylrhamnoside, luteolin mono-C-glycosie, Mangiferin and the tritepenoïd hydrocarbon 22 (29)-hopene (8). However, no reports are available on the phytochemical aspects of the Tunisian species. Thus, due to the medicinal importance of *Aspleniaceae* family and the absence of previous studies related to *A. adiantum-nigrum* and *A. trichomanes* in Tunisia, the aim of the present work is to investigate volatile profiles of both ferns using GC–MS and GC–FID analysis. Total phenolic content and antioxidant activity of crude extracts were also assessed.

Experimental

Plant material and isolation of volatile oils

Fronds of native specimens of the black spleenwort (*Asplenium adiantum-nigrum*, Figure 1A and B) and the maidenhair spleenwort (*Asplenium trichomanes*, Figure 1C and D) were collected in natural oak forests of the Kroumiria region (Northwest of Tunisia; coordinates Lat. 36°54′14.60″ N, Long. 08°42′10.15″ E at elevations from 595 to 717 m a.s.l for the black spleenwort and Lat. 36°46′56.06″ N, Long. 08°41′12.70″ E at an elevation about 716 m a.s.l. for the maidenhair spleenwort). The identification of collected specimens was authenticated by Dr Ridha El Mokni, botanist in the Laboratory of Botany and Plant Ecology, Faculty of Sciences, University of Bizerta, Jarzouna, Bizerta, Tunisia, where voucher specimens have been deposited ([ASPL/01-A.ad.nig/002], [ASPL/09-A.tri/005]). Two hundred grams of each dried plant were subjected to hydrodistillation for 3 h in a Clevenger type apparatus. The essential oils were dried over anhydrous sodium sulfate and stored in sealed vials at −4°C for further analysis. The yields of essential oils are 0.025% (for *A. adiantum-nigrum*) and 0.030% for *A. trichomanes*.

Preparation of crude extracts

Powders from the dried aerial parts of each plant (20 g) were extracted by maceration in 100 mL of ethyl acetate during 3 days. The extract solutions thus obtained were filtered and concentrated under reduced pressure using a rotary evaporator (65°C) to isolate the extract deprived from solvent.

GC–FID and GC–MS analysis

GC analyses of the essential oils were performed using a gas chromatograph (Agilent 7890A; Palo Alto, CA, USA), equipped with a 30 m × 0.25 mm i.d. with a 0.25-µm stationary film thickness DB-5 capillary column (Agilent J&W) and a flame ionization detector (FID). The following temperature program was used: from 60 to 246°C at the rate of 3°C min^{-1} and then held 246°C for 20 min (total analysis time 82 min). Other operating conditions are the following: carrier gas helium (purity ≥ 99.9999%—Air Liquide Italy); flow rate 1.0 mL min^{-1}; injector temperature 250°C; and detector temperature 300°C. Injection of 1 µL of the diluted sample (1:100 in hexane, w/w/) was performed with 1:10 split ratio using an autosampler (Agilent, Model 7683B).

GC–MS analyses were carried out using a gas chromatograph (Agilent 6890N) equipped with a 30 m × 0.25 mm i.d. with 0.25 µm stationary film thickness HP-5 ms capillary column (Agilent J&W) coupled with a mass selective detector having an electron ionization device, EI and quadrupole analyzer (Agilent 5973). The temperature program was the same used for GC. Other chromatographic operating conditions are the following: carrier gas helium (purity ≥ 99.9999%); flow rate 1.0 mL min^{-1} and injector temperature 250°C. Injection of 1 µL of diluted sample (1:100 in hexane, w/w/) was performed with 1:20 split ratio, using an autosampler (Agilent, Model 7683B). The MS conditions were as follows: MS transfer line temperature 240°C; EI ion source temperature, 200°C with ionization energy of 70 eV; quadrupole temperature 150°C and scan rate, 3.2 scan s^{-1} at *m/z* scan range (30 to 480). A software MSD ChemStation (Agilent, rev. E.01.00.237) was used to handle and process chromatograms and mass spectra.

Figure 1. (A) *A. adiantum-nigrum* L. in its natural habitat. (B) Detailed fronds of *A. adiantum-nigrum* L. in their adaxial face. (C) *A. trichomanes* L. in its natural habitat. (D) Detailed fronds of *A. trichomanes* L. in their adaxial face (photos courtesy of Ridha El MOKNI). This figure is available in black and white in print and in color at *JCS* online.

