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Article

Arbutus unedo: Innovative Source of Antioxidant, Anti-Inflammatory and Anti-Tyrosinase Phenolics for Novel Cosmeceuticals

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Abstract: Phenolic compounds are valuable cosmetic ingredients. They display skin protective potential and play an important role in preserving cosmetic formulations due to their ability to neutralize free radicals. Considering this fact, the current study aims to obtain a phenolic-enriched fraction from *Arbutus unedo* for topical application in cosmeceutical products. The chemical composition and the antioxidant, anti-inflammatory, and anti-tyrosinase activities of different extracts from the plant were investigated and compared. Samples were obtained by maceration, reflux, and ultrasound using water and ethanol. The findings indicated that the extraction methods impacted the phytochemical composition of the extracts. The high-performance liquid chromatography with diode-array detection (HPLC–DAD) analysis showed a wide range of phenolic compounds, comprising phenolic acids and flavonoids. Among the extracts, the water reflux had significant levels of both total polyphenols, flavonoids, and tannins and possessed the most important content on hyperoside. It displayed the most significant antioxidant activities with high antiradical and reducing power, as well as strong total antioxidant activity. It possesses a promising whitening effect with high anti-tyrosinase activities. Furthermore, it shows no cytotoxicity and moderate anti-inflammatory activity. Finally, due to its high yield efficiency and activities, water reflux was selected to formulate a cosmeceutical oil-in-water nanoemulsion that displayed optimal pH and stability.

Keywords: *Arbutus unedo*; phytocosmetics; phenolic compounds; antioxidants; anti-inflammatory; anti-tyrosinase; cosmetic formulation; nanoemulsion

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1. Introduction

Skin aging is characterized by a progressive loss of physiological properties and regenerative potential due to intrinsic (genetic factors and hormones) and extrinsic factors caused by the environment [1]. The most harmful of the external factors is UV radiation, known as photoaging, which induces the production of reactive oxygen species (ROS) that can result in oxidative stress and induces aging skin [2], contributing to wrinkle, roughness, dryness, elasticity loss, and pigmentation [3]. Nowadays, cosmetics incorporating natural active compounds are gaining great interest compared to synthetic ingredients [4] due to their capacity to limit the intrinsic aging processes of the skin and to counteract extrinsic processes [5]. Plants' anti-aging effects are mainly attributed to their antioxidant metabolites. Topical antioxidants can protect the skin from free radical damage, and daily use can influence the biological functions of the skin and reverse photo-damage, helping skin reparations [6]. Moreover, plant secondary metabolites can

modulate the activity of enzymes involved in skin aging. Tyrosinase is an enzyme often a target for the cosmetic sector. It is a copper-containing enzyme that catalyzes melanin biosynthesis in human skin. Melanin provides protection against environmental parameters, in particular, UV radiation. However, the hyperpigmentation induced by excessive melanin production may lead to skin disorders such as age spots and melasma and post-inflammatory hyperpigmentation leading to flaw and premature aging appearance [7]. Therefore, targeting tyrosinase activity could be a recommended approach to treating disordered pigmentation problems and developing cosmetic products. [7]. However, synthetic ingredients used in cosmetics and medical preparations are not widely accepted by consumers due to their adverse side effects. Kojic acid is a well-known tyrosinase inhibitor but exhibits cellular toxicity [8]. Therefore, further research is required to identify safe and effective natural tyrosinase inhibitors.

Antioxidants inhibit or quench free radicals and slow the oxidation of oxidizable biomolecules like fats, proteins, and DNA. Antioxidants consist of enzymatic and non-enzymatic forms. Enzymatic antioxidants include the primary enzymes catalase, superoxide dismutase, and glutathione peroxidase. The secondary enzymes consist of glutathione reductase and glucose-6-phosphate dehydrogenase. Non-enzymatic antioxidants are vitamin C, vitamin E, plant phenolic compounds, carotenoids, and glutathione [9].

Phenolics are metabolites with variable phenolic structures and one or more hydroxyl groups and aromatic rings in free or glycosylated form. They are well-known for their precious health advantages and are components of various cosmetic and pharmaceutical applications depending on their bioactivities. Polyphenols have an important role as cardioprotective and neuroprotective, antidiabetic, and anticancer substances. They have antioxidant and anti-inflammatory activities that prevent UV-induced ROS generation in fibroblasts and keratinocytes and the production of pro-inflammatory mediators. [10]. They can restore the proper function of the skin (e.g., DNA repair and viability of human keratinocytes and dermal fibroblasts) after UVA and UVB-caused damages [10–13]. All these precious benefits make phenolics prime candidates for anti-aging therapies as antioxidants. Furthermore, antioxidant testing is a complex topic due to the lack of a standardized test. Two general categories, namely those associated with lipid peroxidation and electron or radical scavenging tests, are widely used for various antioxidant analyses. The former includes the β -carotene bleach test, the lipid peroxidation test with TCA-TBA solution, and the latter includes the ABTS (2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical cation decolorization test, DPPH (1, 1-Diphenyl-2-picryl-hydrazyl) radical scavenging test, ferric reducing antioxidant power test, superoxide anion scavenging activity test, ferrous ion chelation test, etc. The choice of a suitable test is, therefore, crucial to examine the antioxidant activity of biological extracts [14].

Arbutus unedo, also called the strawberry tree, belongs to the Ericaceae family. It grows in the Canary Islands, north-eastern Africa, western Asia, and Europe. This species has been traditionally used as food by using fruits to make marmalades, liquor, jams, and jellies. In traditional medicine, the leaves are used for their astringent, diuretic, urinary anti-septic, antidiarrheal and purgative properties and are useful in the treatment of diabetes, rheumatism and inflammation, and skin diseases [15].

Several pharmacological studies pointed to biological properties such as antitumor, antimicrobial, spasmolytic, antioxidant, and neuroprotective. Phytochemical reports underlined the presence of tannins, flavonoids, phenolic glycosides, irroids, tocopherol, anthocyanins, carotenoids, terpenoids, and fatty acids [16]. It has been shown that genetic variability and environmental factors, as well as extraction techniques, may greatly affect the phytochemical content of *A. unedo* and, thus, its biological capacity [17]. Thus, the selection of extraction method is crucial, depending on the sample matrix and the targeted compound to be recovered. Although *Arbutus* from the European region has been studied, mainly from Portugal, Croatia, and Italy [16–19], there is a scarcity of the phytochemistry and bioactivities of *Arbutus unedo* from Tunisia. Therefore, the objective of this study was to examine the effect of different extraction methods (maceration, reflux, and ultrasound

extraction) on the pharmacological potentials of *Arbutus unedo*, including its antioxidant, anti-inflammatory, and anti-tyrosinase activities. A topical emulsion containing a selected phenolics-enriched extract associated with the highest activities was further developed. Its stability via physicochemical means was evaluated.

2. Materials and Methods

2.1. Plant Material

Leaves of *Arbutus unedo* were collected from Ain draham (North Tunisia). A specimen was deposited at the Center of Biotechnology of Borj Cedria (voucher number AU002020.10). The air-dried leaves were finely ground with an A10 knife mill (Ika-Werk, Staufen, Germany) and stored in the dark.

