



Phenolic Composition, Antioxidant and Anti-Ulcer Activity of the Tunisian Medicinal Herbs *Urtica Dioica* L .

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Abstract: *Urtica dioica* plants constitute valuable sources of chemical substances with potential therapeutic effects. The leaf extract of *U. dioica* was one of the herbal remedies which the experimental, clinical and trials have complemented each other. This study was intended to characterize the phenolic composition, antioxidant, anti-inflammatory and antiulcer properties of EtOH extracts of *U. dioica* (UDE). The antioxidant properties of UDE were evaluated using different antioxidant tests, including total antioxidant (TAA), reducing power and DPPH radical scavenging activities. Phenolic composition was also determined through RP-HPLC analysis correlated with gastro protective effect using model of ethanol/HCl- induced acute gastric ulcer in Wistar rats.

UDE proved potential TAA, FRAP and DPPH radical scavenging activities as rescued by lower IC₅₀ and EC₅₀ values with a noteworthy anti-inflammatory activity inhibiting NO^{*} release with an IC₅₀ value of 40 mg/ml. On the other hand, High-performance liquid chromatography with photodiode-array detection analysis showed the presence of hydroxybenzoic, hydroxycinnamic acid derivatives and flavonoids. In turn, pretreatment with UDE especially at 1000 mg/Kg induced a remarkable inhibition of gastric lesions and acidity, correlated to high healing and protection percentages. Our results showed that *U. dioica* leaves are an interesting source of bioactive compound, justifying their use in folk medicine, to treat human health in particular for inflammation and gastric diseases.

Keywords: *Urtica dioica* ; phenolic pools, anti-inflammatory activity, anti-ulcer activity

1. INTRODUCTION

Urtica dioica L. is an herbaceous plant belonging to the family of Urticaceae that has been used for centuries against a variety of diseases [1]. Thanks to its high content of nutriment and bioactive compounds such as polyphenols, vitamins and minerals, it possesses a great nutritional value and a large number of pharmacological effects, including antioxidant [2], anti-proliferative, anti-

inflammatory [3], immunostimulatory, analgesic, hypotensive, anticancer, [4] anti-infectious, antiulcer activities and cardiovascular disease prevention. It has recently been shown to have anti-Alzheimer activity. The most common *Urtica dioica* L. preparations usually include the dry extract, crude dried powder, infusion (herbal tea), decoction or fresh juice. *Urtica dioica* L. root is mainly used for mictional disorders related to benign prostatic hyperplasia while the leaves are used for arthritis and rheumatism. Furthermore, *Urtica dioica* L. leaves are rich in protein, fat, carbohydrates, vitamins, minerals and trace elements. Proteins make up of 30% of the dry mass [5]. Moreover, the protein content of the leaves widely covers the needs of amino acids, especially the essential amino acids for humans [6] (Rutto et al 2013). Content of mineral substances is about 20% of the dry mass [7].

Urtica dioica L. is rich by iron, zinc, calcium, magnesium, phosphorus and potassium. The proportions of different compounds given in the literature are different. The origin and time of sample collection may be responsible for that. For nutritional value, the vitamin composition is very varied. It contains both fat-soluble vitamins A, D, E and K, and also significant amounts of water-soluble vitamins, such as vitamin C and the B vitamins (B1, B2, B3, B9). Wetherilt et al [8] found that 100g of fresh leaves contained 0.01 mg vitamin B1 (thiamine), 0.23 mg of vitamin B2 (riboflavin), 0.62 g of vitamin B3 (Niacin), 0.068 mg vitamin B6, 238 mg of vitamin C, 5 mg of provitamin A (β -carotene) and 14.4 mg of vitamin E (α -tocopherol). In Turkish folk medicine *Urtica dioica* herbs are used to treat stomachache [9]. In addition, this herb is used to treat rheumatic pain and for colds and cough [10] and is used against liver insufficiency [11]. In Germany and Japan, this plant was sold as herbal drug for prostate diseases and as a diuretic [2]. The leaf of *Urtica dioica* has laxative and diuretic properties and it is useful for treatment of pleurisy, asthma and spleen illnesses. The nettle leaves are used as a nutritious supplement and as weight loss aid [12]. The common phytochemical compounds from *U. dioica* are flavonoids, tannins, volatile compounds and sterols [13]. The leaves of *U. dioica* are rich in flavonoids, as well as phenolic compounds, organic acids, vitamins and minerals. The root contains lectins, polysaccharides, sterols and lignans. The stinging action is due to the liquid contained in nettle's hairs. This liquid contains at least three compounds that could be the cause of its allergic reactions: acetylcholine, histamine and serotonin [14].

