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Phytochemical Analysis, Antioxidant and In Vitro Anti-Inflammatory Activity of Leaves Ethanol Extracts from a Moroccan Plant *Urtica urens L.*

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Abstract: *Urtica urens* L., popularly known in Moroccan as "Hurriyqa", belongs to the Urticacea plant family. This study aimed to evaluate the antioxidant capacity and anti-inflammatory activity as well as total phenolics, flavonoids, and tannins of *Urtica urens* leaves were determined. Ethanolic extracts of their leaves were prepared by maceration. Ethanol extract of leaves plant (*Urtica urens*) was evaluated for its antioxidant activity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay and anti-inflammatory activity by using the Bovine Serum Albumin (BSA) protein denaturation assay. Ethanolic extracts of their leaves were prepared by maceration. Phytochemical tests of ethanolic extract from leaves of *Urtica urens* revealed the presence of an appreciable number of flavonoids, phenolics, and tannins. The extract had a strong ability to scavenge DPPH radicals with an IC_{50} value of 0,44 ± 0.88 mg/L. Also, the ethanolic extracts of *Urtica urens* L. possess significant anti-inflammatory activity. The results showed that ethanol extract at a concentration range of 31.25-250 µg/ml significantly ($p<0.05$) protects the heat-induced protein denaturation. The results obtained in the present study indicate that ethanol extracts of *Urtica urens* L. can be used as an alternative anti-inflammatory drug.

Keywords: *Urtica urens L.*; leaf extract; flavonoids; phenolicstannins; antioxidant activity; anti-inflammatory potential; BSA assay.

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1. Introduction

Urtica urens L. is a member of the Urticaceae, a perennial plant with stinging hairs frequently used in Moroccan folk medicine to treat anxiety, arthritis, rheumatism, and diuretics. toothache, scabies, and pruritus [1-3]. Recent studies demonstrate that *Urtica urens* have anti-inflammatory [4-6], antioxidant [4,5,7,8], anxiolytic [9], antibacterial [10], and antifungal activities [11]. The principal constituents identified in the plant material are flavonoids, phenolic acid, catechins, anthocyanins, and isoflavones [12-14]. Moreover, few studies have reported that its leaves have shown great antioxidant and anti-inflammatory properties [4,5]. Additionally, to the extent of our knowledge, the biological and pharmacological activities of the species of *Urtica urens* from Morocco have not been explored well, especially their antioxidant and anti-inflammatory activities. Besides, no studies have been conducted on ethanolic extracts of leaves of the Urens urens variety collected from Morocco. Therefore, the

present study aimed to evaluate the total phenolics, flavonoids, and tannins contents, the antioxidant capacity by DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay, and anti-inflammatory activity by inhibiting protein denaturation with bovine serum albumin (BSA) assays of leaves in ethanol extracts, using ultrasound treatment.

2. Materials and Methods

2.1. Plant material.

Samples of *Urtica Urens*, locally known as "Hurriyqa", were collected from Beni metal city located in the north-central area of Morocco (in 2022) and identified at the Plant Biology Laboratory of Ben M'sik Faculty of Sciences at Hassan II University in Casablanca. The plant was washed under running water and dried in the laboratory in the open air. After it had dried well, we crushed and sieved it to better extract the active ingredients of the plant.

2.2. Ultrasonic extracts preparation.

The extracts were prepared by weighing 2.5 g of dry leaves of *Urtica urens*, added to 50 mL of absolute ethanol, and placed in a 250 mL Erlenmeyer conical flask. The ultrasound bath was operated at a frequency of 20 kHz with a maximum input power of 120 W for 15 minutes at room temperature. The filtrate was separated from the residue using Whatmann filter paper and evaporated using a rotary evaporator at 40°C [15]. Thereafter, the dried extract was stored at 4°C until use.

The yield was expressed as a percentage and was given by the following formula:

$$\boldsymbol{Yield}\ (\%) = \frac{\textbf{Weight of dried extract}}{\boldsymbol{Dry\ weight\ of\ plant\ materiel}}\ \boldsymbol{x}\ \mathbf{100} \quad (1)$$

2.3. Phytochemical screening.

Phytochemical tests were carried out for tannins, saponosides, anthraquinone, quinone, polyphenols and flavonoids. They were carried out according to standard methods [16].

Research on tannins and polyphenols was carried out in reaction to ferric chloride. 1 gram of plant extract was dissolved in 25 ml of distilled water. The mixture was maintained under agitation for 30 minutes and then filtered through the filter. The filtrate was collected, and 3 drops of a 1% alcoholic solution of ferric chloride were added. The formation of a blue-black or black precipitate indicates the presence of gallotannins, while a green or dark-green coloration indicates the presence of catechin tannins.