2.2. Extraction Procedure

Plant material was subjected to different extraction procedures, reflux: 30 g of *Arbutus unedo* leaves powder was extracted using 300 mL of boiling water under reflux for 30 min, static maceration: 10 g of powder was separately extracted by solvents for 12 h at room temperature, ultrasound: ten grams of powdered were deposited in capped glass vials, mixed with 100 mL solvents and immersed into the ultrasonic bath (SONOREX DIGIPLUS, BANDELIN, Berlin, Germany) (180 W, 40 kHz frequency) for 30 min. For maceration and ultrasound extractions, water, ethanol, and ethanol: water (50:50) were used as solvents. After filtering through the Whatman No.1 filter paper, solvents were removed.

2.3. Total Phenolics Evaluation

The number of total polyphenols in *A. unedo* extracts was carried out by spectrophotometric analysis using the Folin-Ciocalteu method [20]. A sample of 125 μ L of the extract was mixed with 500 μ L of distilled water and 125 μ L of Folin–Ciocalteu reagent. The mixture was stirred before the supply of 1.25 mL of 7% Na_2CO_3 , and distilled water was added to reach a final volume of 3 mL, and the mix was thoroughly shaken. The mixture was incubated in the dark for 90 min. The absorbance was measured at 760 nm. The standard used was gallic acid at different concentrations to establish a calibration curve. The polyphenol contents were expressed as micrograms of gallic acid equivalents per gram of dry matter (mg GAE/g DW).

2.4. Total Flavonoids Measurement

The total flavonoid content of extracts was performed according to the protocol described by Bourgou et al. [20]. Each sample (250 mL) was mixed with 75 mL of the solution of NaNO_2 (5%; *w/v*), and then 150 mL $\text{AlCl}_3 \times 6\text{H}_2\text{O}$ (10%; *w/v*) was added. This mixture was supplied with 500 mL of NaOH (1 M), and the final volume was adjusted to 2.5 mL with distilled water. The mixture was then carefully stirred. Absorbance was assessed at 510 nm. Flavonoid contents were expressed as mg catechin equivalent per gram of dry residue (mg CE/g DW) using the calibration curve of (+)-catechin, with concentrations ranging from 0 to 500 μ g/mL.

2.5. Evaluation of Total Condensed Tannins

Total tannin contents were performed according to Sun et al. [21]. H_2SO_4 solution (1.5 mL) was added to a 50 μ L aliquot of extract. The extract solvent was used as a blank, and the absorbance was measured at 500 nm. Total condensed tannins were expressed as mg catechin/g DW. A calibration curve of catechin with a 50 to 400 μ g/mL concentration range was used for this purpose.

2.6. Identification of Phenolic Compounds

The system of high-performance liquid chromatography (consisting of a vacuum degasser, an autosampler, and a binary pump with a maximum pressure of 400 bar; Agilent 1260, Agilent Technologies, Waldronn, Germany) with a 4.6×100 mm reversed-phase C18 analytical column with a particle size of $3.5 \mu\text{m}$ (Zorbax Eclipse XDB C18, Agilent Technologies GmbH, Böblingen Germany) was used to analyze phenolic compounds in *A. unedo* extracts. The diode array detector was configured over a scan range of 200–400 nm. The temperature of the column was 25°C . Two microliters of the sample were injected. The mobile phase was composed of a mixture of methanol (solvent A) and milli-Q water with 0.1% formic acid (solvent B). The identification of phenolic compounds was performed by comparing their retention times with those of the standards. Gallic, caffeic, *p*-coumaric and ellagic acids were used as phenolic acid standards, while the flavonoid standards were arbutin, catechin, epigallocatechin, rutin, and hyperoside. The detected phenolic compounds were determined using the calibration curves of the corresponding standard solutions. The content of each compound was expressed in micrograms per gram of residue ($\mu\text{g/g DR}$).

2.7. Antioxidant Activity

2.7.1. Total Antioxidant Activity (TAA)

The total antioxidant capacity of *A. unedo* extracts was carried out, according to Bourgou et al. [20]. The extract was homogenized with 1 mL of reagent solution (0.6 N sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The mixtures were cooled to room temperature, and the absorbance was read at 695 nm. The activity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW).

2.7.2. ABTS Radical Scavenging Assay

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assays were carried out by mixing 950 μL of the diluted ABTS solution and 50 μL of extract increasing concentrations. After incubation for 6 min, absorbance was measured at 734 nm. ABTS scavenging ability was expressed as IC_{50} value (the extract's concentration resulting in 50% inhibition of absorbance).

2.7.3. DPPH Radical Scavenging Assay

DPPH quenching ability of extracts were evaluated according to Bourgou et al. [20]. Samples were added to a 0.2 mM solution of DPPH. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517 nm. The results were given as half-maximal inhibitory concentration (IC_{50}) and presented in $\mu\text{g/mL}$.

2.7.4. Iron Reducing Power

The sample extract was supplied with sodium phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (10 g/L) [20]. The mixture was then incubated at 50°C for 20 min. Trichloroacetic acid (100 g/L) was then added. After centrifugation, the upper layer was mixed with deionized water and ferric chloride (0.01 g/L). The generated blue-green color was measured at 700 nm.

2.7.5. Chelating Effect

The chelating activity was evaluated according to Zhao and others [22]. Different concentrations of the sample were added to $\text{FeCl}_2 \times 4\text{H}_2\text{O}$ solution (2 mM) and incubated at room temperature for 5 min. Then, the reaction was initiated by adding ferrozine (5 mM), and the absorbance of the solution was then measured spectrophotometrically at 562 nm. Results were expressed as IC_{50} .

2.8. Cell Culture

RAW 264.7 murine macrophage cells (American Type Culture Collection, Manassas, VA, USA) were cultured at 37 °C in a humidified atmosphere of 5% carbon dioxide; the RPMI medium supplemented with 10% of fetal bovine serum (*v/v*), 100 µg/mL of streptomycin and 100 U/mL of penicillin.

2.9. Cell Viability Assay

Cell viability was assessed using a Resazurin assay [23]. RAW 264.7 (2.10^5 cells/mL) cells were cultured in 24-well and incubated for 24 h. RAW 264.7 cells were treated with *A. unedo* extracts at different concentrations. Indeed, the extracts were dissolved in DMSO and then diluted with the culture medium into different concentrations to make the final DMSO concentration at less than 0.1% (*v/v*) in order to avoid solvent toxicity. After 24 h of treatment, the fluorescence was measured using an automated 96-well Fluoroskan Ascent FITM plate reader (Thermo-Labsystems) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.10. Anti-Inflammatory Activity

The anti-inflammatory activity of the studied *A. unedo* extracts was evaluated on the murine macrophage RAW 264.7 cell line by measuring NO generated by the cells using the Griess reagent. Cells were plated in 24-well plates at a 2×10^5 cells/mL and incubated for 24 h. Then the cells were treated with Lipopolysaccharide (LPS) (1 µg/mL) in the absence or presence of various concentrations of samples. After a 24 h LPS stimulation, the cell-free supernatants were collected, and nitric oxide (NO) levels were assessed using Griess's reagent. The absorbance was evaluated at 540 nm, and the final nitrite concentration was determined using a sodium nitrite standard curve (0–50 µM).

