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Original Article

Phytochemical Screening, Antioxidant and Anti-inflammatory Activities of Polyphenolic Extracts of Strawberry-tree Fruits (*Arbutus unedo* L)

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Abstract

Introduction: *Arbutus unedo* L., is an evergreen plant belonging to the Ericaceae family, an endemic species of the Mediterranean flora. The aim of this study was to characterize the polyphenolic extract of *A. unedo* fruits with phytochemical analysis followed by evaluation of antioxidant and anti-inflammatory activities.

Materials and Methods: Antioxidant activity was determined using the scavenging activity of DPPH free radical and ferric-reducing antioxidant power assay. Then, the anti-inflammatory potential of *A. unedo* extracts was evaluated using Human Red Blood Cells (HRBC) membrane stabilization, and egg albumin denaturation assays.

Results: The highest total phenolic, flavonoid, and tannin content was recorded in the methanolic extract with 61.96 ± 5.33 mg GAE/g, 51.16 ± 0.57 mg QE/g, and 2.40 ± 0.14 mg CE/g, respectively. On the other hand, the best radical scavenging activity (IC_{50} = 0.459 ± 0.022 mg/ml) and the highest reducing power activity (EC_{50} = 0.471± 0.022 mg/ml) were exhibited by the methanolic extract *A. unedo*. Whereas regarding the anti-inflammatory activities, *A. unedo* aqueous extract exerted the highest HRBC stabilization of 70.86 ± 0.61% and Egg albumin denaturation inhibition of 70.06 ± 0.68%.

Conclusions: Overall, the results suggest that aqueous and methanolic extracts of *A. unedo* fruits can be used as future ethnomedicinal antioxidants and anti-inflammatories due to their rich content of bioactive molecules.

Keywords: *Arbutus unedo*, Polyphenols, Oxidative Stress, Inflammation, Phytochemical Screening

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Introduction

In the last decades, herbal medicines have received much attention in Western and Eastern countries as sources of biologically active substances, notably for the discovery of antioxidant, anti-inflammatory, anticarcinogenic, and cytotoxic agents.[1,2] These plants are a natural reservoir of secondary metabolites, which exert various biological activities within the plant and on other organisms.[3] The strawberry tree (*Arbutus unedo* L, family Ericaceae), is one of the most common fleshy fruited species in the Mediterranean basin[4-6] producing berries with high nutritional value due to its rich content of bioactive components such as phenolic compounds, sugar, fatty acids, vitamins, and carotenoids.[7] The fruit is used medicinally to treat gastrointestinal disorders, urological problems, skin problems, kidney disease, and cardiovascular applications.[8] Their leaves are used medicinally to treat urological problems, dermatologic problems, kidney diseases, hypertension, cardiac diseases, diabetes, diuretic, anti-inflammatory, and anti-diarrheal.[8,9] It is a well-known fact that oxidative (or redox) status plays a central role in biological processes.[10] Oxidative stress can be identified as an imbalance between the equilibrium of prooxidants and antioxidants; favoring prooxidants induces oxidative damage. The presence of a low amount of reactive oxygen species can be helpful, but an excessive amount is harmful and can cause oxidative damage to biomolecules, leading to many chronic diseases.[11] In addition, these ROS usually occur due to the leakage of electrons from the electron transport chain to oxygen during aerobic respiration.[12] Polyphenols, commonly called antioxidant compounds, are important in maintaining health and protecting against diseases such as cancer.[13] Moreover, the search for new sources of novel anti-inflammatory agents is increasingly in demand due to the growing number of indications of inflammatory-mediated diseases.[14] Mostly, lysosomal enzymes

released during inflammation produce a variety of disorders that lead to tissue damage by damaging macromolecules and lipid peroxidation of membranes that are believed to be responsible for certain pathological conditions such as heart attacks, septic shock, and rheumatoid arthritis.[15] To the best of our knowledge, few studies have described the biological activities of fruit extracts of *A. unedo*. This study aimed to estimate the antioxidant and anti-inflammatory activity of *A. unedo* fruit extracts.