The aim of the present study was to investigate antioxidant activity by using different antioxidant tests including total antioxidant activity, reducing power and free radical scavenging activities. An important goal of this research was to examine anti-inflammatory and gastro-protective activity of *U. dioica* L. ethanolic extract.

2. MATERIAL AND METHODS

2.1. Plant material and preparation of extracts

Urtica dioica was collected in May 2018 from D Errihan locality in Seliana governorate (154 km from capital of Tunis; 36.31°9027' N and 962°4003' E; superior semi-arid bioclimatic stage; mean annual rainfall: 500-600 mm). The harvested plants were identified at the Biotechnology center of the Technopark of Borj-Cedria by Pr Abderrazek SMAOUI (**Figure 1**).



Figure 1 : Photograph of *Urtica dioica* L. plants

An aliquot of 100 g of dry powder was extracted with 1000 mL of ethanol solution for 30 min under a magnetic stirring. The solution was filtered and centrifuged at 4500 g for 15 min and the supernatant was dried in a rotavap at 50° C and lyophilized and stored at 21° C, until use.

2.2. Total phenolic compounds (TPC)

TPC was evaluated according to Folin-Ciocalteu colorimetric assay. Indeed, 0,125 mL of ethanolic extract of *U.dioica* was affixed to 60 μ L of distilled water and 15 μ L of the Folin-Ciocalteu reagent. After shuddering, 150 μ L of sodium carbonate (7%) was added. After one hour of incubation at room temperature, the optical density (OD) at 750 nm was determined. Results were expressed as mg of gallic acid equivalent per g of dry weight (mg GAE/ g DW) using a calibration curve [15].

2.3. Total flavonoid contents (TFC)

Two hundred fifty μ L of ethanolic *U. dioica* extract was mixed with 75 μ L of sodium nitrite (5%; w/v). Subsequently, 150 μ L of Aluminium chloride hexahydrate (10%; w/v) and 500 μ L of sodium hydroxide (1 M) were added after 6 min of incubation. After adjusting the volume to 2500 μ L with H₂O, the absorbance was determined at 510 nm. TFC were expressed as mg (+)-catechin equivalent/g DW (mg CE/ g DW). The calibration curve range was 50-500 μ g/mL ($R^2 = 0.99$) using catechin as a standard [16].

2.4. Analytical RP-HPLC/UV

The identification of phenolic compounds was done using HPLC system equipped with a reversed phase C18 analytical column of 4.6 x 100 mm and 3.5 μ m particle size (Zorbax Eclipse XDB C18). The DAD detector was set to a scanning range of 200-400 nm. Temperature of column was maintained at 25°C. The volume of injected extract was 2 μ l and 0.4 ml/min was the mobile phase flow-rate. Mobile phase B was milli-Q water constituted of 0.1% formic acid and mobile phase A was methanol. The optimized chromatographic condition was as follows: 0-5 min: 10% A- 90% B; 5-10 min: 20% A-80% B; 10-30 min: 30% A-70% B; 30-40 min: 50% A- 50% B; 40-45 min: 60% A- 40% B; 45-50 min 70% A-30% B; 50-55min: 90% A- 10% B; 55-60 min: 50% A- 50% B and at 60min 10% A- 90% B. Phenolic compounds identification were obtained by comparing their retention time and the UV spectra with those of pure standards