2.11. Tyrosinase Inhibition Assay

The mushroom tyrosinase activity of *A. unedo* extracts was measured according to Momtaz et al. [24]. L-DOPA and tyrosine have been used as substrates, while kojic acid and arbutin as standard inhibitors. The reaction mixture contained 70 µL of extract (dissolved in dimethyl sulfoxide (DMSO) and further diluted in potassium phosphate buffer (50 mM, pH 6.5) and 30 µL of tyrosinase (333 units/mL in phosphate buffer, pH 6.5). After 5 min of incubation at room temperature, the substrate was added. Absorbance was read at 492 nm. The IC₅₀ value was determined as the concentration of tyrosinase inhibitor to inhibit 50% of its activity under the assay conditions.

2.12. Development of Formulation

Oil-in-water (O/W) emulsion was elaborated using the formulation given in Table 1 in two phases (aqueous and oily) according to Khairi et al. with modification [25]. The oily phase was heated to 70 °C using a heating plate with constant stirring until a homogeneous mixture obtaining. The aqueous phase was heated at the same temperature in a beaker until complete melting. Subsequently, the water phase, and the oil phase were placed in a 100 mL glass vessel and homogenized using an Ultraturrax homogenizer at 15.000 rpm. The freshly prepared cosmetic emulsion was then subjected to the following studies.

Table 1. Composition of the O/W formulation containing *A. unedo* phenolic extract.

Components	% (w/w)
Aqueous phase	
Glycerin	1
<i>A. unedo</i> extract	1
Xanthan gum	0.1
Phenoxyethanol	0.1
Distilled water	75.3

Oily phase	
Almond oil	15
Glyceryl monostearate	7.5

2.13. Encapsulation Efficiency Measurement

The percentage of phenolic compounds held within the emulsion was assessed following the Regan and Mulvihill method [26]. About 3 g of the emulsion was mixed with 3 g of phosphate buffer solution (pH 7) and centrifuged at 4500 rpm for 30 min. Then, the lower phase was collected. The percentage of encapsulated compounds (E) was identified by using Equation:

$$E (\%) = (1 - C2/C1) \times 100$$

where $C2$ is the concentration of phenolic compounds found in the aqueous phase after centrifugation, and $C1$ is the initial concentration of phenolic compounds in the inner aqueous phase [26,27].

2.14. Preliminary Stability Tests

2.14.1. Centrifugation Test

Analytical centrifugation was performed (3000 rpm) for 30 min at room temperature. The appearance and homogeneity characteristics were assessed by macroscopic analyses.

2.14.2. Thermal Stress

Consecutive storage was done for eight days at 4 °C and 40 °C in a drying and heating oven [28].

2.14.3. pH Analysis

The determination of pH was measured by a pH meter at 22 ± 2 °C.

2.14.4. Particle Size and Zeta Potential Evaluation

Measurement of the mean droplet diameter and zeta potential was performed by Zetasizer® (Zetasizer Nano-ZS/Malvern Instruments, Worcester, UK). Laser Diffraction Particle Size Analyzer (LS13320, Beckman Coulter, Inc., Brea, CA, USA) after formulations dilution with distilled water 200-fold.

2.15. Statistical Analysis

An analysis of variance (ANOVA) was performed to compare the effect of methods of extraction on the measured traits, and Duncan's test means comparison was used to determine significant differences among means of measured traits.

3. Results and Discussion

3.1. Yield of *Arbutus unedo* Leaves Extracts

Many researchers are currently focusing on replacing harmful solvents with environmentally friendly substitutes. The use of green and sustainable solvents such as ethanol and water, combined with the use of low environmental impact technologies, is a promising holistic approach to the development of "green" extraction processes. Ethanol is recognized as a green solvent and offers greater industrial safety. On the other hand, water has a low environmental impact and is inexpensive in terms of production, transport, and disposal. On the other hand, the possibility of modifying the physico-chemical properties of water by varying the conditions (temperature, pressure, etc.) has increased the interest in its use as an extraction solvent. In this study, ethanol and water were used in order to evaluate and optimize the extracting ability of phenolic compounds from the leaves of *Arbutus unedo*, as well as their influence on the biological activities of this specie, by

varying the extraction process. The extraction of phenolic compounds by “green” non-toxic solvents was carried out by two conventional (or classic) methods, maceration and reflux extraction, and an unconventional (or innovative) namely extraction by sonication (Table 2).

Table 2. Mean values of Yield, total phenolic, tannin, and flavonoid contents of *A. unedo* leaves extracts from different extraction methods (mean \pm standard deviation, $n = 3$).

	Yield (%)	Total Phenolics (mg GAE/g DW)	Total Flavonoids (mg CE/g DW)	Total Tannins (mg CE/g DW)
Reflux water	38	73 \pm 0.7 ^a	51 \pm 1.9 ^a	54 \pm 3.8 ^a
Maceration water	28	79 \pm 3.6 ^a	22 \pm 0.9 ^c	29 \pm 2.6 ^c
Maceration ethanol	5	32 \pm 1.0 ^d	11 \pm 0.4 ^e	17 \pm 1.3 ^d
Maceration ethanol 50%	35	67 \pm 2.7 ^b	18 \pm 1.6 ^d	34 \pm 1.1 ^{bc}
Ultrasound water	29	48 \pm 1.5 ^c	19 \pm 0.3 ^{cd}	30 \pm 1.4 ^c
Ultrasound ethanol	20	76 \pm 2.1 ^a	21 \pm 0.4 ^{cd}	38 \pm 2.6 ^b
Ultrasound ethanol 50%	30	74 \pm 1.2 ^a	27 \pm 1.3 ^b	32 \pm 1.0 ^{bc}

For each column, different letters indicate significant differences between extracts using Duncan’s test at a 0.05 probability level.

Yield results are significantly different and vary markedly depending on the solvent and extraction technique (Table 2). Generally, *Arbutus unedo* seems rich in polar compounds. In fact, the best performance was observed using the reflux extraction technique and water as solvent (38%), followed by extraction with 50% ethanol by maceration and sonication with yields of 35% and 30%, respectively. Cold water extraction and sonication have also shown high yields (28% and 29%). Absolute ethanol, especially by maceration, proved to be the least efficient with the lowest yields (5%). The variation in yields agrees with data from the literature, which indicates that the yield depends on the extraction solvent’s nature and polarity [29]. Our results suggest that the yield increases with increasing solvent polarity.

