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Variability of Phenolic Compounds and Biological Activities among Wormwood Extracts Originated from Different Bioclimatic Zones

Hanene Ghazghazi[1], Chedia Aouadhi[2], Imen Klay[3], Hanen Chakroun[4] and Leila Riahi[3*]

[1]Laboratory of Management and Valorisation of Forest Resources, National Research Institute of Rural Engineering, Water and Forestry (INRGREF), Tunisia.
[2]Laboratoire d'Epidémiologie et Microbiologie Vétérinaire, Groupes de Bactériologie et Développement Biotechnologique, Institut Pasteur de Tunis, Université El Manar, Tunisia.
[3]University of Manouba, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, 2020, Ariana, Tunisia.
[4]Department of Biology, University of Dammam, College of Science, Saudi Arabia.

Authors' contributions

This work was carried out in collaboration between all authors. Authors HG and LR designed the study, dressed the methodology, achieved the phytochemical analyses and wrote the first draft of the manuscript. Author CA achieved the antimicrobial analyses. Authors IK and HC managed the literature searches and performed the statistical analysis. All authors participated in the writing of the manuscript, read and approved the final manuscript.

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ABSTRACT

Aim: Wormwood is an aromatic spice widely used as flavor, antioxidant and antimicrobial agents. In this study the variability of total phenolics, total flavonoids, antioxidant, antibacterial and antifungal activities among leaves extracts originated from four Tunisian regions were assessed.
Study Design: All the analyses were achieved in triplicate.
Methodology: Fresh leaves of *Artemisia absinthium* L. were harvested from four regions of Tunisia representing four different bioclimatic areas ranging from humid to arid zone. Phenolic and flavonoid

**Corresponding author: E-mail: riahi.0311@yahoo.fr;*

contents were assessed based on colorimetric methods. The antioxidant activities of the studied extracts were tested based on DPPH and ABTS methods. The antibacterial and antifungal activities of *Artemisia absinthium* phenolic extracts were determined against ten indicators microorganisms by measuring the diameter of the growth-inhibition zone in millimeters, the minimum inhibitory concentration and the minimum bactericidal concentration.

Results: Considerable levels of bioactive phenolic compounds were revealed for the studied extracts. The highest levels of phenolic compounds were recorded for Gafsa site (Total Phenolic Content=127.5±5.22 mg GAE/g DW, Total Flavonoid Content=34.26±2.48 mg RE/g DW) while the lowest contents were observed for Ghar Dimaou locality (Total Phenolic Content =94.23±4.81 mg GAE/g DW, Total Flavonoid Content=26.95±1.98 mg RE/g DW). Similarly, considerable levels of antioxidant capacities were revealed based on both DPPH and ABTS tests with a significant variation according to the studied site. All the investigated extracts presented antimicrobial capacities against the ten tested strains with a variation according both to the extract origin and to the tested microbial strain. Especially, the highest toxicity was recorded against the species *Aspergillus niger* (MIC: 3.12 µg/ml, MBC: 6.25 µg/ml) and *Candida albicans* (MIC: 6.25 µg/ml, MBC: 12.5 µg/ml). Comparable levels were observed for bacterial strains with slight more effectiveness against Gram positive strains.

Keywords: Artemisia absinthium L.; phenolics; antioxidants; antimicrobials.

1. INTRODUCTION

Phenolic compounds are the most important group of plant secondary metabolites. These compounds play crucial role for plant adaptation and protection against various biotic and abiotic stresses [1]. These plant secondary metabolites present a broad spectrum of biological properties with beneficial effects on human health in different manner. According to Cartea et al. [2], phenolic compounds are considered as potentially protective factors against cancer and heart diseases which is due in a part to their antioxidative capacity. Among a wide range of biological activities, plant phenolic compounds are known to present significant antioxidant [3] and antimicrobial activities [4].

Based on the last considerations, the interest given to plant phenolic compounds has increased considerably in the recent years. Especially, the demand in natural antioxidant and antimicrobial agents derived from plant material has led to a significant increase in investigations regarding plant resources. Among the main objectives of such studies is the discovery of new natural additives with a broad spectrum of antioxidant and antimicrobial activity which can be used in food, cosmetic and pharmaceutical industries to substitute synthetic agents being restricted due to their carcinogenicity [5]. Thus, plants with high contents of secondary metabolites have paid a special attention for economical exploitation. Based on recent reports, leaves accumulated the highest phenolic contents compared to others plant organs [6].

