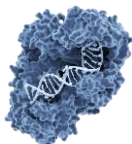

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Original Research Article

HPLC-UV-MS/MS Profiling of Phenolics from *Euphorbia nicaeensis* (All.) Leaf and Stem and Its Antioxidant and Anti-Protein Denaturation Activities

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Abstract: This work focuses on the leaves and stems of *Euphorbia nicaeensis* All. to confirm its historical use by the Moroccan population. The phytochemical profile of the plant by HPLC-UV-MS/MS was identified for the first time, and the biological activities of each plant organ was evaluated separately by maceration and ultrasonic-assisted extraction. The evaluation of antioxidant activity based on DPPH assay and hydrogen peroxide scavenging assay showed that leaves could be used as a natural source of antioxidants as they provide a potent antioxidant effect (DPPH IC_{50} = 23.47±0.62 µg/ml), (H_2O_2 IC_{50} = 110.27 ± 3.59 µg/ml) compared to stems. These results were proven by HPLC-UV-MS/MS analysis and revealed that *E. nicaeensis* leaves are richer in phenolic compounds, especially quercetin and derivatives, known for their antioxidant properties. In contrast, the stems could be considered a potential anti-inflammatory agent considering their solid anti-inflammatory activity. The

most potent effects were obtained at a concentration of 2 mg/ml, which induced 83.98% and 82.04% inhibition against bovine albumin and egg albumin denaturation, respectively, compared to the control. In addition, the stem phytochemical profile indicated the presence of some compounds with anti-inflammatory effects, such as fargesin and nuciferine. Likewise, the findings showed that ultrasound-assisted extraction was more effective than maceration.

Keywords: *Euphorbia nicaeensis* All., Maceration, Ultrasound extraction, Antioxidant activity, Anti-inflammatory activity, HPLC-UV-MS/MS

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1. Introduction

Aromatic and medicinal plants have long been regarded as a vital source of therapeutics and curative remedies due to the presence of bioactive compounds and phytochemical constituents. Medicinal herbs have proven to be the basis of traditional medicine worldwide. Curing and healing human diseases has always been related to using entire plants or parts of them in remedy preparation [1].

Moroccan population has traditionally employed aromatic and medicinal plants; over time, people have gained knowledge of old cosmetics and pharmacopoeia. Oral communication is still used to pass on essential information about these activities from generation to generation [2]. However, several medicinal plants are currently underexploited in Morocco despite their ancestral use in treating human diseases.

Euphorbia nicaeensis All. is one of these plants on which this investigation focuses. It is a species of Euphorbiaceae family, a perennial spurge herbaceous plant that prefers calcareous soils. It grows in sunny and dry places and is distributed in the Mediterranean and central Europe with a strong morphological variability in leaves and bracts [3].

E. nicaeensis is an abundant species in the rocky lawns of Morocco [4], latex is the most traditionally used part of the plant, and it has been used in the past to attack warts and erase dead flesh. The population was aware of the vesicant properties of latex on the skin, eyes, and mucous membranes [5]. There are also other uses of leaves and stems against bacterial infections, hepatitis, tuberculosis, typhoid, and other diseases [6–8].

Numerous studies have proven the traditional use of *E. nicaeensis* and its anthelmintic, antifungal, and anticancer activities due to the jatrophane diterpenoid isolated from the root extracts and latex [9–11]. Previous studies have also evaluated some biological activities of *E. nicaeensis* aerial parts and detected its anti-inflammatory properties [12,13]. This study aims to assess the phytochemical profile of *Euphorbia nicaeensis* by HPLC-UV-MS/MS analysis for the first time and to evaluate the anti-inflammatory properties and antioxidant activity of leaves and stems. Therefore, two extraction methods were compared, probe ultrasonic-assisted extraction and maceration.

2. Materials and Methods

2.1. Plant material

The plant was harvested from the Ifrane region (Figure 1). The Lambert coordinates: 33° 32′ 44.4″ N, 5° 19′ 17.89″ W. and the plant have undergone drying after separation of the leaves.

Figure 1. Photo of the species *Euphorbia nicaeensis* All.

2.2. Extraction procedure

2.2.1. Maceration

An amount of 2.5 g of ground plant powder (leaves and stems) was extracted by 50 ml (methanol - water) (80:20) v/v. The maceration extracts were kept under stirring for 24 hours, then filtered under a Buchner funnel and concentrated by rotary evaporation. The residues were collected and stored until further analysis.

2.2.2. Probe ultrasonic-assisted extraction (PUAE)

Probe ultrasonic-assisted extraction (PUAE) is one of the main extraction methods used for plant materials. Sonication causes cavitation and implosion, which causes cell-wall rupture and increases the number of disturbed cells. When disturbed, the solvent penetrates the cell, and the intracellular plant material is absorbed into the solvent [14].

The extraction was done using an ultrasonic probe system (Bioblock scientific, Vibra Cell 75042). The probe was submerged 1.5 cm under the surface of the mixture (methanol-water) (80:20) v/v and 2.5 g of plant powder. The extraction was performed at the maximum power settings of the transducer (100%, 400 W), at 24 kHz, for 15 min, with a 20 s pulse. Following extraction, the extract was filtered and concentrated by rotary evaporation until dryness and was stored for further use.