Regardless of the extraction method, we observed that the yields using 50% ethanol were much higher than those using pure ethanol, which indicated that the extracting power of ethanol is improved by adding water following the increase in its polarity. Moreover, our results are consistent with several works in the literature indicating the efficiency of water in extracting soluble compounds from *Arbutus unedo* leaves. In effect, Oliveira et al. [30] reported a significant yield difference as a function of the polarity of the solvent used, varying from 2.8% using petroleum ether to 32.4% using boiling water. These authors showed an average yield of 15% using ethanol. Similarly, Malheiro et al. [18] worked on 19 *Arbutus* ecotypes Portuguese and noticed hot water extraction yields ranging from 27% to 61%. However, Orak et al. [31] noticed that ethanol and water showed yields close to 40% and 39%.

3.2. Effect of Solvent and Extraction Method on Total Phenolics, Flavonoids, and Tannins Contents

The choice of solvent is crucial in the extraction process as it determines the selectivity and then impacts the chemical composition and functional properties of the final extract. This choice usually depends on the solubility of the target compound. Since solubilization involves electrostatic repulsions and attractions between the solvent and the solute, a polar solvent would be the best for the solubilization and extraction of polar molecules, while a less polar solvent would be suitable for less polar compounds.

3.2.1. Total Phenolic Contents

The impact of solvents and extraction methods on polyphenol content has been extensively studied. Despite the fact that aqueous alcoholic solvents are generally considered the most suitable for phenolic extraction, no universal extraction method is adequate for every phenolic [31]. Table 2 provides a summary of the total contents of phenolics, flavonoids, and tannins in *A. unedo* leaves. According to these results, the maximum total phenolic content was noted in water extract obtained by maceration (79 mg GAE/g DW) as well as ethanol, ethanol 50% ultrasound extracts, and reflux water (76, 74 and 73 mg GAE/g DW, respectively) and the lowest in ethanol extract by maceration (32 mg GAE/g DW). In this case, Oliveira et al. [30] reported a higher amount of total polyphenol in Portuguese *A. unedo* leaves (172.21 and 192.66 mg EAG/g DW, respectively, for aqueous and ethanolic extracts). Tenuta et al. [16] examined four methods for bioactive component extraction from fresh and dried leaves of *A. unedo* and observed a significant impact of solvents and extraction techniques on the content of phytochemicals. Indeed, ethanol 60% by maceration was better than decoction to extract polyphenols from dried leaves with a content of 329 mg/g. These differences observed in plant extracts are probably due to geographical factors, extraction method, and maturity level of the plant at the time of harvest effects [31]. Besides, the contents of total polyphenols are known to be considerably influenced by various extrinsic and intrinsic factors, particularly environmental factors.

Furthermore, we noticed in our study that the extraction technique greatly influenced the phenolic contents of *A. unedo* leaves. In fact, ultrasonic extraction using ethanol 50% seems to enhance the phenolic contents extraction compared to extraction by maceration using this solvent (Table 2), while water use shows a negative effect in ultrasonic extraction. It has been reported that, for some plant materials, excessive extraction time in the water would lead to the degradation of some target compounds, resulting in reduced concentrations. [32].

3.2.2. Total Flavonoids

The results in Table 2 show variability in the flavonoid content of *A. unedo* leaves by varying the extraction process and type of solvent. Reflux extraction was the most efficient method to extract flavonoids from *A. unedo* leaves, displaying the highest total concentration of 51 mg CE/g DW (Table 2).

Considering the extraction solvent, we notice that compared to ethanol, water is much more effective in extracting flavonoids with high levels ranging from 19 to 51 mg CE/g DW where reflux extraction showed the highest content. This indicates that heating improves the extractive power of water. The beneficial effect of increasing the temperature would be linked to an increase in the transfer of matter, inducing an increase in the diffusion of molecules [32]. The ethanolic extracts showed lower but interesting flavonoid contents. Indeed, pure ethanol used in ultrasonic extraction has a content of 27 mg CE/g DW followed by 50% ethanol using the same process (21 mg CE/g DW). While the lowest content was found with the maceration extract with 100% ethanol (11 mg EC/g DW). The richness of the aqueous extract of *A. unedo* on flavonoids compared to ethanol extracts is confirmed by the work of Jurica et al. [33], who showed that the leaves' aqueous extract has lower levels of total polyphenols than the methanolic extract but was richer in flavonoids. Moreover, compared to data from the literature, the Tunisian *Arbutus* was richer in flavonoid content than *Arbutus* from Algeria and Portugal [34,35]. Flavonoids have the ability to act as antioxidants in biological systems.

3.2.3. Condensed Tannins

Tannins can be widely found in plants and have several health benefits. As shown in Table 2, *Arbutus unedo* leaves were rich in condensed tannins. Indeed, regardless of the solvent involved and the extraction process adopted, water reflux had the highest content (54 mg CE/g DW). Thus, extraction using hot water leads to obtaining an *Arbutus* extract

rich in tannins. As for polyphenols and total flavonoids, ultrasound improves tannin extraction using ethanol as a solvent. Indeed, the ultrasound extraction with pure ethanol and 50% ethanol were richer in tannins (38 and 32 mg CE/g DW, respectively) compared to pure ethanol by maceration (17 mg CE/g DW).

The various results suggest that using water as a solvent at a high temperature ensures the extraction of the maximum level of tannins.

3.3. Phytochemicals Identification

Although the total contents of bioactive molecules are valuable data regarding the phytochemical profile of a plant, they do not allow the separate quantification of the main bioactive compounds. For this reason, a detailed assessment of the phenolic compounds in the seven different extracts was performed by HPLC-DAD analysis, and the results are displayed in Table 3. The main compounds in the extracts of *Arbutus unedo* leaves were flavonoids and phenolic acids. The obtained data highlighted the richness of extracts on hyperoside (quercetin 3-*O*-galactoside) with amounts ranging from 30 to 56 mg/g DR. The highest content was observed in reflux water (56 mg/g DR) and ultrasound ethanol (55 mg/g DR). As previously reported in the literature, hyperoside was the most predominant phenolic compound in Croatian *Arbutus unedo* leaves [19].

Table 3. Identification and quantification (mg/g DR) of the main polyphenols present in *A. unedo* extracts (mean \pm standard deviation, $n = 3$).