Among a variety of recognized industrial plant species is cited *Artemisia absinthium* L. (*Asteraceae* family) well known as wormwood. *A. absinthium* is a perennial small aromatic shrub, distributed in Europe, Asia and North Africa [7]. Compared to others important commercial plant species, wormwood leaves are cited to possess significant health benefits [8]. The present study aims to investigate the variability of the concentration of phenolic metabolites in *A. absinthium* L. leaves harvested from four different localities of Tunisia characterized by various climatic and soil types. In the second part of this work the *in vitro* variation of antioxidant, antibacterial and antifungal activities of the studied extracts were evaluated.

2. MATERIALS AND METHODS

2.1 Plant Material and Characteristics of the Studied Sites

Fresh leaves of *A. absinthium* L. were harvested from four regions of Tunisia (Ghar Dimaou, El Kef, Kasserine and Gafsa) representing four different bioclimatic areas ranging from humid to arid zone. As listed in Table 1, the studied sites presented different geographical, bioclimatic and soil characteristics. The collected plant materials were air dried for seven days in the shade at room temperature. After drying, samples were ground to a fine powder, with particle size less than 5 mm in diameter, to be used for the preparation of extracts.

Table 1. Climate, geographic and soil characteristics of the four investigated sites

Locality	Bioclimatic area	Latitude	Longitude	Altitude (m)	Soil type	Mean rainfall (mm/year)
Ghar Dimaou	Humid	36° 26′ N	8° 26′ E	200	Clay-sandstone	800–1200
El Kef	Semi Arid	36° 11′ N	8° 42′ E	780	Marlstone-clay	400–600
Kasserine	Superior Arid	35° 10′ N	8° 50′ E	656	Limestone	200–400
Gafsa	Inferior Arid	34° 25′ N	8° 47′ E	298	Sand-phosphateous	100–200

2.2 Extract Preparation

The ground plant material (5 g of each sample) was extracted with 10 ml methanol: water (80:20) for 24h in a water bath shaker at room temperature. The obtained macerates were centrifuged at 2500 rpm for 10 min and then filtered. The solvent was evaporated completely by using rotary evaporator under reduced pressure to obtain dry leaf extracts used in the phytochemical analyses.

2.3 Total Phenolic and Flavonoid Contents

The amount of total phenol contents (TPC) of wormwood leaves extracts were determined using the Folin-Ciocalteu method as described by Singleton et al. [9] based on colorimetric method. The absorbance was measured at 760 nm and the standard curve calibration solutions were prepared using gallic acid. The results are given as mg gallic acid equivalent per 1 gram dry weight (mg GAE/g DW).

The aluminum chloride colorimetric method as described by Popova et al. [10] was used to estimate the total flavonoid contents (TFC). The absorbance was measured at 430 nm and the concentration of total flavonoid in the test sample was determined from the calibration curve using rutin as standard. Results were expressed as mg rutin equivalent per 1 gram of dry weight (mg RE/g DW).

2.4 Antioxidant Activities

The antioxidant activity of the studied extracts based on DPPH test was measured in term of hydrogen donating or radical scavenging ability using the DPPH method [11]. The ability to scavenge the DPPH radical was calculated using the following equation: Scavenging effect (%) = [(A0- A1)/A0] ×100, where A0 and A1 respectively stand for the absorbance of the control and the sample. The sample concentration providing 50% inhibition (IC50) obtained by plotting the inhibition percentage against extract concentrations were determined.

The scavenging of $ABTS^{+}$ radicals were measured according to the method described by Re et al. [12]. The percentage of ABTS radical scavenging activity of extract was calculated from the equation (Ac−At/Ac)×100 where Ac is the absorbance of the control and At is the absorbance of the plant extract. The concentration of the sample required to provide 50% of inhibition (IC50) were calculated.

2.5 Antimicrobial Activity

The leaves phenolic extracts of *A. absinthium* were tested for their antibacterial and antifungal activities against ten indicators microorganisms including seven bacteria reference pathogenic (*Escherichia coli* ATCC8739, *Salmonella typhimurium* NCTC6017, *Staphylococcus aureus* ATCC29213, *Pseudomonas aeruginosa* ATCC27853, *Aeromonas hydrophila* EI, *Listeria monocytogenes* ATCC7644 and *Bacillus cereus* ATCC1247), two fungi (*Aspergillus niger* and *Aspergillus flavus*) and one yeast (*Candida albicans* ATCC2091) species. Bacterial strains were grown in trypto-caseine soja agar (TSA) and incubated at 37°C for 24 h. Fungal species were grown on potato dextrose agar (PDA) at 28°C for 72 h while *Candida albicans* was grown on sabouraud dextrose agar (SDA) plate at 30°C for 48h. The antibacterial and antifungal activities of *A. absinthium* phenolic extracts were determined measuring the diameter of the growth-inhibition zone in millimeters, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) as described by Ghazghazi et al. [13]. Gentamicin was used as positive control in the antibacterial assay while Amphotericin B was used as positive control in the antifungal analysis.