Materials and Methods

Plant Collection

The fruits of *A. unedo* were collected at the end of November 2020 in Tizi-Ouzou, Algeria at their optimal stage of maturation. The plant was identified by experts in these fields, using standard literature and taxonomic keys. The samples were packed in plastic boxes and stored in a cold system before extraction. All the selected berries presented a healthy external appearance.

Preparation of Polyphenolic Extracts

Hot Extraction (Decoction)

The preparation of the methanolic extract (MetEx) consisted of introducing 25 g of the sample into the cellulosic cartridge; the latter was inserted into the extractor of the Soxhlet assembly, which was topped with a refrigerator and 250 ml of methanol in the mounting flask, after boiling for 4 h at 65 °C. In addition, the methanol is also performed using rotavapor at 45 °C.[16]

Cold Extraction (Maceration)

20 grams of powder were dissolved in 200 ml of distilled water After 24 h of maceration at room temperature, this operation was performed three times.[17,18] The resulting solution was filtrated and then evaporated to obtain *A. unedo* L. extract. The extract obtained (AqEx) was stored at a temperature of 4 °C until use.[19] The extraction yield (%) was calculated using the following formula[20]:

Yield (%) = Weight of extract (g)/Weight of dried plant x 100

Phytochemical Screening

Qualitative Analysis

Qualitative analysis of phytochemical content was performed using the following published methods with minor modifications.[21-27]

Quantitative Analysis

Determination of Total Phenolic Content (TPC)

The amount of phenol in the extract was determined by the Folin-Ciocalteu reagent method.[28] The assays were performed as follows: 1250 µl of the Folin Ciocalteu reagent (diluted 10-fold in distilled water) were mixed with 250 µl of the gallic acid solution and 1000 µl of 7% Na2CO3, the final mix was slightly stirred; after 1 h reaction at room temperature, the absorbance was measured at 765 nm. The extracts solutions were mixed with the safme reagents and treated as indicated above. All determinations were carried out in triplicate, and the results were expressed as mg of gallic acid equivalent (GAE).[29]

Determination of Flavonoid Content (FC)

The protocol was optimized from the study of Kumar et al.[30] An aluminum chloride complex-forming assay was used to determine the flavonoid content of the extracts. Quercetin was used as the standard and flavonoid content was determined as the quercetin equivalent. 1000 µl of the quercetin dilution was mixed with 300 µl of 4% sodium nitrate and allowed to stand for 5 minutes. Then, 300 µl of 10% aluminum chloride solution was added and allowed to stand for 6 minutes, after which 2000 µl solution of 1 M sodium hydroxide was added sequentially and completed volume to 10 ml. The absorbance of this reaction mixture was recorded at 510 nm on a UV spectrophotometer. The flavonoid content was calculated as quercetin equivalents (mg QE/g). All the procedures were performed in triplicate.

Determination of Condensed Tannins (CT)

Condensed tannins of the extracts were determined using the vanillin assay described by Hayet et al.,[31] with some modifications. To 500 µl of standard (catechin) or extract, 3000 µl of vanillin/methanol (4%) solution was added and mixed. Then, 1500 µl of concentrated HCl was added and allowed to react at room temperature for 15 min. The absorbance at 500 nm was measured against a blank. The total concentration of condensed tannins was expressed in micrograms of catechin equivalents per milligram of dry matter with reference to the catechin calibration curve.

Antioxidant Activity

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) Assay

The scavenging potency of the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical of the extract was determined by Diomande Awa,[32] with a small modification. The absorbance at 517 nm was measured to assess the remaining amount of DPPH. Ascorbic acid (Vit C) was applied as a standard. The ability to scavenge DPPH radicals was calculated using the following equation:

Inhibition (%) = (A control – A test) / A control x 100

Where: A control = The absorbance of the control reaction

A test = The absorbance of the extract

The results were expressed as the half-maximal inhibitory concentration (IC_{50}) and compared with the standard. All measurements were fulfilled in triplicate and mean values were calculated.