	Phenolic Acids					Flavonoids			
	Gallic Acid	Caffeic Acid	<i>p</i> -Coumaric Acid	Ellagic Acid	Arbutin	Catechin	Epigallocatechin	Rutin	Hyperoside
Reflux water	0.42 \pm 0.1 ^b	0.07 \pm 0.00 ^e	0.39 \pm 0.00 ^d	1.27 \pm 0.05 ^{bc}	1.2 \pm 0.2 ^{bc}	ND	0.96 \pm 0.2 ^a	ND	56.06 \pm 1.8 ^a
Maceration water	0.18 \pm 0.0 ^c	0.05 \pm 0.00 ^e	0.27 \pm 0.04 ^d	0.83 \pm 0.1 ^d	2.78 \pm 1.2 ^a	0.58 \pm 0.1 ^b	0.19 \pm 0.04 ^b	1.55 \pm 0.4 ^c	31.32 \pm 3.1 ^b
Maceration ethanol	0.09 \pm 0.0 ^c	0.27 \pm 0.1 ^d	0.59 \pm 0.01 ^c	1.26 \pm 0.2 ^{bc}	2.26 \pm 0.7 ^a	ND	0.77 \pm 0.1 ^a	2.07 \pm 0.3 ^b	34.24 \pm 6.1 ^b
Maceration ethanol 50%	0.08 \pm 0.0 ^c	0.31 \pm 0.08 ^{bc}	0.28 \pm 0.03 ^d	1.06 \pm 0.01 ^{cd}	1.87 \pm 0.4 ^{ab}	1.22 \pm 0.6 ^a	0.34 \pm 0.1 ^b	2.14 \pm 0.01 ^{ab}	33.97 \pm 4.5 ^b
Ultrasound water	1.83 \pm 0.6 ^c	0.38 \pm 0.02 ^b	1.45 \pm 0.9 ^a	1.51 \pm 0.45	0.73 \pm 0.02 ^c	0.58 \pm 0.03 ^b	0.18 \pm 0.07 ^b	ND	30.03 \pm 5.5 ^b
Ultrasound ethanol	0.18 \pm 0.05 ^c	0.28 \pm 0.1 ^d	0.96 \pm 0.2 ^b	1.50 \pm 0.1 ^{ab}	0.85 \pm 0.1 ^c	ND	0.16 \pm 0.04 ^b	2.66 \pm 0.7 ^a	55.05 \pm 2.1 ^a
Ultrasound ethanol 50%	0.12 \pm 0.05 ^c	0.59 \pm 0.2 ^a	0.02 \pm 0.01 ^e	1.36 \pm 0.1 ^{ab}	2.16 \pm 0.1 ^{ab}	1.22 \pm 0.6 ^a	0.34 \pm 0.1 ^b	2.56 \pm 0.2 ^{ab}	41.88 \pm 6.6 ^b

ND: not detected. For each column, different letters indicate significant differences between extracts using Duncan's test at 0.05 probability level.

Moreover, samples contained interesting amounts of arbutin. The highest quantity was obtained in water maceration extract (3 mg/g DR) followed by ethanol maceration and ethanol 50% ultrasound by about 2 mg/g DR. This hydroquinone- β -D-glucopyranoside is common in the leaves of several plant species, particularly in the Ericaceae. [36]. Jurica et al. [33] compared the effectiveness of different solvents (methanol, methanol 50%, ethyl acetate, and dichloromethane) and techniques for arbutin extraction from *A. unedo* leaves and reported that ultrasound-assisted extraction with methanol was the most suitable extraction procedure for the recovery for arbutin. The arbutin content in *A. unedo* leaves has been reported to vary according to the origin due to the effect of climate and soil characteristics, ranging from 0.6 mg/g to 12.4 mg/g [36,37].

Other flavonoids detected in our study were rutin, catechin, and epigallocatechin (Table 3). Among the phenolic acids, ellagic acid was the most abundant; the highest quantities were recovered in water and ethanol ultrasound extracts (1.5 mg/g DR). Our results agree with several studies which have established that the main phenolic compounds

present in *A. unedo* leaves are catechin, epicatechin, catechin gallate, quercetin, gallic acid, ellagic acid and *p*-hydroxybenzoic acid [34,38–40].

3.4. Effect of Solvent and Extraction Method on Antioxidant Activities

To understand the mechanism of action of the antioxidants present in the extracts of *Arbutus unedo* leaves, four different methods were used, and the results are depicted in Table 4.

Table 4. Mean values of Antioxidant activity of *A. unedo* leaves extracts from different extraction methods.

	TAA (mg GAE/g DW)	DPPH IC ₅₀ (µg/mL)	ABTS IC ₅₀ (µg/mL)	Reducing Power EC ₅₀ (µg/mL)	Chelating Power EC ₅₀ (mg/mL)
Reflux water	194 ± 5.2 ^a	7 ± 1.1 ^d	58 ± 3.2 ^e	82 ± 5.2 ^c	40 ± 3.1 ^a
Maceration water	86 ± 3.2 ^b	17 ± 1.5 ^a	66 ± 5.5 ^d	112 ± 7.4 ^a	33 ± 4.2 ^b
Maceration ethanol	32 ± 2.1 ^e	9 ± 1.0 ^c	114 ± 7.2 ^a	83 ± 4.4 ^c	NA
Maceration ethanol 50%	52 ± 2.1 ^d	10 ± 1.1 ^b	68 ± 4.1 ^d	64 ± 3.2 ^d	NA
Ultrasound water	63 ± 3.2 ^c	9 ± 1.0 ^c	101 ± 4.3 ^b	97 ± 4.3 ^b	34 ± 4.4 ^b
Ultrasound ethanol	65 ± 4.2 ^c	9 ± 0.7 ^c	102 ± 5.1 ^b	60 ± 3.7 ^d	NA
Ultrasound ethanol 50%	59 ± 2.2 ^{cd}	6 ± 1.1 ^d	94 ± 4.4 ^c	62 ± 3.2 ^d	NA

NA: not active. For each column, different letters indicate significant differences between extracts at 0.05 probability level using Duncan's test).

The results showed that the nature of the solvent and the extraction technique influence the total antioxidant activity of *A. unedo* (Table 4). Water was found to be the most powerful for extracting antioxidant compounds. In particular, water reflux extraction exhibited a very high value of 194 mg GAE/g DW. Water cold and ultrasound extractions also showed interesting activities (86 mg GAE/g DW and 63 mg GAE/g DW, respectively), while ethanol and ethanol 50% maceration presented the lowest activity, around 32 and 52 mg EAG/g DW. We noted that the ultrasound technique improves the activity in ethanol extracts (TAA = 65 and 59 mg GAE/g DW in ethanol and ethanolic 50%, respectively).

Moreover, the results showed that the significant total antioxidant activity of water reflux extract was positively correlated to its richness in flavonoids and tannins. Indeed, this extract showed the highest levels of these compounds, which suggests that flavonoids and total tannins participated actively in the antioxidant activity of *Arbutus unedo* extracts.

The scavenging activity measured by the DPPH assay showed that the different extracts from *A. unedo* exhibited high capacity with low IC₅₀ values that do not exceed 17 µg/mL (Table 4). The ethanolic extract (50%) obtained by sonication and water reflux extract showed the highest trapping power of the DPPH (6 and 7 µg/mL, respectively). It should be noted that apart from the cold aqueous extract, the antiradical capacities obtained are higher than the synthetic antioxidant standard BHT (IC₅₀ value is equal to 11.5 µg/mL).