The saponosides were detected by foam Index. 1 gram of extract was dissolved in 50 ml of distilled water. The mixture was maintained under agitation for 15 minutes and then filtered through the filter. The filtrate was stirred vigorously for 10 seconds and let stand for 10 minutes. The persistence of the foam at a height of 2 to 3 cm indicated the presence of the saponosides.

The detection of free quinone substances was performed with the reagent of Bornstraëgen. 1 gram of extract was triturated in 5 ml of hydrochloric acid. The solution was heated for 30 min in a boiling water bath. After cooling, the hydrolyzate was extracted with 25 ml of chloroform. After filtration, 3 ml of 25% ammonia NH_4OH was added. The appearance of a color ranging from red to violet indicates the presence of quinones.

The search for anthraquinones was carried out from the reagent of Bornstraëgen. 1g of each extract was dissolved in 25 ml of chloroform for 5 seconds and then filtered. After filtration, 3 drops of 10% potassium hydroxide solution were added. The appearance of a red coloration indicates the presence of anthraquinones.

2.4. Determination of total flavonoid content.

The total flavonoid content was determined using the aluminum chloride colorimetric method described by Dewanto et al. [17]. The ethanol extract was mixed with 0.3 mL of 10% AlCl3, followed by adding 0.3 mL of $NaNO_2$. The reaction mixture was kept at room temperature for 5 min. The absorbance of the reaction mixture was recorded at 510 nm. The total flavonoid content was calculated from the linear regression equation of the calibration curve, using quercetin as standard. The total flavonoids were expressed in mg equivalent to quercetin/g dry weight. Each extract was tested with 3 repetitions and reported as mean ± SD.

2.5. Determination of total phenolic content.

The total phenolic content was determined using the Berrani method using the Folin-Ciocalteu reagent [18]. The ethanol extract was mixed with 100 µl of 50% Folin–Ciocalteu reagent. The mixture was incubated for 3 minutes, and then 2 mL of 2% Na_2CO_3 solution was added. The reaction mixture was incubated again for 1h at room temperature. The absorbance of the reaction mixture was recorded at 750 nm. The standard calibration was made using gallic acid. The total phenolics were expressed as mg gallic acid equivalent/g dry weight. Each extract was tested with 3 repetitions and reported as mean ± SD.

2.6. Determination of total tannin.

The total tannin content was determined using the vanillin assay described by Julkumen-Titto [19]. To 50 µL of such extract, 1500 µL of vanillin/methanol (4%) solution was added and mixed. Then, 750 µL of concentrated HCl was added and allowed to react at room temperature for 20 min. The absorbance at 500 nm was measured against a blank. The standard calibration was made using catechin. The total tannin was expressed in mg of catechin equivalents/g weight. Each extract was tested with 3 repetitions and reported as mean ± SD.

2.7. DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay.

The free radical scavenging assay of different extracts was determined using the DPPH method following the procedure of Khattak et al. (2008) with modifications [20]. The extract of various concentrations was mixed with 1 ml of 0.1 mM DPPH in methanol. The reaction mixture was incubated for 30 min at room temperature in dark conditions. The absorbance of the reaction mixture was measured at 517 nm. Ascorbic acid was used as the positive control. DPPH scavenging capacity was calculated by using the following formula:

$$Scavenging\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{\text{Absorbance of control}}\ x\ 100 \quad (2)$$

2.8. Bovine serum albumin assay (BSA).

Anti-inflammatory tests were carried out using the protein denaturation inhibition method with bovine serum albumin (BSA). This method was performed as described by Qamar

et al. [21]. BSA solution (5%, w/v) was prepared in Tris Buffered Saline. The reaction mixture (500 µL) consisted of a 50 µL test sample of different concentrations (31.25, 62.5, 125, 250 µg/mL). The pH was adjusted to 6.3 using 1N HCl. The sample extracts were incubated at 40°C for 25 minutes. After cooling, turbidity was measured at 660 nm. The experiment was performed in triplicate. The positive control was sodium diclofenac (concentrations of 31.25, 62.5, 125, 250 µg/mL) and was assayed in a similar manner. The percentage inhibition of protein denaturation was calculated using the following equation:

$$\%\ inhibition\ of\ protein\ denaturation = \frac{Absorabance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \quad (3)$$

2.9. Statistical analysis.

Results were expressed as average ± SEM (Standard Error Mean) and statistically analyzed using Minitab.16. A statistical analysis was performed by using a one-way analysis of variance (ANOVA) followed by a Dunnet Multiple comparison test (control Vs. test). Significant differences were considered when $p < 0.05$.