2.3. Phytochemical screening

Phytochemical screening of *Euphorbia nicaeensis* All. leaves and stems were performed using standardized laboratory protocols. The presence or absence of phenols, tannins [15], flavonoids [16], saponins [17], flavonol, carbohydrates, alkaloids, gums, amino acids [18], sterols and polyterpenes [19], was analyzed accordingly.

2.4. Total phenolic content (TPC)

The phenolic compounds were measured using Folin-Ciocalteu method [20]. In brief, 50 µl of Folin-Ciocalteu reagent was added to the sample, followed by 150 µl of Na_2CO_3 after 10 min, and the volume was made up to 1 ml with water. The absorbance was measured in a spectrophotometer reader at 760 nm after 2 hours of incubation and compared to the Gallic acid calibration curve (5 – 100 µg/ml), R^2= 0.998. The results were expressed as mg Gallic acid equivalents /g dry weight (mg GAE/g DW).

2.5. Total flavonoid content (TFC)

According to [21], 250 µl of the extract solution was mixed with 1 ml of distilled water and 75 µl of $NaNO_2$ solution (5 %). After 6 min, 75 µl of $Alcl_3$ solution (10 %) was added. The mixture was allowed to stand for 6 min before adding 1 ml of NaOH solution (4 %) and bringing the final volume to 2.5 ml with distilled water. The mixture was properly vortexed and allowed to stand for 15 min in darkness. The absorbance was measured at 510 nm and compared to the catechin standard curve (5 – 400 µg/ml) R^2= 0.997. The results were expressed as mg of catechin equivalent per g dry weight (mg CE/g DW).

2.6. Flavonol content (FC)

The content of flavonols was determined according to the method described by [22]. An aliquot of 500 µl of extract was added to 500 µl of Aluminium chloride (20 mg/ml) and 1.5 ml of sodium acetate (50 mg/ml). The mixture was properly vortexed and left to stand in darkness for 2.5 h. The absorbance was read at 440 nm, and the result was expressed referring to the absorbance of standard quercetin solution prepared in the same conditions (5 – 200 µg/ml), R^2= 0,995. The flavonol content is expressed in mg Quercetin equivalents per g dry weight (mg QE/g DW).

2.7. Condensed tannin content (CTC)

The assay was executed as reported previously [23]. An aliquot of 100 µl of each extract was added to 1.5 ml of Vanillin (4 %) and 750 µl of concentrated hydrochloric acid (HCL). The mixture was vortexed, left to stand in the dark for 20 min, and the absorbance was recorded at 500 nm. The condensed tannin content was counted related to the Catechin calibration curve (5 – 400 µg/ml) R^2= 0.985, elaborated in the same manner. The results are expressed as mg of Catechin equivalent per 100 g dry weight (mg CE/100g DW).

2.8. Lipid-soluble pigment content

According to [21], 150 mg of vegetal powder (leaves and stems) was mixed for 1 min with 10 ml of Acetone/Hexane (4:6). The mixture was filtered through Whatman filter paper grade 4. The absorbance was recorded at 453,505,645, and 663 nm. The content of β-carotene, lycopene, chlorophyll a, and chlorophyll b was calculated according to the following equations, expressed in µg per g dry weight (DW):

$$\beta\text{-carotene} = 0.216 \times A663 - 1.220 \times A645 - 0.304 \times A505 + 0.452 \times A453$$

$$\text{Lycopene} = -0.0458 \times A663 + 0.204 \times A645 - 0.304 \times A505 + 0.452 \times A453$$

$$\text{Chlorophyll a} = 0.999 \times A663 - 0.0989 \times A645$$

$$\text{Chlorophyll b} = 0.328 \times A663 + 1.77 \times A645$$

A453, A505, A645, and A663 are the absorbance measured at 453,505,645, and 663 nm, respectively.

2.9. In vitro antioxidant activity

2.9.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay

The radical scavenging ability of the extract was carried out as described by [24–26]. 500 μl of extract solution was mixed with 1 ml of DPPH solution (0.1 mM in methanol) freshly prepared. The mixture was vortexed properly and left to stand in the dark for 60 min. The reduction of DPPH was measured at 515 nm, and the scavenging effect was estimated based on the percentage of scavenging DPPH radicals using the following equation:

$$\%\ \textbf{DPPH scavenging activity} = \frac{\boldsymbol{ADPPH - As}}{\boldsymbol{ADPPH}} \times \mathbf{100}$$

ADPPH is the absorbance of DPPH and AS is the absorbance of DPPH when the sample has been added at different concentrations. The IC_{50} value is the concentration that scavenges 50 % of DPPH radicals, and it is calculated from the scavenging effect percentage graph. Ascorbic acid was used as a standard.

2.9.2. Scavenging of hydrogen peroxide assay

The performance of hydrogen peroxide (H_2O_2) scavenging was investigated as follows [27]. The phosphate buffer (50 mM, 7.4 pH) received a 40 mM H_2O_2 solution. In order to quantify the mixture's absorbance spectrophotometrically at 230 nm, all experimental samples were combined with 0.6 ml of H_2O_2 solution. The mixture was then incubated for 10 minutes. Ascorbic acid served as the standard, while phosphate buffer functioned as the control. Hydrogen peroxide scavenging (%) was calculated using the formula below.

$$\%\ \textbf{Hydrogen peroxide scavenging activity} = \frac{\boldsymbol{A0 - As}}{\boldsymbol{A0}} \times \mathbf{100}$$

Where As is the absorbance in the presence of the ascorbic acid standard or samples, and Ao is the absorbance of the blank. Ascorbic acid was used as a standard.