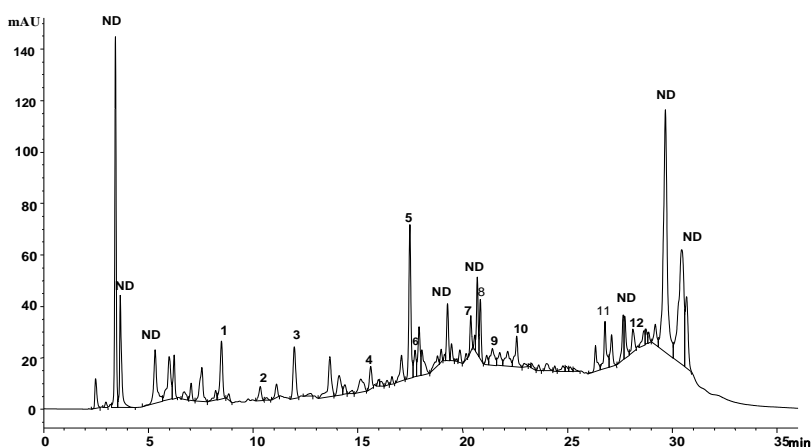


Figure 2. RP-HPLC chromatograms of *U. dioica* Tunisian herbs. Signal was monitored at 254 nm. The peak numbers correspond to: 1: gallic acid; 2: resorcinol; 3: catechol; 4: chlorogenic acid; 5: caffeic acid; 6: syringic acid; 7: ferulic acid; 8: luteolin7-O-glucoside; 9: quercetin; 10: kaempferol; 11: 4'-5-7-trihydroxyflavone; 12: pinostrobin

2.5. Evaluation of antioxidant capacities

Total antioxidant capacity (TAC) was assessed according to Ben Mansour et al. [17]. Briefly, 100 μ L of diluted *U.dioica* was mixed with 1000 μ L of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After cooling, an incubation at 95°C for 90 min. The absorbance was measured at 695 nm and TAC was expressed as mg gallic acid equivalent / g DW (mg GAE. g⁻¹ DW).

2.6. DPPH test

The aptitude of quenching 2,2-diphenyl-1-picrylhydrazyl was estimated according to the method of Wasli et al. [18]. In brief, 50 μ L of *U.dioica* leaves different concentrations were mixed with 250 mL

of a methanolic solution of DPPH and allowed to react in the dark for 30 min. Then the absorbance of the resulting solution was read at 517 nm. The antiradical activity was expressed as IC₅₀ (mg/mL), the extract dose required to cause a 50% decrease of the absorbance at 517 nm.

2.7. Ferric reducing antioxidant power (FRAP) assay

FRAP was traduced by the altering of test solution from yellow to green due the reduction of Fe³⁺. In brief, 1mL of *U.dioica* with a concentrations ranging from 1 to 5 mg/mL was mixed with 2.5 mL of Na₃PO₄ buffer (pH 6.6, 0.2 M), and 2.5 mL of K₃Fe (CN)₆; (1% w/v). After incubation in a water bath at 50°C for 20 min ; 2.5 mL of TCA (10 %, w/v) were inserted followed by a vigorous centrifugation of 10 min at 650 g. At the final step, 2.5 mL of supernatant was blended with 2.5 mL of dionized water and 0.5 mL of FeCl₃ solution (0.1 %, w/v), as described by Ben Mansour et al. [17] . The absorbance was assessed at 700 nm against blank sample and ascorbic acid was used as a positive control and results was expressed as EC₅₀ values (mg/mL)