3. Results

3.1. Extraction yield and phytochemical screening.

The results obtained have shown that the extraction yield of ethanol extract from leaves of U. urens using ultrasound-assisted extraction represents 9.46 %. Phytochemical tests showed that the leaves of *Urtica urens* contained phenolics, flavonoids, and tannins. The results of phytochemical screening are presented in Table 1.

Table 1. Phytochemical screening of ethanolic leaf extract of *Urtica urens.*

Secondary Metabolite	Test Results
Flavanoids Substances	+
Phenolic Substances	+
Aponosides Substances	-
Anthraquinones Substances	-
Tannins Substances	++

3.2. Antioxidant capacity.

The experimental results on the Antioxidant capacity, total phenols, flavonoids, and tannin contents are presented in Table 2. The quantitative phytochemical analyses showed that the ethanolic extract of *Urtica urens* had the highest values of total phenolic (24.21 mg GAE/g), flavonoids (17.91 mg quercetin/g), and tannins (11.51 mg GAE/g) (Table 2). In this study, the DPPH free radical scavenging test was used to assess the antioxidant activity of ethanolic extracts from leaves of *Urtica urens*. The results of this test are presented in Figure 1. The percentage inhibitions of the extract at different concentrations (0.25, 0.5, 1, and 2 mg/mL) were 50.7, 66.11, 75.11 %, and 76.61 %, respectively (Figure 1). However, those extracts displayed a good total antioxidant activity that increased with increasing concentration. Therefore, the results indicated that ethanolic extracts were the strongest radical scavengers (IC_{50} =0.44 mg/mL).

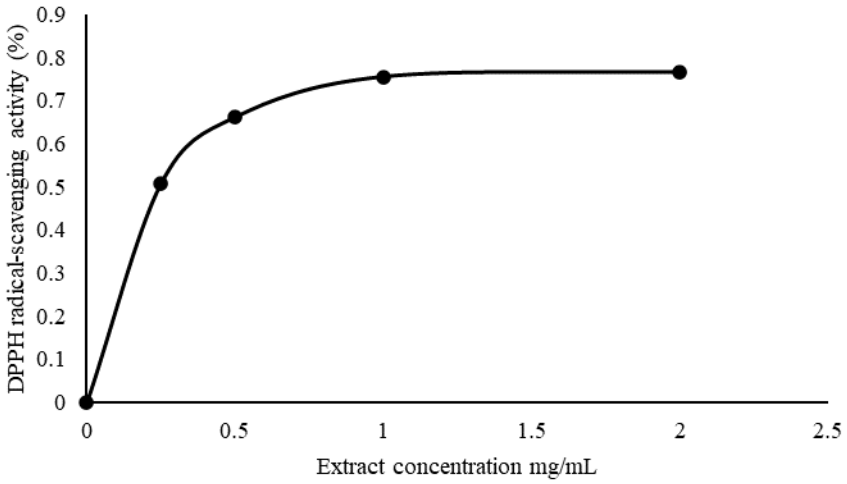

Figure 1. Antioxidant activity for *Urtica urens* extracts obtained by ultrasound extraction.

Table 2. Total antioxidant capacity, amount of phenolic flavonoids, and tannin compounds of *Urtica urens* extract.

Extract	IC_{50} (mg/ml)	Total flavonoids (mg QE/g)	Total phenolic (mg GAE/g)	Total tannin (mg CE/g)
Ethanolic extract	0,44±0.88	17.91 ± 1.01	24.21 ± 4,47	11,51 ± 3,26

The values represent the means of three measurements ± standard deviation.

3.3. BSA anti-denaturation assay.

Urtica urens leaves' anti-inflammatory activity was studied using the Bovine Serum Albumin (BSA) protein denaturation technique. Table 3 shows the percent inhibition of protein denaturation for different concentrations of *Urtica urens* and sodium diclofenac ethanol extracts. The ethanol extract of *Urtica urens* at different concentrations showed significant anti-inflammatory activity at different concentrations comparable with standard sodium diclofenac. It was observed that ethanolic leaf extracts exhibited concentration-dependent inhibition of the denaturation of protein, where the inhibition of the denaturation of the BSA increased with the concentration of the extract. The highest anti-denaturation activities were observed at 250 µg/mL for leaf extracts (94,87%). Also, sodium diclofenac, a standard antiinflammation drug, showed a maximum inhibition of 84.88% at 250 µg/ml concentration.