2.10. In vitro anti-inflammatory activity

2.10.1. Bovine Serum Albumin Assay (BSA)

Protein denaturation is one of the causes of inflammatory and rheumatic diseases, as reported by a previous study [28]. Any compound that provides greater than 20% inhibition of protein denaturation is considered a potential anti-inflammatory agent and could be useful for treating several diseases [29].

BSA assay of leaf and stem extracts was determined using the method reported by [27]. 0.45 ml of bovine serum albumin was mixed with 0.05 ml of samples at various concentrations (200 - 2000 µg/ml). The mixture was incubated for 25 min at 40°C, and phosphate buffer saline (2.5 ml; pH 6.3) was added to tubes. The control received phosphate buffer solution (0.05 ml) instead of extract. The absorbance was measured using a spectrophotometer at 660 nm, and the percentage inhibition of BSA denaturation was calculated using the following equation:

$$\%\ \textbf{inhibition of BSA denaturation} = \frac{\boldsymbol{A1 - A2}}{\boldsymbol{A1}} \times \mathbf{100}$$

Where A1 = absorbance of the control and A2 = absorbance of the test sample.

2.10.2. Chicken Egg albumin assay (CEA)

Using the technique described by [30], the ability of our extracts to inhibit protein denaturation was examined. To 2.8 ml of PBS solution, 2 ml of extract and 0.2 ml of chicken egg albumin were added. All samples were kept at room temperature for 15 minutes before being heated to 70 °C for 10 minutes. Diclofenac was employed as standard, and the absorbance was measured at 660 nm. The equation below was used to determine the inhibition of egg albumin denaturation:

$$\%\ \textbf{inhibition of egg albumin denaturation} = \frac{\boldsymbol{A1 - A2}}{\boldsymbol{A1}} \times \mathbf{100}$$

A1 is the control's absorbance and A2 is the test sample's absorbance.

2.11. Phenolic profile analysis by HPLC-UV-MS/MS

Phenolic compounds were qualitatively analyzed using a liquid chromatography system coupled with a triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The suggested technique was carried out using a Kinetex C18 reversed-phase column (100 x 4.6 mm, 2.6 m particles). From solvent A (0.1% formic acid aqueous solution) and solvent B (methanol), gradient separation was created following the method described by [31]. Retention time and spectrum matching with nine standards were used to identify the phenolic compounds, along with the NIST-MS/MS library.

2.12. Statistical analysis

T-test was used to evaluate the statistical differences between extraction methods (maceration and probe-assisted ultrasound extraction) and organs (leaves and stems). Experiments were run in triplicate, and the results are expressed as mean values of three analyses. Data are statistically significant at $p<0.05$ and highly significant at $p<0.001$. Pearson correlation coefficient (r), DPPH (IC_{50}), and H_2O_2 (IC_{50}) were determined using GraphPad prism 8.0.2.

3. Results

3.1. Phytochemical screening

The results of phytochemical screening of *Euphorbia nicaeensis* All. leaves and stems are shown in Table 1. Phenols, tannins, saponins, and carbohydrates are strongly present in all plant organs. Flavonoids, flavonol glycosides, sterols, and polyterpenes are highly present in leaves and small amounts in stems. Alkaloids and amino acids are present in tiny quantities, quinones and mucilage are absent in all plant organs.

Table 1. Phytochemical screening results of leaves and stems of *Euphorbia nicaeensis* All.

Phytochemical compounds	Leaves	Stems
Phenols / Tanins	(++)	(++)
Flavonoids	(++)	(+)
Flavonol glycosides	(+)	(+/-)
Alkaloids	(+/-)	(+/-)
Saponins	(++)	(++)
Carbohydrates	(++)	(++)
Quinones	(-)	(-)
Gums and mucilages	(-)	(-)
Amino acids	(+)	(+)
Sterols and Polyterpenes	(++)	(+)

(+): Presence of phytochemicals, (-): Absence of phytochemicals, (++): Presence in significant amounts, (+/-): Presence in small amounts.

3.2. Total phenol (TPC), Total flavonoid (TFC), Flavonol content (FC), and Condensed tannin content (CTC)

The carried-out tests indicated that the leaves and stems of *Euphorbia nicaeensis* are rich in phenolic compounds. The percentage yield of leaf and stem extracts by maceration (3.32%, 1.68%), and ultrasonic-assisted extraction (12.69 ± 0.17%, 4.62 ± 1.55%), respectively, showed that the highest extraction yield of both leaves and stems was obtained with probe ultrasonic-assisted extraction (PUAE). The (TPC) values of PUAE extract of leaves and stems (80.13 ± 1.61 mg GAE/g DW, 77.79 ± 2.29mg GAE/g DW), respectively, were statically higher (t-test, $p<0.05$) than that of maceration extracts (68.69 ± 0.91mg GAE/g DW, 68.31 ± 1.83 mg GAE/g DW). This data also reveals that the leaves are richer in flavonoids, condensed tannins, and flavonols than the stems as presented in Table 2.

Table 2. Total phenol, flavonoids, condensed tannins, flavonols contents and extraction yield of maceration extracts and probe ultrasonic-assisted extract of *Euphorbia nicaeensis* All. leaves and stems.