2.8. NO[•] production

RAW 264.7 cells were seeded in 24- well plates at 5×10⁴ cells/well. After 24 h of incubation, cells were pretreated for one hour with 50, 100, 150 and 200µg/mL of PRE or WCP before 24h-stimulation with 1µg.mL⁻¹ of lipopolysaccharide (LPS). Griess reagent (0.75%N (naphthyethylene) diamine, 0.8% sulfanilamide in 0.5N HCl) was used to determine the accumulation of nitrite in culture supernatant. The test was performed by mixing 100 µL of cells' supernatant with the same volume of Griess solution. The absorbance was then determined at 540 nm and the content of nitrite was calculated referring to NaNO₂ standard curve [17]

2.9. In vivo Anti-ulcer activity

Healthy male Wistar rats weighing were procured from Tunis Pasteur Institute and kept under standard conditions (20-22°C, 45-50% relative humidity; 12 h light:12 h dark cycle). All animals were handled according to the guidelines of the Tunisian Society for the Care and Use of Laboratory Animals. The study was approved by the University of Tunisia Ethical Committee. Gastric ulceration was induced in rats orally with 0.2 mL of ulcerogenic solution containing 0.3 M of HCl and 60% of ethanol (HCl/EtOH). Groups (n=6) of rats were pre-treated with 30 minutes before the ulcerogenic procedure in the following manner.

Group I: Control rat received vehicle solution

Group II: Rats received standard drug famotidine (20mg/Kg)

Group III: Rats treated with 250 mg/Kg of UDE

Group IV: Rats treated with 500 mg/Kg of UDE

Group V: Rats treated with 1000 mg/Kg of UDE

After 21 days of treatment, animals are sacrificed and assessed for the gastric mucosal damage. The stomach was opened along the greater curvature, washed under running water and the glandular portion of the stomach was examined. The length in mm of each lesion was measured under a dissecting microscope and the ulcer index (UI) was measured as follows:

Ulcer index (UI): Average number of severity score x Percentage of animals with ulcers / number of animals

The other parameters were determined as follows:

The percentage of ulceration (% UP) = (UI * 100) / 3

The healing percentage (% HP) = UI (control group) – UI (treated group with extract or famotidine) x 100 / UI (control group)

2.10. Statistical analyses

Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test was performed. The statistical tests were applied using Graph Pad Prism, version 6 and the significance level was p < 0.05.

3. RESULTS AND DISCUSSION

3.1. Phenolic pools and antioxidant activities of ethanolic extract from *U.dioica* plants

As shown in Table 1 ethanolic extract of *U. dioica* exhibited a noteworthy amount of total phenolics and flavonoids in the order of 6 mg/g GAE DR and 0.87 mg/g CE DR respectively (**Table 1**).

In turn, RP-HPLC analysis was used to characterize and quantify phenolic compounds. The HPLC profile of *Urtica dioica* leaf extracts proved the omnipresence of twelve phenolic metabolites (**Fig. 2, Table 1**). They include gallic acid, resorcinol, catechol, chlorogenic acid, caffeic acid, syringic acid, ferulic acid, luteolin-7-*O*-glucoside, kaempferol, quercetin, 4'-5-7-trihydroxyflavone and pinostrobin .

These compounds have been identified in consistent with their RT and the spectral characteristics of their peaks compared to those of standards, as well as by spiking the sample with standards (**Table 1**).

Table1. Phenolic compounds identified and quantified by analytical RP-HPLC/UV from ethanolic extracts of *U. dioica* Tunisian herbs . Values are the means of three replicates and standard deviation.