Table 3. % inhibition of protein (BSA) denaturation from ethanolic leaf extracts of *Urtica urens* and positive control (Sodium Diclofenac).

		Concentration (µg/mL)			
		31.25	**62.5**	**125**	**250**
% Inhibition	**Ethanolic Extract**	74,35 ± 5,14	81,19 ± 1,48	88,88 ± 1,48	94,87 ± 1,48
	Sodium Diclofenac	75,33 ± 4,04	80 ± 4,04	82,22 ± 4,04	84,88 ± 4,04

The values represent the means of three measurements ± standard deviation.

4 . Discussion

The extraction yield of the ethanol extract from leaves of *Urtica urens* using ultrasound-assisted extraction was 9.46%, which was less than that obtained with maceration extraction, as reported by Mekhahli and Djedouani, 2021 [22]. However, the yield of the leaves of *Urtica urens* in ethanol solvent obtained in this study was higher compared to other studies that used other solvents (butanol R=3.33%, ethyl acetate, R=2.56%, aqueous R= 3.83% (Daoudi et al., 2015). Therefore, ethanol had the ability to attract polyphenols, flavonols, tannins, terpenoids, and alkaloids [23,24]. The choice of ethanol (less toxic) as the extraction solvent using

ultrasound-assisted treatment had the advantage of obtaining a better extraction yield. Moreover, the ultrasound-assisted extraction technique used in this study also allows greater penetration by the solvent into the materials to be extracted.

The present study suggests that several phytochemicals and antioxidant properties are present in the leaf extracts of *Urtica urens*. The phytochemical analysis evinced the quantitative values of *Urtica urens* extracts: 24.21 mg GAE/g to phenolic compound, 17.91 mg quercetin/g to flavonoids and 11.51 mg GAE/g to tannins. These results showed that the ethanol extract leafs using ultrasound treatment had appreciable amounts of phenolics, flavonoids, and tannins, which are known to possess potent antioxidants, anticancer, antifungal, antibacterial, antidiabetic, and anti-inflammatory properties [25-36]. The richness of *Urtica urens* extracts on phenolic and flavonoid compounds was higher than those of Tunisia, as reported by Mzid M et al. [4]. Besides, the ethanol extract presented less content of tannin compounds than those reported in ethanol extract leaves from Tunisia [4]. However, this variation in the content between the studied extracts can be explained by various parameters and conditions such as genetic variation, growth conditions, and type of extract.

One of the major and well-documented causes of inflammation activity is the denaturation of proteins. The present study seeks to evaluate the in vitro anti-inflammatory potential of the ethanolic leaf extracts of *Urtica urens L*. In vitro, anti-inflammatory activity was performed using the Bovine Serum Albumin (BSA) protein denaturation technique. This study results indicate that the ethanol extracts of *Urtica urens* effectively inhibited the denaturation of BSA in vitro and were even more significant than the reference anti-inflammatory drug, Diclofenac sodium. Therefore, this extract possesses significant anti-inflammatory properties.

Earlier literature reported that the *U. urens* extract could act as a more potent anti-inflammatory agent compared with *U. dioica*, *U. membranacea*, and *U. urens* aerial parts [5, 37,38].

However, further investigations of *Urtica urens* will be necessary to purify each bioactive compound, which may show increased activity. The results obtained in the present study indicate that ethanol l extracts of *Urtica urens L* can be used as a potential source of anti-inflammatory agents, which can be used to treat various diseases such as cancer, neurological disorders, aging, and inflammation.

5. Conclusions

The results obtained have shown that the extraction yield of ethanol extract from leaves of *U. urens* represents 9.46 %. Phytochemical screening results on the ethanolic extract of *Urtica urens* leaves revealed the presence of flavonoids, phenolics, and tannins. Additionally, the quantitative phytochemical analyses of the ethanol extract leaves showed the presence of an appreciable number of phenolics, flavonoids, and tannins, which are known to exhibit medical and physiological activities. The results indicated that ethanolic extracts were the strongest radical scavengers (IC_{50} =0.44 mg/mL). Protein denaturation is a well-documented cause of inflammation; thus, it is used to confirm the anti-inflammatory activities of plant extract. It was effective in inhibiting heat-induced albumin denaturation. This study has shown that the ethanolic extracts of *Urtica urens* possessed a significant anti-inflammatory activity. Maximum inhibition of 94.87% was observed at 250 µg/mL. These activities may be due to the strong occurrence of polyphenols.

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Conflicts of Interest

The authors declare no conflict of interest.

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