Constituents of the samples were identified by comparing mass spectral fragmentation patterns with those of a computer library (9, 10) and linear retention indices (RI) based on a homologous series of C_8–C_{26} *n*-alkanes with those reported in the literature (9, 10). Table I shows the chromatographic results, expressed as GC peak area percentages calculated without any response factor correction.

Determination of total phenolic contents

Aliquots of 0.2 mL of ethyl acetate extracts (1 mg mL^{-1}) were mixed with 1 mL of Folin–Ciocalteu reagent. After 5 min incubation at room temperature, 0.8 mL of sodium carbonate solution (7.5%) was added. Samples were incubated at room temperature for 1 h, and the absorbance was measured at 765 nm versus a blank sample. A calibration curve was performed in parallel under the same operating conditions using gallic acid as a positive control. The results are expressed as mg of gallic acid equivalent per gram of dry extract (mg GAE/g) (11).

DPPH radical-scavenging activity assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay: DPPH free radical assay was adopted to measure the free radical-scavenging activity as reported previously (12). The antioxidant effects were tested for crude ethyl acetate extracts from both *Asplenium* ferns. A volume of 1.0 mL of each ethanol solution prepared at different concentrations (0.12, 0.25, 0.5 and 1 mg mL^{-1}) was mixed with an equal volume of ethanolic solution of DPPH (0.1 mM). The decrease in absorbance at 517 nm was measured after 30 min incubation at room temperature. The inhibition percentage of the DPPH radical by the tested sample was calculated according

Table I. Chemical Composition of Essential Oils Obtained by Hydrodistillation from *A. adiantum-nigrum* and *A. trichomanes* Harvested in Kroumiria Oak Forests, Northernwest of Tunisia

No.	RI	RI_{litt}	Compound name	*A. adiantum-nigrum* L.	*A. trichomanes* L.	Identification	Class
1	976	974	1-Octen-3-ol	0.5	–	MS, RI	Alcohols
2	1100	1096	Linalool	0.3	tr.	MS, RI	OM
3	1104	1100	*n*-Nonanal	5.4	2.0	MS, RI	Aldehydes
4	1142	1141	Camphor	tr.	3.7	MS, RI	OM
5	1189	1186	α-Terpineol	0.3	–	MS, RI	OM
6	1191	1187	1-Dodecene	0.4	0.5	MS, RI	Alkenes
7	1292	1289	Thymol	0.7	0.7	MS, RI	OM
8	1315	1315	2*E*,4*E*-Decadienal	0.7	0.7	MS, RI	OM
9	1341	1340	Piperitenone	0.8	tr.	MS, RI	OM
10	1373	1376	2-Butyl-2-octenal	tr.	0.8	MS, RI	Aldehydes
11	1392	1392	1-Tetradecene	0.8	1.5	MS, RI	Alkenes
12	1400	1400	*n*-Tetradecane	0.4	0.5	MS, RI	Alkanes
13	1484	1487	*E*-β-Ionone	0.9	0.7	MS, RI	OS
14	1512	1512	2,4-Di-*t*-Butylphenol	2.7	3.1	MS, RI	Alcohols
15	1526	1525	Dihydroactinidiolide	1.2	tr.	MS, RI	OM
16	1592	1588	1-Hexadecene	1.5	2.7	MS, RI	Alkenes
17	1600	1600	n-Hexadecane	0.4	0.5	MS, RI	Alkanes
18	1623	1626	Benzophenone	0.4	0.5	MS, RI	Ketones
19	1677	1680	1-Heptadecene	0.6	0.7	MS, RI	Alkenes
20	1698	1697	2-Pentadecanone	0.4	tr.	MS, RI	Ketones
21	1778	1777	*p*-Cresol octanoate	tr.	0.3	MS, RI	Esters
22	1792	1792	1-Octadecene	1.6	2.7	MS, RI	Alkenes
23	1800	1800	*n*-Octadecane	0.4	0.6	MS, RI	Alkanes
24	1838	1843	Neophytadiene	0.3	tr.	MS, RI	DH
25	1844	1844	Hexahydrofarnesyl acetone	4.4	4.6	MS, RI	DO
26	1879	1879	1-Hexadecanol	1.0	0.8	MS, RI	Alcohols
27	1925	1927	Cyclohexadecanolide	0.8	1.0	MS, RI	Esters
28	1947	1949	Isophytol	0.6	tr.	MS, RI	DO
29	1973	1973	Palmitic acid	34.5	–	MS, RI	Fatty acid
30	1993	1992	1-Eicosene	2.1	2.6	MS, RI	Alkenes
31	2081	2081	1-Octadecanol	0.6	tr.	MS, RI	Alcohols
32	2098	2105	γ-Palmitolactone	0.8	tr.	MS, RI	Esters
33	2111	2111	Phytol	7.5	14.4	MS, RI	OD
34	2193	2189	1-Docosene	0.8	1.3	MS, RI	Alkenes
35	2300	2300	Tricosane	0.4	tr.	MS, RI	Alkanes
36	2349	2349	4,8,12,16-Tetramethylheptadecan-4-olide	0.7	2.5	MS, RI	Esters
37	2393	2396	1-Tetracosene	0.5	tr.	MS, RI	Alkenes
38	2500	2500	Pentacosane	tr.	2.5	MS, RI	Alkanes
39	2729	2732	1-Pentacosanal	–	2.2	MS, RI	Aldehydes
40	2793	2798	1-Hexacosanol	tr.	2.7	MS, RI	Alcohols
41	2822	2822	Squalene	2.2	4.4	MS, RI	Triterpene
42	2831	2830	1-Hexacosanal	–	7.8	MS, RI	Aldehydes