Besides, the results relating to the antiradical activity against the ABTS radical are in line with those of antiradical activity against the DPPH radical (Table 4) and indicate that water reflux extract exhibits the best activity displaying a low IC₅₀ (58 µg/mL). However, this capacity becomes lower when the solvent used is ethanol. Ethanol 50% (IC₅₀ = 68 µg/mL), ethanolic extracts presented moderate antiradical activity since the IC₅₀ varies from 94 to 114 µg/mL. Our results agree with other reports, which underlined a significant antiradical potential of *Arbutus* extracts. Moderate scavenging activity has been reported for the Portuguese and Turkish *A. unedo*, with IC₅₀ values ranging from 73 to 487.2 µg/mL [30,31,41]. Indeed, the high antiradical potential of the various extracts, especially that of the water extract by reflux, could be due to their richness in flavonoids, in particular, hydroperoxide present with levels varying from 28 to 37 mg/g DW (Table 3). The antioxidant activity of hydroperoxide has been described as being related to the hydroxyl groups of the

A and B rings and the glycosides bound to the C ring [42]. Liu et al. [43] found that hyperoxide could effectively protect PC12 cells from ROS-induced cytotoxicity, including hydrogen peroxide and tert-butyl hydroperoxide, without being damaging. Flavonoids are known to be powerful scavenger agents and effective hydrogen donors, acting as primary antioxidants and stabilizing radicals. In addition, *A. unedo* leaf extracts are rich in *p*-coumaric acid. This last one is one of the most active free radical scavenging hydroxycinnamic acids [44]. Finally, the antiradical activity of the *Arbutus* could also be due to the presence of arbutin. Indeed, this flavonoid has been reported as a powerful radical scavenger [45].

According to the results illustrated in Table 4, we noticed that the reducing powers of *A. unedo* leaf extracts were significantly different and varied markedly depending on the solvent and extraction technique. Hence, the ethanolic extracts, as well as that of the water reflux method, presented the lowest values of the EC₅₀ (varying from 60 and 83 µg/mL), therefore, the strongest reducing activities of the iron. Indeed, we noted that the pure ethanol extract of the ultrasound is distinguished by the best-reducing power of iron (EC₅₀ = 60 µg/mL). As for the previous tests, the water reflux extract showed a very high reducing power with an EC₅₀ of 83 µg/mL. On the contrary, lower activity was observed in the aqueous extract obtained by maceration, of which the EC₅₀ value is equal to 112 µg/mL. Our results reveal that the Tunisian *Arbutus* exhibits an important reducing power. The lower reducing activity was noted by Malheiro et al. [18] in aqueous extract leaves of 19 Portuguese *A. unedo* genotypes. These authors explained that the antioxidant power of the *Arbutus* would be due to its richness in reducing phenolic compounds.

Concerning the chelating power of iron which was measured by the inhibition of the formation of ferrozine-Fe²⁺ complex, our study revealed a low chelating capacity of ferrous iron; the aqueous extracts showed moderate activity displaying EC₅₀ values ranging from 33 to 40 mg/mL (Table 4).

Our results revealed a strong antioxidant potential of the Tunisian *A. unedo*. In particular, the aqueous extraction under reflux was distinguished by a very high power to trap free radicals as well as a high total and reduced antioxidant activities.

3.5. Effect of Solvent and Extraction Method on Anti-Tyrosinase Activity

A. unedo leaves extracts were analyzed for tyrosinase inhibition activity. Tyrosinase is a copper enzyme that plays a main role by catalyzing the first two steps in melanogenesis. Firstly, it converts L-tyrosine into L-DOPA by hydroxylation, and secondly, this compound is converted into o-dopaquinone by oxidation which polymerizes spontaneously to form melanin, which is the key molecule for skin color [46]. Melanin overproduction can produce hyperpigmentation disorders, such as lentigo, melasma, and hyperpigmentation. As a consequence, tyrosinase inhibitors are promising potential skin-whitening agents [5]. There are well-known tyrosinase inhibitors such as hydroquinone; nevertheless, their adverse effects are a serious concern, which leads to the search for natural compounds that have tyrosinase inhibitory effects [47].

The inhibition of tyrosinase by *A. unedo* extracts was assessed on its two catalysis functions, i.e., monophenolase (the inhibition of L-tyrosine hydroxylation to L-DOPA) and diphenolase (L-DOPA oxidation to dopaquinone) activities. Interestingly and considering monophenolase inhibition, all the extracts were able to inhibit tyrosinase activity efficiently, although to a different extent (Table 5). Ethanol and ethanol 50% maceration extracts exhibited the strongest activities (IC₅₀ = 90 µg/mL), followed by ethanol 50% ultrasound (IC₅₀ = 150 µg/mL) and decoction (IC₅₀ = 200 µg/mL), whereas ultrasound ethanol was the less potent inhibitor for monophenolase activity.

Table 5. Mean values of Anti-tyrosinase activities of *A. unedo* extracts.

	Monophenolase Inhibition (IC ₅₀ in µg/mL)	Diphenolase Inhibition (IC ₅₀ in µg/mL)
Reflux water	200 ± 2.5 ^d	2500 ± 9.5 ^a
Maceration water	340 ± 5 ^b	1600 ± 10.0 ^c
Maceration ethanol	90 ± 2.0 ^f	450 ± 5.0 ^f
Maceration ethanol 50%	90 ± 1.0 ^f	500 ± 5.0 ^e
Ultrasound water	290 ± 6.5 ^c	2000 ± 8.0 ^b
Ultrasound ethanol	390 ± 4.5 ^a	900 ± 7.5 ^d
Ultrasound ethanol 50%	150 ± 2.5 ^e	400 ± 5.0 ^f
Arbutin	100 ± 3.5 ^f	NA
Kojic acid	4.7 ± 0.1 ^g	0.018 ± 0.00 ^g

NA: not active. In each column, the same letters mean non-significant differences using Duncan's test at 0.05 probability level.

Some extracts displayed moderate diphenolase activity. Ethanol 50% obtained by ultrasound and maceration as well as ethanol maceration showed the highest activities with IC₅₀ values of 400, 450, and 500 µg/mL, respectively. The other extracts were rather ineffective (IC₅₀ > 1 mg/mL). These results confirm previous observations on moderate diphenolase *A. unedo* activity. Recently, Deniz et al. [48], have investigated the enzyme inhibitory activity of 92 herbal ethanol extracts and showed that *A. unedo* ethanol 80% leaves extract by maceration showed 32% diphenolase inhibition at 666 µg/mL.

These data pointed out the high potency of *A. unedo* monophenolase activity and revealed strong lightening capacity. This remarkable activity is likely to result from high arbutin content in the different extracts (Table 3), which act as the major tyrosinase-modulating compound. In our study, this phenolic exhibited high monophenolase activity (IC₅₀ = 100 µg/mL, Table 5) while it was inactive in diphenolase activity. Recently, the effect of α-arbutin on the monophenolase and diphenolase activities of tyrosinase was analyzed and reported that this compound inhibits monophenolase activity and activates diphenolase activity [49]. In addition, it has been shown that arbutin is also safe and can potentially prevent melanin formation without cytotoxicity [50]. Data on the antioxidant capacity of arbutin are emerging, and these antioxidant properties are proposed to contribute to the skin-lightening effect of the molecule.

There are several other compounds present in extracts from *A. unedo* plants with tyrosinase-modulating properties. Accordingly, Huang et al. [51] explained that ellagic acid inhibited tyrosinase activity in a reversible manner and was a mixed tyrosinase inhibitor. Moreover, catechins and *p*-coumaric acid were reported to inhibit tyrosinase by acting as alternative substrates [52].