2.6 Data Analysis

It is noted that all the previous assays were conducted in triplicate. Results for each studied parameter were expressed as mean ± SD (n=3). The significance of the differences between the obtained results were assessed by ANOVA procedure (at $P < 0.05$) followed by Duncan's multiple range test.

3. RESULTS AND DISCUSSION

3.1 Total Phenolic and Flavonoid Contents

The concentration of phenolic constituents in *Artemisia absinthium* L. leaves were evaluated among four studied sites characterized by contrasted pedoclimatic conditions. According to our obtained results the total phenolic (TPC) and flavonoid contents (TFC) contents vary according to the site of harvest. The highest levels of phenolic compounds were recorded for Gafsa site (TPC=127.5±5.22 mg GAE/g DW, TFC=34.26±2.48 mg RE/g DW) while the lowest contents were observed for Ghar Dimaou locality (TPC=94.23±4.81 mg GAE/g DW, TFC=26.95±1.98 mg RE/g DW). El Kef and Kasserine localities highlighted comparable contents (Fig. 1).

The means of phenolic contents recorded for Tunisian wormwood leaves (TPC=107.51±14.18 mg GAE/g DW, TFC=30.43±3.02 mg RE/g DW) are comparable to levels reported for the same species in South Korea [8], Turkey [14] and others species of the genus *Artemisia* such as *Artemisia alba* [15], *Artemisia vulgaris* and *Artemisia campestris* [3].

According to our findings environmental factors seem to influence the contents in secondary metabolites for Tunisian wormwood leaves. Indeed, the synthesis of phenolic compounds increase significantly for samples harvested from stressed conditions. Indeed Gafsa site (Inferior Arid) which presents the highest amounts of total phenolic, flavonoid and condensed tannins contents is a transition region between the Mediterranean and the Saharan area in Tunisia and is characterized by heat and drought stresses. The mean rainfall in this region varies from 100 to 200 mm/year and the soil is sand-phosphateous. On the other side, Ghar Dimaou locality (Humid) where the mean rainfall vary from 800 to 1200 mm/year and a clay-sandstone soil rich in organic material shows the lowest production of phenolic compounds. Furthermore El Kef (Semi Arid, 400-600 mm/year, marlstone-clay soil) and Kasserine (Superior Arid, mean rainfall of 200-400 mm/year, limestone sol) which are the closest geographical localities highlighted comparable amounts of phenolic concentrations in their leaves.

Our findings corroborate previous investigations which reported that in addition to genetic factors phenolic compound concentrations in plants are significantly affected by environmental changes including soil type, temperature and rainfall among others [16]. Temperature and rainfall are among the most important bioclimatic factors which define the levels of phenolic compounds in plant leaves [17]. Furthermore, a relatively high correlation was recorded between soil type and phenolic composition. The levels of phenolic compounds in plant leaves were influenced by soil nutrients (Als, Ks, Ss, Nas and Mns). Therefore, plants of the same species grown in

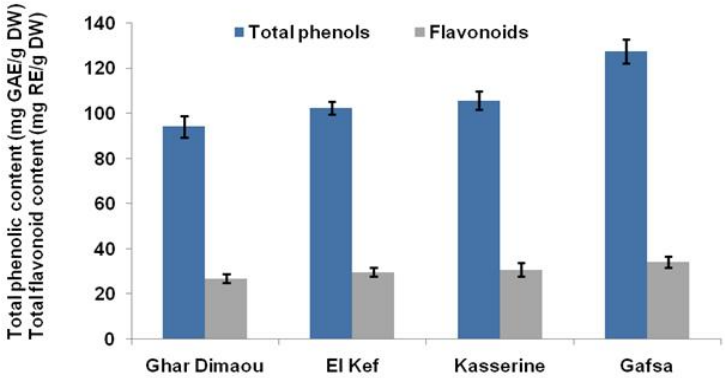

Fig. 1. Total phenolic and flavonoids contents in the leaves extracts of Tunisian *Artemisia absinthium* L. according to the four studied localities

different environments may differ in the composition of their secondary metabolites as an evolutionary response to different environmental pressures. Recent reports had suggested that levels of secondary plant metabolites production is higher in plants cultivated under abiotic stress than plants grown under optimal conditions [18]. Thus, for industrial plants with economic value such as *Artemisia absinthium* L. their cultivation under biotic stressed conditions could increase the production of secondary metabolite products including an array of phenolic compounds with various applications in food, medicine and cosmetic industries.