RI, retention index determined on a HP5-MS fused silica column relative to a series of *n*-alkanes (C_8–C_{26}); RI_{litt}, retention index reported from the literature (9, 10); OM, oxygenated monoterpene; OS, oxygenated sesquiterpene; DH, diterpene hydrocarbon; OD, oxygenated diterpene.

to the formula of Yen and Duh (13).

$$\%\text{RSA} = \left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right) \times 100,$$

where A_{control} is the absorbance of the control sample ($t = 0$ h), A_{sample} is the absorbance of a tested sample at the end of the reaction ($t = 1$ h), and IC_{50} value is the concentration of each extract to scavenge 50% of the DPPH radicals present. Quercetin is used as a standard.

Results

Chemical composition

In this study, the essential oils from two pteridophytes growing in Tunisia were prepared by hydrodistillation. Results of the chemical composition analysis via GC-FID and GC–MS are presented in Table I.

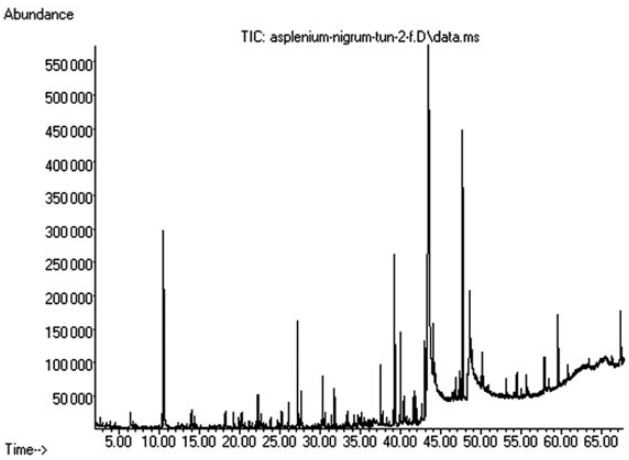

Figure 2. GC chromatogram of the volatile oil *A. adiantum-nigrum*.

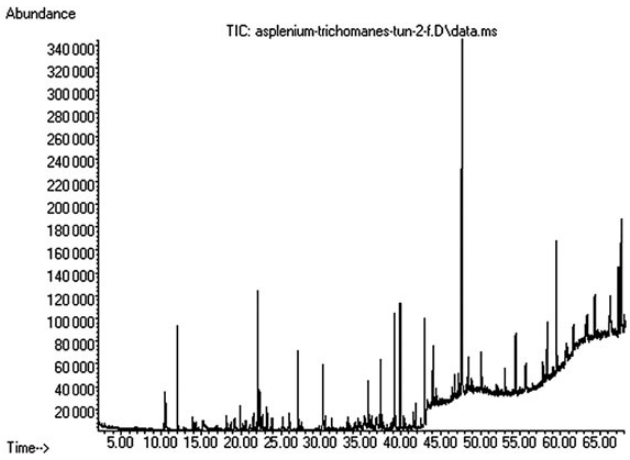

Figure 3. GC chromatogram of the volatile oil *A. trichomanes*.