3.6. Effect of Solvent and Extraction Method on Cytotoxic and Anti-Inflammatory Activities

Due to the significant side effect profiles of drugs, the use of natural compounds for preventing or reducing inflammation has recently received a lot of attention. The cytotoxicity of *A. unedo* extracts was studied using the resazurin assay. RAW 264.7 cells were treated with different concentrations of extracts (25–300 µg/mL), as shown in Figure 1. Independently of technique, both water and ethanol of *A. unedo* extracts exhibited no cytotoxic effect for the tested concentrations up to 300 µg/mL since cell viability exceeded 80%. Interestingly, *A. unedo* extracts, especially water reflux and ethanol extract by maceration at 50 µg/mL, caused an increase in cell viability by 11% compared to the control. Based on this, concentrations ranging from 25 to 150 µg/mL were selected for further studies.

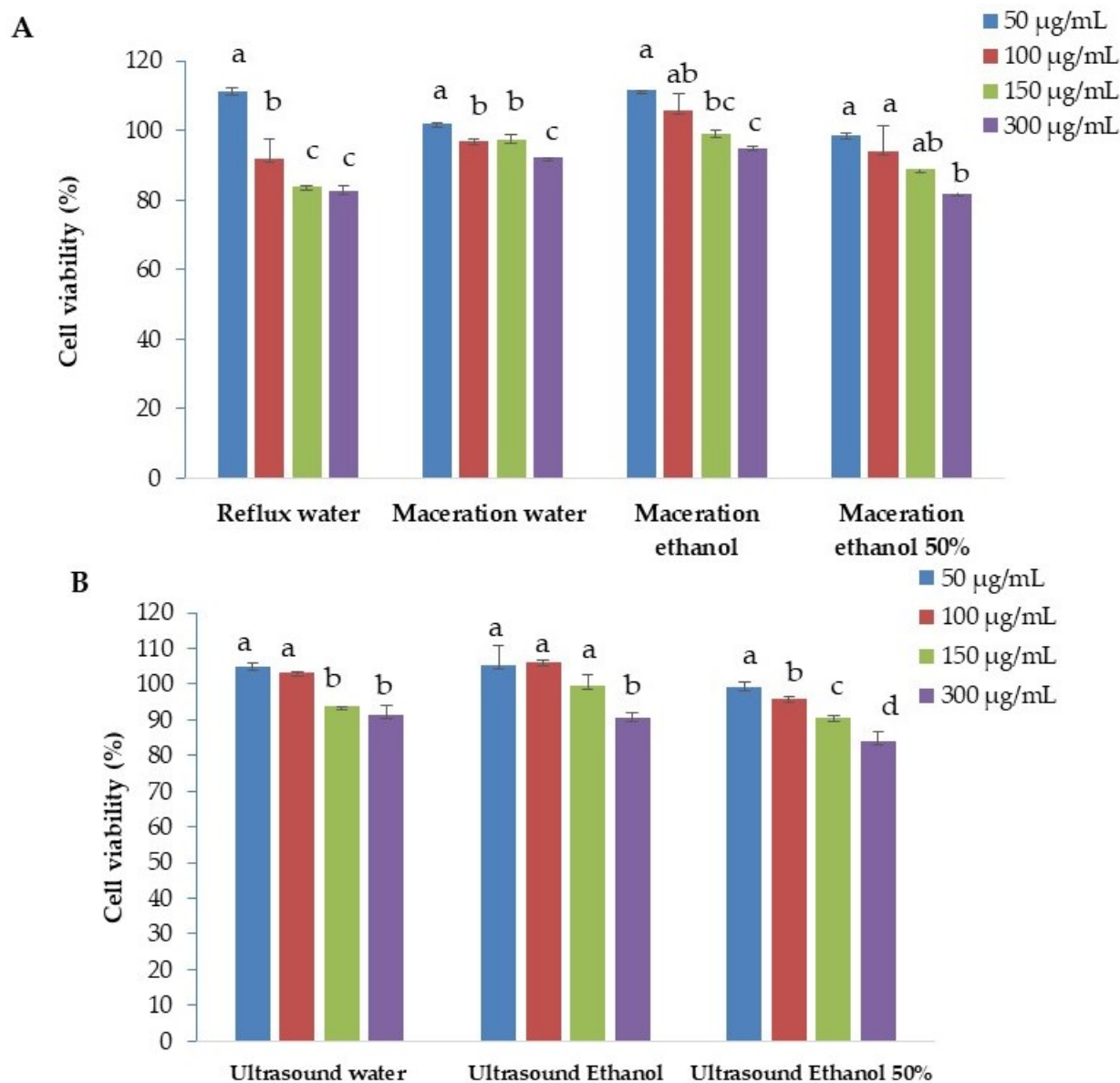


Figure 1. Cell viability (%) evaluation using resazurin assay for *Arbutus unedo* extracts obtained by reflux and maceration (A) and ultrasound (B) extraction on RAW 264.7 cells. Cells were incubated, for 24 h, with 50–300 µg/mL extract. The percent cell viability was determined by comparison to the untreated control. Data were displayed as mean ± SD calculated from triplicate results. For each extraction method, different letters between extract concentrations mean significant differences using Duncan's test at a 0.05 probability level.

Nitric oxide (NO) is a strong mediator in numerous cellular processes, such as the regulation of neurotransmission, vasodilatation, inhibition of platelet adherence, host defense mechanisms, and inflammation [53]. LPS can activate macrophage cells to initiate proinflammatory mediators, including TNF- α , IL-6, and NO. Therefore, the utilization of NO inhibitors constitutes a substantial therapeutic advance in the treatment of inflammatory diseases. Noncytotoxic *A. unedo* extracts concentrations were examined to explore their potential to inhibit NO production in LPS-treated RAW 264.7 macrophages. Extracts showed anti-inflammatory activity in a concentration-dependent manner (Figure 2). The most active extracts were ethanol maceration and water extract obtained by ultrasound with percentage NO production inhibition of 37 and 35%, respectively, at a high dose of 150 µg/mL. Some studies highlighted the potential activity of *A. unedo* extracts as anti-inflammatory candidates. Tenuta et al. [16] showed that *A. unedo* ethanolic and

hydroalcoholic macerations were able to reduce nitrite production in HFF1 cells stimulated with interleukin-2 β .