Phenolic compounds	TPC (mg GAE/RS)		TFC (mg CE/RS)	
	6		0.87	
	RT	CE	CC (R ²)	Quantification (µg/g)
HPLC identified compound				
Gallic acid	8.2	y=22.28x+1.68	1 .00	0.35
Resorcinol	10.3	y = 9.33 x – 2.17	0.96	0.46
Catechol	12.0	y=3,632x+1,80	0.99	1.76
Chlorogenic acid	15.6	y=9,02x-1,55	1.00	0.67
Caffeic acid	17.4	y=23.49x+5.57	0.98	4.14
Syringic acid	17.7	y =22.34 x-1.56	1.00	0.80
Ferulic acid	20.3	y=20.50 x-8.72	0.99	0.77
Luteolin-7- <i>O</i> -glucoside	20.8	y=7.43 x+13.16	0.99	1.27
Kaempferol	22.6	y = 9.87x - 4.30	0.99	0 .11
Quercetin	24.01	y= 9.58x-7.41	0.99	0.36
4'-5-7-trihydroxyflavone	26.80	y= 49.68 x - 24.6	1.00	1 .47
Pinostrobin	28.12	y = 7.42x - 1.11	0.97	0.76

Table2. Antioxidant activities of the ethanolic extracts prepared from *U. dioica* plants .

	<i>U.dioica</i>	Standard
TAA (mg EAG/ g DR)	1.51±0.17	-
DPPH (IC ₅₀ mg/mL)	2.0±0.065	0.003 ± 0.00
FRAP (EC ₅₀ mg/mL)	2.6±0.43	0.0018 ± 0.00

Caffeic acid was detected to be the major phenolic component in *U. dioica*, (4.14 µg/g DR). Previous studies reported its presence as potential compound in Romanian species [19]. caffeic acid, and ferulic acid also have antioxidant activity and may protect cells against damage caused by free radicals. Catechol was the second most abundant phenolic compound accounting for 1.76 µg/g DR that represent about 17% of total quantified phenolic pools (Table 1). 4'-5-7-trihydroxyflavone, the third predominant component in *U. dioica* , was depicted at an amount of 1 .47µg/g DR.

On the other hand, the present study analyzed the antioxidant properties of EtOH leaf extracts from *U. dioica* through total antioxidant capacity (TAC), their ability to trap free radicals, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), along the capacity to reduce ferric Fe to ferrous form (FRAP assay). Considering the multiple aspects of antioxidants and their reactivity, the application of a single method is in general consider to be a very limited approach to estimate the antioxidant properties of plant extracts,As presented in **Table 2**, *U.dioica* represented a noteworthy antioxidant activity (1.51 mg EAG/ g DR) with an important antiradical activity against DPPH radical with an IC₅₀ value equal to 2 mg/mL and 2.6 mg/mL for FRAP test.

3.2. Anti-inflammatory and anti-ulcer activities of ethanolic extract from *U.dioica* plants

The ability of EtOH extracts from *U.dioica* to inhibit cellular NO generation was assessed using LPS-stimulated RAW 264.7 macrophages. As shown in **Table 3**, *U.dioica* exhibited a potential aptitude to inhibit LPS-induced NO[•] secretion at concentrations ranging from 1 to 200 mg/ mL with IC₅₀ values of 40 mg/mL. Scientific research has highlighted the nettle's ability to decrease the inflammatory response, through multiple mechanisms whose consequences are the reduction of synthesis of lipid mediators and pro-inflammatory cytokines. Indeed, leaf extracts inhibit the biosynthesis of arachidonic acid cascade enzymes, in particular the cyclooxygenases COX-1 and COX-2, thereby blocking the biosynthesis of prostaglandins and thromboxanes [20].

Table3. Anti-inflammatory activity of ethanolic extract from *U. dioica* plants. The results are expressed as concentration of inhibition of NO. L-NAME is used as a positive control. Values are the means of three replicates and standard deviation.