Thus, 35 volatiles were identified in *Asplenium adiantum-nigrum* oil representing 77.5% of the whole constituents (Figure 2). The oil composed of 19.9% of terpenoids from which 12.5% are oxygenated diterpenes. High amounts of nonterpenic derivatives (carboxylic acids, aldehydes, ketones and hydrocarbons) were detected with an abundance of 57.6% of the characterized volatiles. Palmitic acid (34.5%) was identified as the major component of *A. adiantum-nigrum* oil.

In *A. trichomanes*, 29 components were identified representing 69.5% of the total oil volatiles (Figure 3). Furthermore, 29.4% are terpenoids. Phytol (14.4%) was identified as the chemotype of the analyzed oil followed by hexacosanal (7.8%), hexafarnesylacetone (4.6%) and squalene (4.4%) as the predominant components. We noted that nonterpenic compounds (aldehydes, esters and hydrocarbons) were present at a percentage of 40.1%. We recall that terpenoids are hydrocarbons and oxygenated derivatives of plant origin built from isoprene unit, they have the general formula $(C_5H_8)_n$ (14).

DPPH radical-scavenging activity and phenolic contents

Native ferns from *Aspleniaceae* were reported for their richness in phenolic compounds such as flavones, flavonols and phenolic acids. These phenolic derivatives can react as antioxidant agents because they possess interesting free radical-scavenging effects. The aim of our study is the evaluation of the total phenolic content and the antioxidant activities of *A. adiantum-nigrum* and *A. trichomanes*. The results presented in Table II showed that the maximum amount of extractable total phenolic derivatives (55.4 mg GAE/g) was recorded for *A. trichomanes*. The free radical-scavenging activity test showed that *A. trichomanes* ethyl acetate extract exhibited the highest antioxidant activity, DPPH $IC_{50} = (0.59 \pm 0.05)$ mg mL^{-1}, thus increasing of phenolic content induces the improvement of antioxidant effects.

Discussion

In this study, the chemical composition, the antioxidant activities and the total phenolic contents of two native ferns within the *Aspleniaceae* family harvested in Tunisia were studied. Essential oils from both *A. adiantum-nigrum* and *A. trichomanes* presented a high amount of nonterpenic compounds (hydrocarbons, alcohols, acids and aldehydes). The percentage of terpenic components ranged from 19.9% in *A. adiantum-nigrum* to 29.4% in *A. trichomanes* palmitic acid, detected as the predominant compound in *A. adiantum-nigrum* oil, was completely absent in *A. trichomanes*. The abundance of phytol in both essential oils varies from 7.5 to 14.4% in both species. Thus, a considerable variability in the chemical composition of essential oils collected in Kroumiria (Northwest of Tunisia) was observed and may be justified by either environmental and/or genetic factors. Furthermore, no relationships were observed between the chromatographic profiles of *A. trichomanes*. growing in Tunisia and that of the same species cultivated in France (4). It is worth noting that the Tunisian sample is richer in terpenoids (19.9%) in comparison with the earlier work on the French species (4.3%) (4). The chemical variability observed in our sample seems to be linked to exogenous (ecological) factors like altitude, humidity and soils. Levels of phenolics in ethyl acetate extracts of both ferns: *A. adiantum-nigrum* and *A. trichomanes* ranged from 49.3

Table II. Total Phenolic Content and Antioxidant Activity of Ethyl Acetate Extracts of Two *Asplenium* Ferns

Plant	Total phenolic content (mg GAE/g DW)	DPPH IC_{50} (mg mL^{-1})	Quercetin bleaching (inhibition %)
A. adiantum-nigrum L.	49.3 ± 1.7	0.88 ± 0.06	0.069 ± 0.02
A. trichomanes L.	55.4 ± 2.1	0.59 ± 0.05	

to 55.4 mg GAE/g and were considerably higher than the phenolic contents in ethanolic extract of *A. africanum* Desv. cultivated in Gabon (2.21 mg GAE/g) (11).

Conclusion

This study is a first report on the chemical composition, antioxidant effects and phenolic contents of *A. adiantum-nigrum* and *A. trichomanes* growing naturally in Tunisia. The considerable levels of the phenolic contents of ethyl acetate extracts deriving from both fern species (49.3–55.4 mg GAE/g) are encouraging and will give impetus for further detailed phytochemical analysis of those extracts, which may be rich on natural antioxidants.

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