	ELM*	**ESM***	**ELU***	**ESU***	***p*1**	***p*2**	***p*3**	***p*4**
TPC (mg GAE/g DW)	68.69±0.91	68.31± 1.83	80.13± 1.61	77.79±2.29	0.574	0.042	<0.001	<0.001
TFC (mg CE/g DW)	32.83 ±3.84	21.83 ±9.93	49.22 ±0.192	45.27 ±2.5	0.047	0.031	0.004	0.010
CTC (mg CE/100gDW)	1.33±1.1	1.88±1.92	7.61±1.92	1.22±0.92	0.450	0.002	0.001	0.361
FC (mg QE/g DW)	4.80±0.08	1.23 ±0.27	36.14±0.08	5.88±0.33	<0.001	<0.001	<0.001	<0.001
Extraction yield (%)	3.32±0.59	1.68±0.28	12.69±0.17	4.62±1.55	0.024	0.011	<0.001	0.077

ELM: Euphorbia leaf maceration, ESM: Euphorbia stem maceration, ELU: Euphorbia leaves ultrasonic-assisted extraction, ESU: Euphorbia stems ultrasonic-assisted extraction, TPC: Total flavonoid content, TFC: Total flavonoid content, CTC: Condensed tannin contents, FC: Flavonols content, *p*1: *p*-value (ELM – ESM), *p*2: *p*-value (ELU – ESU), *p*3: *p*-value (ELM – ELU), *p*4: *p*-value (ESM – ESU). *Means ± SD from triplicate determinations. *p*-value is considered significant at $p<0.05$ and highly significant at $p<0.001$.

3.3. Lipid-soluble pigment content

As shown in Figure 2, this study revealed that leaves and stems differed significantly (p<0.05) in the content of chlorophyll pigment. The leaves are richer in chlorophyll a and b (0.167±0.001 µg/g DW, 0.108± 0.002 µg/g DW) than the stems (0.016 µg/g DW, 0.049± 0.001 µg/g DW). These findings also showed that leaves have significantly higher (p<0.05) β-carotene content (0.035 ± 0.001 µg/g DW) compared to stems (0.006 ± 0.001 µg/g DW). In addition, low lycopene concentration was detected in both leaves and stems (0.033 ±0.001 µg/g DW, 0.036 µg/g DW), respectively, with no significant difference (p>0.05).

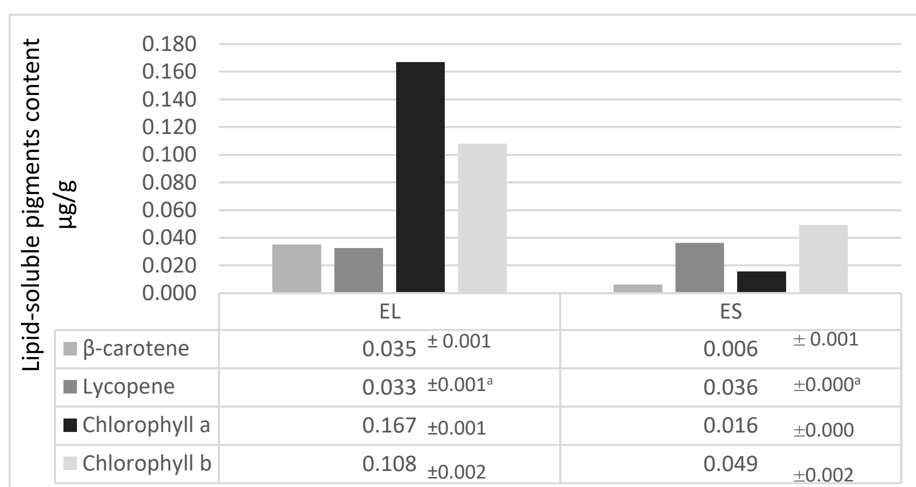

Figure 2. Lipid-soluble pigment content of leaves and stems of *Euphorbia nicaeensis.* Values are means ± S.D of three independent measurements. EL: Euphorbia leaves, ES: Euphorbia stems. The values with the same superscript letters are not significantly different (p>0.05).

3.4. In vitro antioxidant activity

The evaluation of the antioxidant activity of *E. nicaeensis* leaf and stem extracts was screened by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay and scavenging of hydrogen peroxide (H_2O_2) assay. All extracts showed antioxidant activity in Figure 3. The results were positively correlated to the concentration, and the maximum scavenging activity was recorded at the highest concentration for all extracts. Furthermore, it was observed that PUAE provided a strong significant DPPH scavenging effect (p<0.01), translated by a low IC_{50} value of leaf extracts (IC_{50} = 23.47±0.62 µg/ml) closely compared to Ascorbic Acid (IC_{50}

=19.89±0.14 μg/ml). While the maceration leaf extract showed a low antioxidant effect (IC_{50} = 79.76±1.93 μg/ml).