Reducing power Assay

The reducing power of the extract was accomplished as described by Moriasi et al.[33] The absorbance was recorded at 700 nm in a UV–vis spectrophotometer and ascorbic acid was used as a standard. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against the correspondent extract concentration. All measurements were accomplished in triplicate.[34]

Anti-inflammatory Activity

Inhibition of Albumin Denaturation

A solution of 0.2% (w/v) of egg albumin was prepared in a phosphate-buffered saline (PBS, pH 6.4)[13] The method of inhibiting the denaturation of egg albumin was carried out according to the protocol of Habibur et al.[35] With a slight change. The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin, 2.8 ml of PBS (pH 6.4), and 2 ml of varying concentrations (31.25-1000 µg/ml) of extract. A similar volume of double-distilled water served as the control. Then, the mixtures were incubated at 37 ± 2 °C in an incubator for 15 min and then heated at 70 °C for 5 min. After cooling, their absorbance was measured at 660 nm. Diclofenac sodium in concentrations of (31.25-1000 µg/ml) was used as the reference drug and treated similarly for the determination of absorbance.[35] Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated by using the following formula:

Inhibition percentage (%) = (A0 − A/A0) × 100

Where: A0 = absorbance of negative control

A = absorbance of test solution

HRBC Membrane Stabilization Method

The membrane-stabilizing activity was performed as per the method described previously by Dadoriya et al.[36] 10% human red blood cells (HRBCs) suspension was prepared in phosphate buffer. An equal volume (1 ml) of extract (100-500 µg/ml in phosphate buffer), diclofenac sodium (100 µg/ml in phosphate buffer), or phosphate buffer (as control) was separately mixed with phosphate buffer (1 ml), hyposaline (1 ml) and HRBC suspension (0.5 ml). All the assay mixtures were incubated at 37 °C for 30 min and then centrifuged at 3000 rpm. The supernatants were decanted and their optical density was measured at 560 nm by spectrophotometer to estimate the hemoglobin content using phosphate buffer as blank. The percentage of hemolysis was calculated by the formula given below considering the hemolysis of control as 100%.

%Protection = 100-[Optical density (sample) / Optical density (control)] × 100

Statistical Analysis

Data were presented as means ± SD and with linear regression analysis for calculation of IC_{50} and EC_{50} values (excel 2019, GraphPad prism7). Statistical significance (p<0.05) was assessed by one-way analysis of variance ANOVA coupled with Tukey's post hoc test.

Results and Discussion

Phytochemical Screening

Extraction Yield

The yields were 21.4 and 18.9% for AqEx and MetEx, respectively, as show in Table 1. The type of organic solvent used is a very important factor that affects the separation of a selective chemical from plant samples. The main function of polar solvents is to improve and facilitate the permeability of the cell wall of the chemical substances, which allows a better contact between the solvent and the solute, thus increasing the percentage yield of the extraction.[37]

Table 1. Yield (%), Phenolic Content, IC_{50} and EC_{50} values

	Yield (%)	TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg CE/g)	DPPH IC_{50} (mg/ml)	FRAP EC_{50} (mg/ml)
AqEx	21.45 ± 0.07	12.38 ± 0.29	16.13 ± 2.30	1.46 ± 0.04	0.942**** ± 0.03	1.655**** ± 0.03
MetEx	19.35 ± 0.63	61.96 ± 5.33	51.16 ± 0.57	2.40 ± 0.14	0.459**** ± 0.02	0.471**** ± 0.02
Ascorbic Acid	-	-	-	-	0.063**** ± 0.0002	0.085**** ± 0.005

****significant at p<0.0001

Table 2. Phytochemical Screening Results of *A. unedo* Extracts

Chemical Compound	Extract	
	AqEx	MetEx
Reducing sugar	+++	+++
Alkaloids	-	-
Flavonoids	+	++
Tannins	+++	+++
Phenols	+++	+++
Saponosides	-	-
Terpenoid	++	-
Steroid	++	-
Anthocyanin	+	+++
Quinone	-	-

The symbols: +++, ++, +, and – refer to appreciable amounts, moderate, trace, and absent amounts, respectively.