3.2 Antioxidant Activities

The *in vitro* antioxidant activities of the four studied extracts have been evaluated based on DPPH and ABTS tests. Similarly to phenolic content results, considerable levels of antioxidant capacities were revealed by the two used tests with a significant variation according to the studied site (Table 2).

Based on both DPPH and ABTS tests, Gasfa originated extracts showed the highest antioxidant activity ($IC50_{DPPH}$ = 20.12±0.65 µg/ml, $IC50_{ABTS}$=9.16±0.34) while Ghar Dimaou leaves extracts exhibited the lowest antioxidant potential among the studied extracts ($IC50_{DPPH}$ = 28.61±1.02 µg/ml, $IC50_{ABTS}$=14.98±1.01). The antioxidant potential for El Kef and Kasserine leaves extracts are comparable. It is noted that patterns of variation of radical scavenging ability among the four studied sites is in agreement with the different phenolic contents. It is observed that radical scavenging potential increase significantly from humid to arid bioclimatic areas. This can be explained by the chemical variability of the four *A. absinthium* leaves extracts which is defined by local environmental factors.

Our results confirmed previous studies showing an important antioxidant activity of *A. absinthium* L. extracts across its distribution range [8]. It is well established that phenolic constituents such as flavonoids and phenolic acids contribute directly to the antioxidant capacity of plants. Their antioxidant properties were attributed to their abundance in the different plant tissues. This can explain the high levels of antioxidant abilities observed for Tunisian germplasm of *A. absinthium*. Moreover, the significant variation of antioxidant activities among the studied extracts is related to the observed variation in phenolic and flavonoids contents among them. These results agree with findings by Sengul et al. [19] concerning Turkish *Artemisia* spp. extracts.

Drought, chemical composition of soil and others environmental conditions seem to have a significant effect on the yields and phenolic compounds production in Tunisian wormwood leaves which clearly have a significant impact on their antioxidant abilities. Plants growing under abiotic stress conditions accumulate polyphenol compounds more than non stressed ones which present a responsive to protective the plant against oxidative damage. In other report, findings highlighted a significant increase of the antioxidant activity of plant seeds exposed to greater water restriction [20]. According to André et al. [21] environmental conditions influence the quantity of detected phenolic compounds in plant species and thereby affect their antioxidant capacity.

Table 2. Antioxidant activities of Tunisian *Artemisia absinthium* leaves extracts. Means in each column followed by different letters are significantly different (p<0.05)

Localities	DPPH (IC50, µg/ml)	ABTS (IC50, µg/ml)
Ghar Dimaou	28.61±1.02^{c}	14.98±1.01^{d}
El Kef	24.28±0.94^{b}	12.01±0.91^{c}
Kasserine	23.09±0.81^{b}	10.26±0.88^{b}
Gafsa	20.12±0.65^{a}	9.16±0.34^{a}
Means	24.02±3.52	11.60±2.53

3.3 Antibacterial and Antifungal Activities

The antimicrobial activities of *A. absinthium* leaves extracts originated from four different Tunisian localities were assessed against a set of Gram-positive, Gram-negative bacteria, fungus and yeast strains. Their potency was qualitatively and quantitatively assessed by the presence or absence of inhibition zones, MIC and MBC values. According to our results, the investigated extracts of *A. absinthium* presented antimicrobial activity against all the tested strains with a variation according both to the plant extract and to the tested microbial strain (Table 3).

According to our results, the diameters of inhibition (ID) values vary from 8 to 16 mm. The obtained ID values for bacterial strains have

Table 3. Antimicrobial activities of *A. absinthium* L. leaves phenolic extracts. ID: diameter of inhibition (mm), MIC (µg/ml): minimum inhibitory concentration, MBC (µg/ml): minimum bactericidal concentration. G: Gafsa, K: Kasserine, EK: El Kef, GD: Ghar Dimaou. Ant.: Antibiotics, [a]: Gentamicin [b]: Amphotericin B