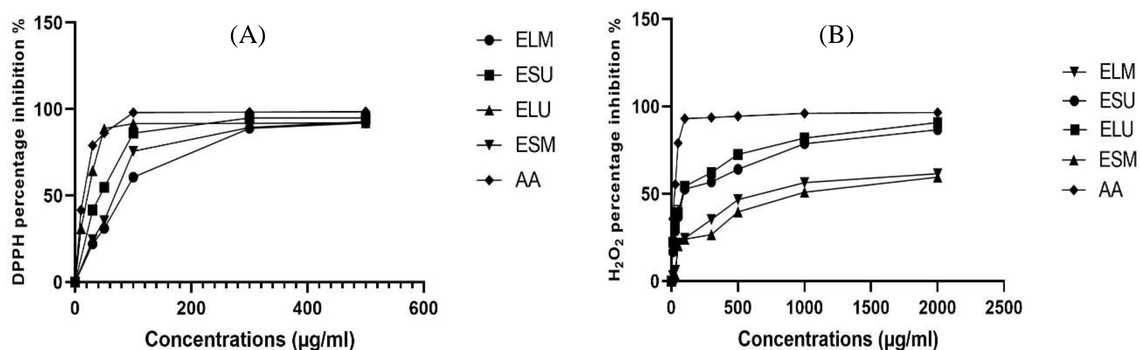

Figure 3. DPPH radical scavenging activity (A) and H2O2 scavenging activity (B) versus concentration (µg/mL). ELM: Euphorbia leaf maceration, ESM: Euphorbia stem maceration, ELU: Euphorbia leaf ultrasonic-assisted extraction, ESU: Euphorbia stem ultrasonic-assisted extraction. The results are expressed as means ± SD of three independent measurements.

The stem extract of PUAE also exhibited a moderate antioxidant effect (IC_{50} = 42.45 ± 2.30μg/ml) compared to the maceration extract (IC_{50} = 61.16 ± 1.53μg/ml). However, it was observed that stem maceration extract has a significantly ($p<0.01$) lower IC_{50} value than leaf maceration extract, which implies a higher antioxidant capacity, as shown in Table 3, similarly, for H_2O_2 scavenging results. The lowest IC_{50} was observed in PUAE leaf extract (IC_{50}=110.27 ± 3.59 µg/ml) followed by stem extract (IC_{50} = 131.51 ± 1.80 µg/ml) in comparison to maceration (IC_{50} = 678.32 ± 7.22 µg/ml, IC_{50} = 1073.63 ± 1.08 µg/ml, respectively).

Table 3. Scavenging activity of leaf and stem extracts of *Euphorbia nicaeensis* All.

Scavenging activity (IC50 µg/ml)		
	DPPH assay	**H_2O_2 assay**
ESM*	61.16 ± 1.53	1073.63 ± 1.08
ELM*	79.76 ± 1.93	678.32 ± 7.22
ESU*	42.46 ± 2.30	131.51 ± 1.80
ELU*	23.48 ± 0.62	110.27 ± 3.59
AA*	19.90 ± 0.14	35.41 ± 0.23

*Means ± SD from triplicate determinations, $p < 0.001$.

The correlation analysis between antioxidant activity and phenolic content of the extracts is presented in Table 4. Pearson's coefficient revealed a positive relationship between antioxidant activity and total phenolics and flavonoids' plant content. The DPPH assay of antioxidant activity and TPC showed a Pearson coefficient of r=0.8827 and r=0.7768 for total flavonoids content with no significant correlation (p>0.05). Moreover, a highly significant correlation was observed between the H_2O_2 assay and the composition of the plant in both TPC ($p < 0.001$) and TFC ($p < 0.05$).

Table 4. Pearson correlation test between total phenolic content, total flavonoids content, and antioxidant activity.

	Pearson's correlation (r)	
	DPPH (1/IC_{50})	**H_2O_2 (1/IC_{50})**
TPC	0.8827	0.9995**
TFC	0.7768	0.9505*

*Significant at p <0.05; **Significant at p<0.001; DPPH: DPPH scavenging activity; H_2O_2: hydrogen peroxide scavenging activity; TPC: Total flavonoid content; TFC: Total flavonoid content.

3.5. In vitro anti-inflammatory activity

According to the results presented in Table 5, all extracts showed an inhibition of Bovine Serum Albumin (BSA) and chicken egg albumin denaturation (CEA), which increased with the sample concentration. The highest inhibitions were observed at the 2000 µg/ml dose for all extracts.

The findings revealed a notable inhibition of serum albumin denaturation (83.98% and 75.39 %, p<0.001) and egg albumin denaturation (82.04% and 78.87%, p<0.001) induced by PUAE extracts of stems and leaves, respectively, at the dose of (2000 µg/ml). The maceration extracts of leaves and stems showed a lower inhibition of bovine serum albumin (38.28% and 43.36%, p<0.001) at the same concentration. The inhibition of egg albumin revealed similar results. Maceration extracts from stems and leaves showed a weak inhibition (49.85%, 62.52%, respectively). While the standard drug diclofenac sodium exhibited potent inhibition (97.73%, p<0.001) at the same concentration when compared to the control.

Stem extracts from maceration and ultrasound-assisted extraction showed a significantly higher level of protein denaturation inhibition ($p<0.05$) than leaf extracts at the concentration range of (2000- 500 µg/ml).

Table 5. In vitro anti-inflammatory activity of *Euphorbia nicaeensis* All.