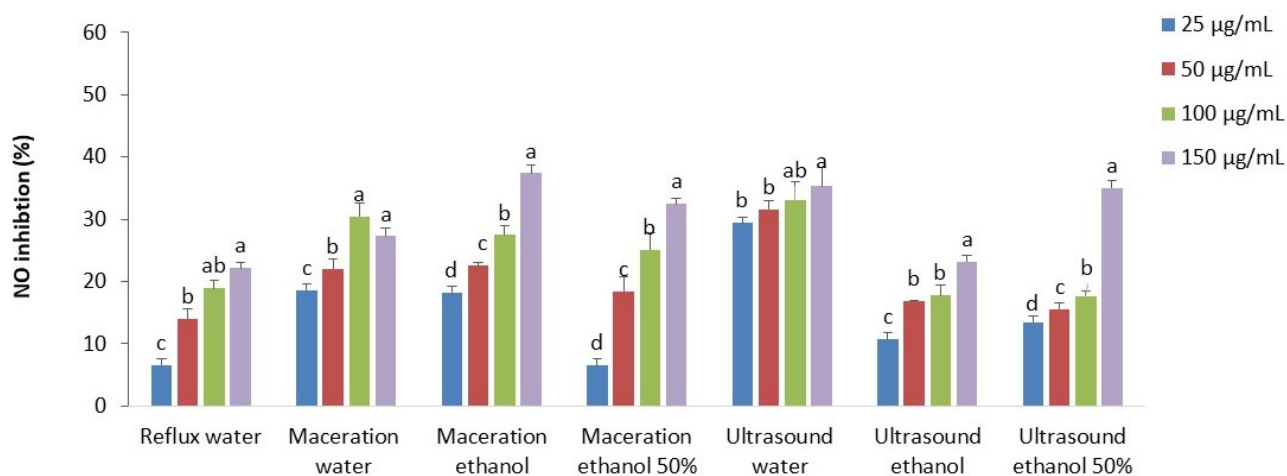


Figure 2. Anti-inflammatory activity of *A. unedo* extracts from different extraction methods (mean \pm SD, $n = 3$ replicates). Cells were incubated with 25–150 $\mu\text{g/mL}$ extract and Lipopolysaccharide (LPS). After a 24 h LPS stimulation, the cell-free supernatants were collected and assayed for nitric oxide (NO) levels. For the same extraction method, different letters (a, b, c, d) mean significant differences between extract concentrations using the Duncan test at the probability level of $p < 0.05$.

Mariotto et al. [54] reported that treatment with an aqueous extract of *A. unedo* decreased acute lung inflammation in an animal model. Among *A. unedo* phenolics, arbutin suppressed LPS-induced production of NO and expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS stimulated BV2 microglial cells in a dose-dependent manner and without causing cellular toxicity [55].

3.7. Characterization of Emulsion Containing *A. unedo* Extract

After evaluating the phytochemical composition and the activities of the seven *A. unedo* extracts, it was found that the reflux water has the best performance with satisfactory compositional characteristics that allowed its use in the elaboration of a cosmetic emulsion based on a high phenolic compounds content and elevated antioxidant and anti-tyrosinase activities. Thus, a formulation that contained 1% of the selected *A. unedo* extract was developed.

Initially, the formulation loading phenolic-enriched *A. unedo* extract was light yellow and homogeneous, while the base formulation was white homogeneous. This color difference is caused by the color of the extract influencing the color of the end product.

On the other hand, total polyphenols retention evaluation showed a satisfactory level of phenolics retention (60.32%) in the nanoemulsion.

Stability tests are crucial because of their predictive nature. The centrifugation test was carried out to obtain information on possible instability processes. Formulations were centrifuged 24 h after preparation at 3000 rpm for 30 min. The emulsion loading phenolic-enriched *A. unedo* extract showed no change in its initial characteristics and stability. The emulsion was described as stable, as no cremation, flocculation, or phase separation was observed during the available time. Similar behavior was observed in the thermal stress test in which the cream did not show changes during the whole process.

Regarding pH analysis, the emulsion loading phenolic-enriched *A. unedo* extract initially presented a pH value of about 6.9 (Table 6). The value for the emulsion subjected to thermal stress for eight days was 6.5. Both values are compatible with skin [56]. Therefore, these emulsions were considered suitable for use.

Table 6. Mean droplet diameter (Z-average), zeta potential (ZP), and pH values of preliminary stability tests of nanoemulsions containing phenolic-enriched *A. unedo* extract (A-NE) and blank nanoemulsion (NE).

	Z-Average (d.nm)		Zeta Potential (mV)		pH	
	T0	T8	T0	T8	T0	T8
A-NE	197 ± 1.05 ^b	243 ± 0.8 ^b	−56 ± 2 ^a	−64 ± 1.0 ^a	6.9 ^b	6.5 ^b
NE	216 ± 0.8 ^a	286 ± 0.5 ^a	−59 ± 1.2 ^a	−70 ± 1.8 ^a	7.6 ^a	7.9 ^a

Mean followed by the same letter at each column are not significantly different using Duncan test at the $p < 0.05$ probability level. Each value represents the mean of three replicates.

Zeta potential and particle size measurement are commonly employed methods to assess the stability of emulsions. Initially, the emulsion possesses 197 nm. After thermal stress for eight days, the sample showed a slight increase in the mean droplet size to 243 nm, as shown in Table 6. However, the radius of the droplets was still within the range of 20–500 nm, which corresponds to the size of a nanoemulsion [57]. The stability of the emulsion is strongly related to the droplet size distribution. Generally, the smaller the droplet size, the more stable the W/O nanoemulsions and the longer their shelf life. The small droplet size of nanoemulsions stabilizes them against gravitational separation and flocculation. Large droplet size can promote Ostwald ripening, which increases droplet size leading to coalescence and creaming [58]. The zeta potential value obtained for the nanoemulsion on day zero was -56 ± 2 mV. Alternatively, the value presented for the nanoemulsion subjected to thermal stress for eight days was -64 ± 1 mV (Table 6). Both values reflect the stability of the emulsion even when subjected to changes in temperature. The zeta potential shows the force of repulsion between adjacent equally charged droplets and consequently proves the important role of the droplets' surface layer in the stabilization process. High values of zeta potential ($> |30|$ mV) indicate resistance to particle aggregation and, therefore, greater stability [59].

4. Conclusions

This study investigated the skin anti-aging activity of *A. unedo* from Tunisia to assess the opportunity of exploiting this plant species as a source of bioactive compounds with numerous applications. Various extraction techniques were compared for the recovery of phenolic compounds from *A. unedo*. Among the different extracts, the reflux water extract was rich in total polyphenols, recovered the highest amounts of flavonoids and tannins, and showed the highest antioxidant activity. Additionally, it showed anti-inflammatory and anti-tyrosinase activities with no cytotoxic effect. Furthermore, the reflux water extract gave the highest yield (38%), which would be advantageous for the cosmetic industry due to its cost-effectiveness. Consequently, in the present study, a cosmetic emulsion containing the reflux extract was developed with high polyphenol content. It is worth noting that in future studies, we will assess the stability of the formulation incorporating the *A. unedo* extract at different times (several weeks) and temperatures.

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Abbreviations

ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); A-NE: A. unedo extract phenolic-enriched nanoemulsion; COX-2: cyclooxygenase-2; DPPH: 2,2-diphenyl-1-picrylhydrazyl; DMSO: dimethyl sulfoxide; HPLC-DAD: high-performance liquid chromatography coupled to a diode-array detector; IL-6: interleukine 6; iNOS: NO synthase; LPS: lipopolysaccharides; NE: blank nanoemulsion; NO: Nitric oxide; O/W: oil in water; ROS: reactive oxygen species; SD: standard deviation; TAA: Total antioxidant activity; TNF α : Tumor Necrosis Factor; Z-average: Mean droplet diameter; ZP: zeta potential.

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