Microorganisms		ID (mm)					MIC (µg/ml)				MBC (µg/ml)			
		EK	G	K	GD	Ant.	EK	G	K	GD	EK	G	K	GD
Gram- bacteria	*Escherichia coli*	9	11	13	12	22[a]	25	12.5	12.5	25	50	25	25	50
	Salmonella typhimurium	8	9	11	10	21[a]	25	25	25	25	50	50	50	12.5
	Aeromonas hydrophila El	10	8	11	12	20[a]	12.5	25	12.5	25	25	50	25	50
	Pseudomonas aeruginosa	9	10	13	13	19[a]	25	25	12.5	25	50	50	25	50
Gram+ bacteria	*Staphylococcus aureus*	10	13	15	15	21[a]	25	25	25	25	50	50	50	50
	Listeria monocytogenes	10	11	16	16	21[a]	25	12.5	25	25	50	25	50	50
	Bacillus cereus	9	10	15	15	18[a]	25	25	25	25	50	50	50	50
Fungus	*Aspergillus flavus*	8	10	10	11	12[b]	25	25	25	12.5	50	12.5	12.5	25
	Aspergillus niger	13	14	11	13	13[b]	6.25	3.12	6.25	6.25	12.5	6.25	12.5	12.5
Yeast	*Candida albicans*	10	11	10	10	16[b]	6.25	12.5	6.25	6.25	12.5	25	12.5	12.5

been shown to be inferior to ID values of Gentamicin antibiotic which exhibited an ID values varying from 18 mm (*Bacillus cereus)* to 22 mm (*Escherichia coli)*. However, Amphotericin B antibiotic showed comparable diameters of inhibition values to those of the fungus *Aspergillus flavus* and *Aspergillus niger* (Table 3).

The lowest minimum inhibitory concentration MIC value (3.12 µg/ml) was recorded for Gafsa extracts against the species *A. niger* while the highest MIC value (25 µg/ml) observed against bacterial strains. On the other hand the minimum bactericidal concentration (MBC) presents a range of variation between 6.25 µg/ml (Gafsa extract) and 50 µg/ml for bacterial species. According to the obtained results, the highest antimicrobial potential was recorded against the fungus species *Aspergillus niger* (MIC: 3.12 µg/ml, MBC: 6.25 µg/ml) with Gasfa extracts. A considerable antimicrobial activity was also recorded against the yeast species *Candida albicans* (MIC: 6.25 µg/ml, MBC: 12.5 µg/ml). Comparable levels were observed for bacterial strains with slight more effectiveness of the studied extracts against Gram positive strains (Table 3).

Our results corroborate findings by Cruz-Galvez et al. [22] who reported a comparable antimicrobial potential of *Artemisia absinthium* L. from central Mexico against a wide range of pathogenic bacteria with a mean diameter of inhibition of 12± 0.81 mm. Antimicrobial activities has been also reported for *A. absinthium* extracts originated from Turkey which showed antibacterial activity against all tested microorganisms with inhibition zone ranging from 6 to 19 mm [19]. Based on results of Erel et al. [14] the species *Artemisia santonicum* and *Artemisia scoparia* presented their highest antimicrobial activity against *Candida albicans* among a set of 9 microbial strains. This is confirmed by investigation of *A. absinthium* extracts originated from Iran where the antimicrobial ability tested against various microorganisms was recorded against *Candida albicans* shown to be the most vulnerable [23]. Based on our results, wormwood phenolic compounds present a valuable source for further investigation aiming to isolate natural antimicrobial agents especially against the fungal and yeast species *Aspergillus niger* and *Candida albicans* well established to cause the most superficial and systemic infections and reported to present high resistance face to various developed antifungal agents [24,25].

Based on our findings there is no clear correlation between the obtained antimicrobial potentials of the studied extracts and their phenolic contents. This corroborates previous reports which highlighted that different microbial species exhibit different sensitivity towards phenolics. This variability of response is recorded sometimes among strains belonging to the same species [26].

The antibacterial activity of plant phenolic compounds is due to the disturbance of the microbial strain cell membranes which inhibit its multiplication. However, based on various scientific investigations, there is no correlation between phenolic compound contents and antimicrobial activity. The resulted antimicrobial potential is sometimes due to some specific phenolic compounds present in the phenolic extract. The antimicrobial activity of plant extract depends generally on the diversity of their chemical constituents [27]. According to previous studies, the antimicrobial capacity of plant extracts depends strongly on synergistic effects between their individual components [28,29].

4. CONCLUSION

The findings of this study revealed that Tunisian germplasm of the species *Artemisia absinthium* L. is rich in different phenolic constituents such total phenols and flavonoids which traduced by a high antioxidant ability of all the investigated samples based on both DPPH and ABTS tests. Furthermore, our results had shown that plants grown in stressed conditions exhibited higher secondary metabolites amounts and then higher antioxidant potentials. All the investigated extracts presented antibacterial and antifungal capacities against all the ten tested strains. Especially, a considerable toxicity was recorded against the species *Aspergillus niger* and *Candida albicans*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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