Treatment	Dosage (µg.ml^{-1})	Bovine serum albumin denaturation		Chicken Egg albumin denaturation	
		Absorbance	% Inhibition	Absorbance	% Inhibition
Control	-	0.917 ± 0.015	-	0.913 ± 0.015	_
ELM	200	0.652 ± 0.0006	28.90	0.286 ± 0.003	72.48
	300	0.591 ± 0.002 [a]	35.54	0.221 ± 0.001	72.08
	500	0.571 ± 0.0026	37.77	0.218 ± 0.003	70.95
	1000	0.570 ± 0.0021	37.89	0.197 ± 0.001	68.80
	2000	0.566 ± 0.001	38.28	0.193 ± 0.001	62.52
ESM	200	0.677 ± 0.0072	26.17	0.279 ± 0.001	73.32
	300	0.570 ± 0.006	37.89	0.223 ± 0.001	63.94
	500	0.541 ± 0.0066	41.02	0.206 ± 0.004	59.82
	1000	0.536 ± 0.0061	42.19	0.192 ± 0.001	52.04
	2000	0.519 ± 0.009	43.36	0.164 ± 0.004	49.85
ELU	200	0.638 ± 0.003	30.47	0.342 ± 0.003	68.72
	300	0.605 ± 0.0096 [a]	33.98	0.285 ± 0.004 [b]	75.77
	500	0.390 ± 0.0046	57.42	0.265 ± 0.003	76.13
	1000	0.276 ± 0.0053	69.92	0.255 ± 0.004	78.43
	2000	0.226 ± 0.001	75.39	0.251 ± 0.001	78.87
ESU	200	0.613 ± 0.0044	33.20	0.458 ± 0.006	69.49
	300	0.537 ± 0.001	41.41	0.438 ± 0.012 [b]	75.62
	500	0.308 ± 0.0053	66.41	0.367 ± 0.002	77.48
	1000	0.208 ± 0.000	77.34	0.329 ± 0.003	78.98
	2000	0.147 ± 0.0035	83.98	0.244 ± 0.002	82.04
Diclofenac sodium	200	0.079 ± 0.0026	91.41	0.054 ± 0.004	94.12
	300	0.062 ± 0.001	93.23	0.047 ± 0.002	94.89
	500	0.057 ± 0.0036	93.75	0.041 ± 0.002	95.47
	1000	0.050 ± 0.0017	94.53	0.035 ± 0.004	96.17
	2000	0.021 ± 0.0044	97.73	0.022 ± 0.004	97.59

The results are expressed as means ± SD of three independent measurements. ELM: Euphorbia leaf maceration, ESM: Euphorbia stem maceration. ELU: *Euphorbia* leaf ultrasonic-assisted extraction, ESU: *Euphorbia* stem ultrasonic-assisted extraction. the values with the same superscript letters are not significantly different ($p>0.05$).

3.6. Phenolic profile analysis by HPLC-MS

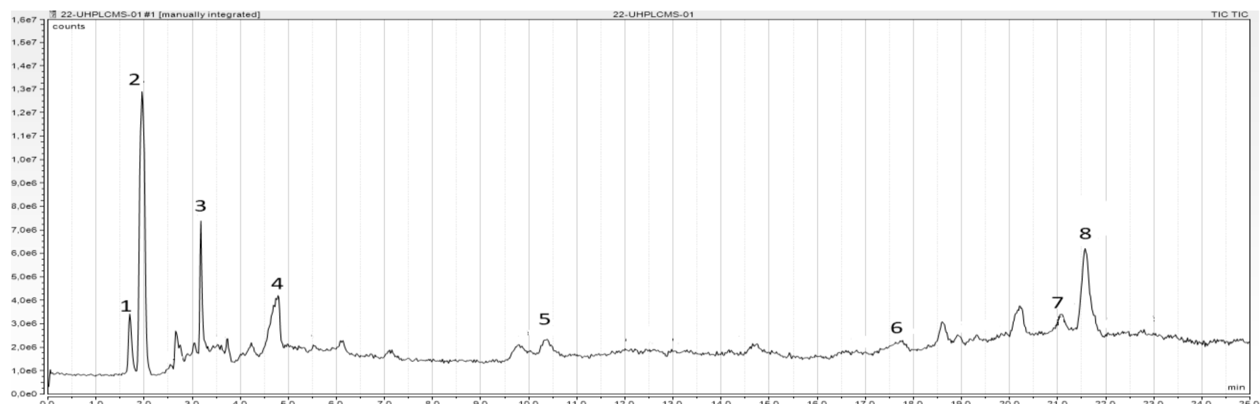

Figure 4. HPLC-MS/MS chromatogram of UAE stem extract.