	1,25	3,5	6,5	12,5	25	50	100	200
fluo	0,178243	0,17477	0,15655	0,12789	0,119605	0,0547055	0,0679264	0,0574371
fluo	0,173472	0,162223	0,146666	0,134459	0,142577	0,065493	0,0620165	0,0641019
fluo	0,159845	0,163709	0,154317	0,141953	0,124138	0,05773	0,0573185	0,0700093
fluo relative	0,1202466 7	0,1167736 7	0,098553 67	0,069893 67	0,061608 67	0,0032908 3	0,0099300 7	0,0005592 3
fluo relative	0,1154756 7	0,1042266 7	0,088669 67	0,076462 67	0,084580 67	0,0074966 7	0,0040201 7	0,0061055 7
fluo relative	0,1018486 7	0,1057126 7	0,096320 67	0,083956 67	0,066141 67	0,0002663 3	0,0006778 3	0,0120129 7
%inhibition	7,7965271 1	4,6831157 4	11,65041 58	37,34300 72	44,77019 21	102,95010 6	91,098075 9	100,50133 1
%inhibition	3,5195085 8	6,5647887 8	20,51104 29	31,45415 05	24,17667 47	93,279525 7	96,396074 7	94,526593 5
%inhibition	8,6965746 2	5,2326467 4	13,65221 47	24,73606 67	40,70653 13	100,23875 8	100,60765 2	89,230835 9
moy	-1	2	15	31	37	99	96	95

Table4. Effect of administration from *U.dioica* ethanolic extracts on gastric ulcer parameters in rats (n = 6/group). GV: Gastric Volume ml; GpH: Gastric pH; UP: Ulceration Percentage, PP: Percentage of protection. Values are the means of three replicates and standard deviation.

	Group 1 Control	Group 2 EtOH/HCl	Group 3 (250 mg)	Group 4 (500 mg)	Group 5 (1000 mg)	Group 6 (FAM)
UP	-	84	60.66	19	18	23.33
PP	-	-	24.66	81.33	82.33	67.83
GV	3.76	1.75	2.96	3.80	3.71	3.86
GpH	1.86	3.10	3.40	2.86	2.70	3.10

According to Francisco et al. (2014), luteolin 7-O-glycoside could modulate significantly cytokine expression, by reducing the LPS-induced IL-1b expression; therefore reinforcing the anti-inflammatory properties, by inhibiting NO and also IL-1b production.

Gastric lesions produced by EtOH-HCl resulted in inflammation of the mucosa and the installation of several hemorrhagic furrows (% of ulceration = 84%). The positive control, famotidine, reduced the the ulcerative percentage to 75% leading to recovery of the gastric mucosa with a percentage of protection and healing rating around 51.2% and 43.6% respectively.

Oral administration of UDE at 1000 mg/Kg reduced significantly the percentage of ulceration (UP = 18%) and showed a better gastric mucosal protective effect than the positive control group with a percentage protection (PP) up to 80% . As well, In pylorus-ligated rats, UDE and famotidine pretreatment reduce the gastric volume and acidity (pH) at different extend in HCl/EtOH-ulcerated rats (Table 4). Results indicate that ulceration caused by the ethanol/HCl mixture resulted in an increase in gastric juice volume to 3.76 mL followed by an increase in acidity accompanied in a

decrease in pH to 1.86. UDE at 1000 mg/Kg caused the greatest reduction (2.70 mL) even greater than standard reference famotidine (3.10 mL). This decrease is also accompanied by a pH recovery to 1.86.

Several studies investigated the mode of gastroprotective action of phenolic compounds. It was reported that the treatment using doses of 50 and 250 mg/Kg of caffeic acid significantly reduced the lesion index and contributed to the reduction of gastric juice and total acidity correlated to a significant increase in gastric pH. Quercetin intake was negatively associated with the risk of gastric cancer by reducing oxidant level [21]. Ben Mansour et al. [17] showed a strong contribution between the protection, recovery and ulceration percentages with gallic acid, quercetin and rosmarinic acid which substantiate the key role of phenolic compounds in fighting gastric disease.

4. CONCLUSION

U. dioica species investigated in our research provided evidence for the value of Tunisian herb species as a potential source of phenolic compounds with compelling anti-inflammatory and anti-ulcer effects which support the notion that phenolic pools largely implicate to the biological activities assessed by *in vivo* tests.

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