Qualitative Analysis

Secondary metabolites were the principal constituents of plants that can be detected by phytochemical screening. Both the preliminary phytochemical screening of the AqEx and MetEx of *A. unedo* fruits revealed the presence of different chemical compounds (Table 2): containing phenols, tannins, reducing sugar at very high concentrations for both and anthocyanins were abundant only in the MetEx. This was observed due to the intense staining caused by the strong reaction between the sample and the reagents. On the other hand, terpenoid and steroid have been detected at moderate concentrations in AqEx and a complete absence of these in MetEx. However, the test showed the presence of flavonoids and anthocyanin traces in AqEx, and a missing of alkaloids, quinone, and saponosides for both extracts. This finding, with the exception of the presence of steroids in AqEx, was in agreement with previous work.[38]

Quantitative Analysis

This study dealt with the quantitative determination of phenolics, flavonoids, and tannins. The results showed that MetEx presented higher phenol, flavonoid, and tannin content than aqueous ones (Table 1). The TPC was expressed as mg of gallic acid equivalent (GAE) per gram of extract and varied from 61.965 ± 5.337 to 12.38 ± 0.296 mg GAE/g in methanolic and aqueous extract, respectively. TPC in *A. unedo* fruits reached similar values to those published by El Cadi et al.[38] On other hand, these values were higher than those found by Asmaa et al. and Bouzid,[39, 40] and lesser than those obtained by Salem et al. and Moualek et al.[17,13] The experimental results showed that the fruit extract of *A. unedo* contains flavonoids ranging from 51.166 ± 0.577 to 16.133 ± 2.309 mg of QE/g of extract in the MetEx and AqEx, respectively, these values similar to those found by El Cadi et al.,[38] and a higher value compared to that published by Moualek et al.[13] So, the tannins content of the studied extract was varied from 2.408 ± 0.144 to 1.46 ± 0.044 EC mg/g in the methanolic extract and aqueous, respectively. Although the quantity of tannins was higher than that published by El Cadi et al.,[38] and similar to that found by Bouzid.[40] However, the variation in the determination of polyphenols, flavonoids, and tannins can be explained by the fact that the content of phenolic compounds was influenced by several factors and parameters such as geographical, climatic conditions of the locality where the fruits are collected, extraction method, solubility and type of solvent used.[31,41,42] In addition, this study demonstrated the presence of various phenolic compounds such as phenols, tannins, and flavonoids. These compounds were commonly present in polar extracts such as hydroalcoholic extracts, and could be the main cause of their considerable radical scavenging activity.[43]

DPPH Scavenging Activity

The DPPH radical scavenging activity (RSA) was recorded in terms of inhibition percent as shown in Figure 1, the parameter used to compare RSA of the extract and ascorbic acid was IC_{50} value, which represents the concentration of antioxidant sufficient to scavenge 50% of DPPH radicals. The IC_{50} value for ascorbic acid was (0.063 ± 0.0002) mg/ml, which was comparatively lower than the IC_{50} [(0.459 ± 0.022) mg/ml, (0.942 ± 0.03)] of the MetEx and AqEx, respectively as shown in Table 1. The IC_{50} of the extract studied exceeds that reported by Brigitte et al.,[44] and Oliveira.[8] In contrast, the methanolic extract has an important antioxidant effect than the aqueous. This finding was in agreement with THE previous work of Masmoudi et al.[45] So, the DPPH entrapment results detect that *A. unedo* fruit extracts may be useful in the manufacture of drugs to help prevent or cure health problems that could lead to systemic actions of oxidizing agents.