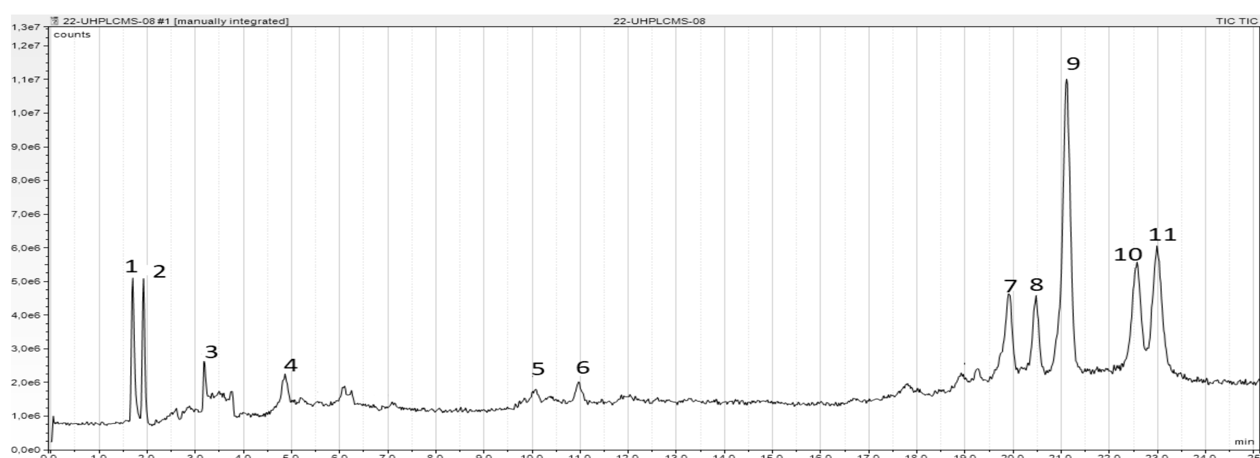

Figure 5. HPLC-MS/MS chromatogram of UAE leave extract.

Based on the previous results of the phenolic compounds assay, UAE leaf, and stem extracts were retained for HPLC-UV-MS/MS analysis. The presence of the compounds identified by HPLC-UV analysis was confirmed using HPLC-MS/MS. Comparisons with reference standards, NIST library, and earlier literature reports helped identify. The retention times of the standards tested did not match the peaks detected in the chromatograms of the leaf and stem extracts (Figures 4 and 5). This is because many are in the form of sugar derivatives that are rarely available. Therefore, mass spectrometry was utilized to characterize the peaks and allowed further identification of the compounds. Comparing the MS/MS data with those in the NIST library and other references revealed several phenolic compounds.

Table 6 demonstrates the abundance of different phytochemicals present in both extracts, with a slight difference in composition, including flavonols, phenolic acids, lignan, calchone, carotenoids, and alkaloids. Fargesin, 2'-hydroxy-3,4,4',6'- tetramethoxychalcone, nuciferin, quercetin 3'-methyl ether, and isoquercetin are detected in both extracts with different peak airs. Isoquercetin was detected as the predominant compound in the leaf extract, followed by quercitrin hydrate and guaiaverin. The stem extract is rich in 2'-hydroxy-3,4,4',6'- tetramethoxychalcone, representing the highest peak (22.04%), followed by Quercetin 3'-methyl ether.

Table 6. HPLC-MS/MS tentative identification of phenolics and derivatives in E. nicaeensis leaf and stem extracts.

Leave					Stem				
N°	**RT**	**Tentative identification**	**Air %**	**Ref**	**N°**	**RT**	**Tentative identification**	**Air%**	**Ref**
1	1.70	Fargesin	5.72	N	1	1.70	Fargesin	3.34	N
2	1.92	2'-hydroxy-3,4,4',6'-tetramethoxychalcone	4.51	N	2	1.96	2'-hydroxy-3,4,4',6'-tetramethoxychalcone	22.04	N
3	3.18	Nuciferine	2.08	N	3	3.18	Nuciferine	8.14	N
4	4.86	Quercetin 3'-methyl ether	3.90	N	4	4.78	Quercetin 3'-methyl ether	10.02	N
5	10.08	5,7,3',4'-Tetramethoxyisoflavone	2.11	N	5	10.38	Lutein	3.65	N
6	10.97	Galloylquinic acid	1.79	[32]	6	17.77	Dilinolenin	3.11	N
7	19.90	Quercetin-galactoside-gallate	8.70	[33]	7	21.11	Isoquercetine	0.94	N
8	20.47	Myricitrin	6.59	N	8	21.52	2'-Hydroxy-2,4,4'-trimethoxychalcone	9.59	N
9	21.11	Isoquercetin	27.49	N					
10	22.57	Guaiaverin	11.78	N					
11	22.99	Quercitrin hydrate	14.49	[31]					

RT: retention time, N: NIST-MS/MS library, Air %: Relative peak area

4. Discussion

Most of the phytochemical screening findings align with a previous investigation conducted in Serbia for *Euphorbia nicaeensis* ssp. glareosa [34]. However, some differences were observed, namely, the presence of alkaloids and the absence of quinones in *E. nicaeensis* All. leaves and stems, compared to this subspecies. This could be due to several parameters, including genetics, differences in harvesting site, rainfall, light, season, harvesting period, topography, soil type, and extraction method [35].

The phytochemical assay showed that the leaves and stems of *E. nicaeensis* are rich in total phenolic, flavonoids, and flavonols and contain a moderate amount of condensed tannins. Flavonoids, particularly flavonols, are effective antifungal agents against various pathogens, including *Candida albicans, Candida glabrata, Candida krusei, Candida parapsilosis, Candida tropicalis, Trichophyton rubrum and Trichophyton beigelii*.[36–40]. Condensed tannins have also been demonstrated to have potent anthelmintic properties against worm infections. These findings support the traditional usage of *Euphorbia nicaeensis* to treat fungal infections and parasite disorders [41]. By comparing the results of total phenolic content (TPC), total flavonoid content (TFC), condensed tannin content (CTC), flavonol content (FC), and the yield of the two extraction methods, it can be concluded that probe ultrasound-assisted extraction (PUAE) is more efficient than maceration. These might be linked to acoustic cavitation processes, which could cause a forceful impact on the solid surface, resulting in an enhanced extraction rate, as already reported [42]. It is therefore inferred that PUAE could be a convenient technique for phenolic compound extraction. However, the maceration extract of stems proved to have a higher antioxidant effect than leaf maceration extract. These results can be interpreted by the variation of the nature and chemical structure of the phenolic compounds present in each sample. In some cases, the highest antioxidant activity may be observed in extracts with a low phenolic content. Synergy may occur between the major antioxidants (phenolic compounds) and other minor constituents of the plant, so this could significantly impact the differences in their antioxidant activity [43].

This study revealed that *E. nicaeensis* All. leaves possess a potent antioxidant effect close to ascorbic acid activity. A higher free radical scavenging and hydrogen peroxide scavenging effect were observed in probe ultrasound-assisted extraction leaf extracts, compared to stems. The difference between leaves and stems in extraction yield and total phenolic contents could partially explain this. Studies have reported that leaves contain a high amount of phenolic compounds than stems [44]. A potent correlation was observed between phenolic compounds and H_2O_2 scavenging activity, as reported by reports [45,46]. However, the inclusion of other substances, particularly sugars, which, according to [47], can interfere with quantification and antioxidant activity assays, may cause a non-significant correlation between TPC and DPPH.

HPLC-UV-MS/MS analysis showed that the tested extracts are rich in phenolic compounds, especially flavonoids (flavonol glycosides). Quercetin and its derivatives are the

most abandoned compounds in the tested extracts, especially in the leaves, and studies have reported their different biological virtues [48,49]. The presence of isoquercetin and quercitrin hydrate represented by the highest peaks, may be among the leading causes of the significant antioxidant properties of the leaves. Fargesin, myricitrin, and guaiaverin also play a crucial role in plant health and display several therapeutic values due to their antioxidant or/and anti-inflammatory properties [50,51].

From this, we can infer that the phenolic compounds of the plant are the major cause of the scavenging effect of the extracts, supporting the results obtained and explaining why the leaves have a higher antioxidant power than the stems. Therefore, leaves are a potential source of natural antioxidants and bioactive compounds.

The results of previous research on different species and subspecies of the Euphorbiacea family have demonstrated that E. nicaeensis All. has a higher DPPH radical inhibition potential than *E. retusa* Forssk. (IC_{50} leaf = 287.52±2.92 µg/ml, IC50 stem=225.87±3.88 µg/ml [52] and *E. hirta* L. (IC_{50} leaf = 803 µg/ml, IC_{50} stem= 1358 µg/ml) [53], as well as *E. heterophylla* (IC_{50} = 194.28±0.22 µg/ml) [54]. The subspecies *Euphorbia nicaeensis* ssp. *glareosa* showed more than 50% inhibition of DPPH (56.5%), which qualifies the plant as moderately active [34]. Yet no previous study on the in-vitro antioxidant activity of *E. nicaeensis* All. has been published.

The aerial part of *E. nicaeensis* All. is recognized as having anti-inflammatory virtue as it contains glyceroglycolipids tested for their anti-inflammatory activity [55]. This study's results agree with what has been previously published. In vitro tests showed that the plant has a strong anti-inflammatory effect, inhibiting the degradation of bovine serum albumin (BSA) and chicken egg albumin (CEA), observed mainly in the stem extracts. Nuciferine (alkaloid) and fargesin (lignan) are among the main compounds identified in the stem extract according to chromatograms and MS/MS identification; they are phytochemical compounds known for their anti-inflammatory virtues [50,56] which proves the results of the anti-inflammatory activities performed.

The inhibition of thermal degradation of proteins by extract can be explained by several mechanisms, depending on the type of plant extract and the protein studied [57]: Proteins can be protected from thermal degradation by antioxidants because they can scavenge reactive oxygen species (ROS) and reduce oxidative degradation [58]. When phenolic chemicals interact with proteins, their properties, such as solubility, digestibility, and thermal stability, may alter. However, extracts rich in phenolic compounds have been employed to inhibit protein denaturation and improve thermal stability [59]. The antioxidant effect of *E. nicaeensis* extracts resulting from the presence of phenolic compounds, specifically quercetin, and derivatives, play an essential role in the stability of proteins and inhibit denaturation caused by heat treatment, as already reported.

Based on the results of anti-inflammatory activity and those reported by a previous study, we can deduce that the aerial part of *E. nicaeensis*, especially the stems, provides a

potent anti-inflammatory activity and could be considered a potential anti- inflammatory agent.

5. Conclusion

This study aimed to investigate the phytochemical profile and determine the antioxidant and anti-inflammatory activities of leaf and stem extracts of *Euphorbia nicaeensis* All. It has been observed that ultrasound-assisted extraction is an efficient and recommended method for phenolic compound extraction. This work also showed that all extracts are rich in phenolic compounds and exhibited efficient antioxidant and anti-inflammatory properties with significant differences between organs.

HPLC-UV-MS/MS confirmed the presence of phenolic compounds, particularly quercetin and its derivatives, present mainly in the leaf extracts. The leaves showed the highest antioxidant activity, while the most potent anti-inflammatory effect was observed in the stem extracts. The phenolic composition indicated the presence of several compounds with anti-inflammatory properties. These findings confirmed *E. nicaeensis* historical medicinal uses and opened up new ways and possibilities of developing this plant in the pharmaceutical and food fields as a new source of bioactive compounds unaffected by commercial breeding. However, it is evident that additional research, such as the molecular identification of the bioactive substances responsible for these biological activities, is required to offer a more satisfactory and clearer perspective to this study.

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