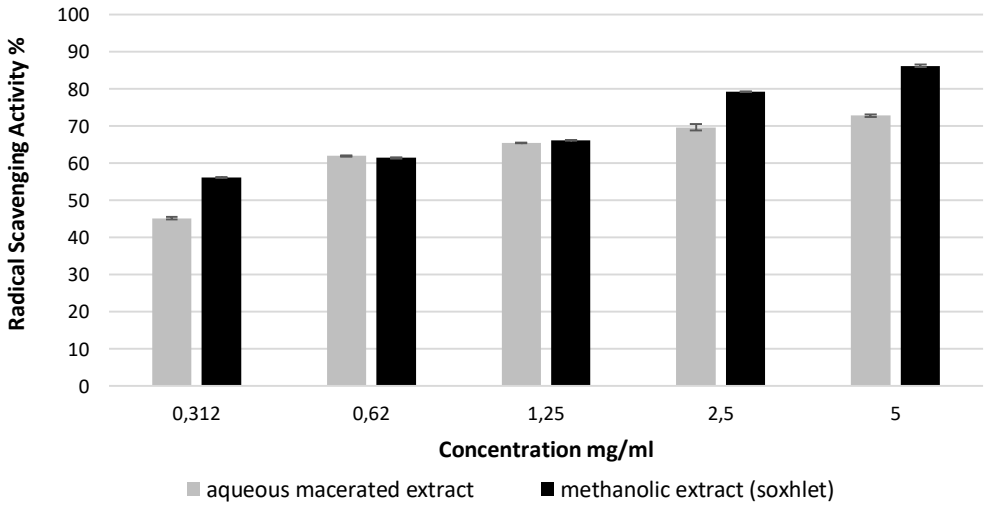

Figure 1. Radical Scavenging Activity of *A. unedo* Extracts.

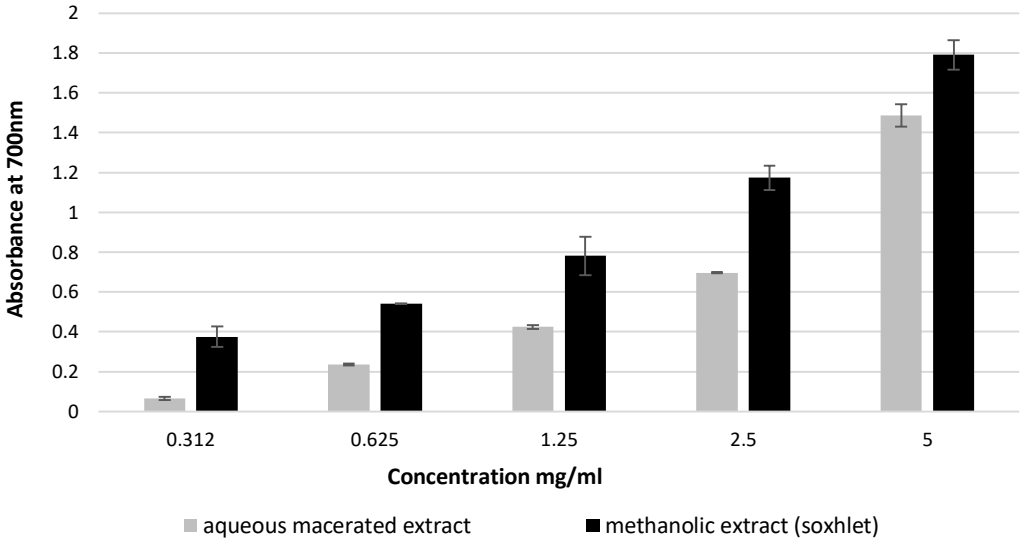

Figure 2. Ferric Reducing Power *A. unedo* Extracts.

Reducing Power Assay

The reducing power of the sample extract, which is related to the antioxidant activity, was evaluated using the ferric reducing antioxidant power (FRAP) test. When the sample contained antioxidant compounds so it was able to reduce Fe (III) from potassium ferricyanide to Fe (II), resulting in a color change of the solution from yellow to light green. Figure 2 showed the reducing power of *A. unedo* extracts as a function of their concentration. In this assay, the MetEx exhibited a reducing power clearly stronger than that of AqEx. The lower EC_{50} value that corresponds to the higher reducing capacity was obtained from MetEx (0.471 ± 0.022 and 1.655 ± 0.032 mg/ml, for MetEx and AqEx, respectively) (Table 1). The EC_{50} of the extract was lower than the one reported by Oliveira and Mendes et al.[8,46] On the other hand, these values were higher than those found by Asmaa et al.[39] This antioxidant activity showed that phenolic constituents of *A. unedo* were good electron donors, being able to terminate the radical chain reaction by transforming the free radicals into more stable compounds. Furthermore, the fruits presenting higher amounts of total phenols (intermediate stage of maturation) achieved a lower EC_{50} value for this method. This relationship was also valid for the other two stages of maturity. The unripe fruits, presenting the lowest content of total phenols, present the higher value of EC_{50} in the reducing power assay, and the ripe fruits, with an in-between phenolic content, also presented an intermediate EC_{50} value.[8] Finally, this study confirmed that antioxidant activity was well correlated with the amount of phenolics present in the extract of *A. unedo* fruits.

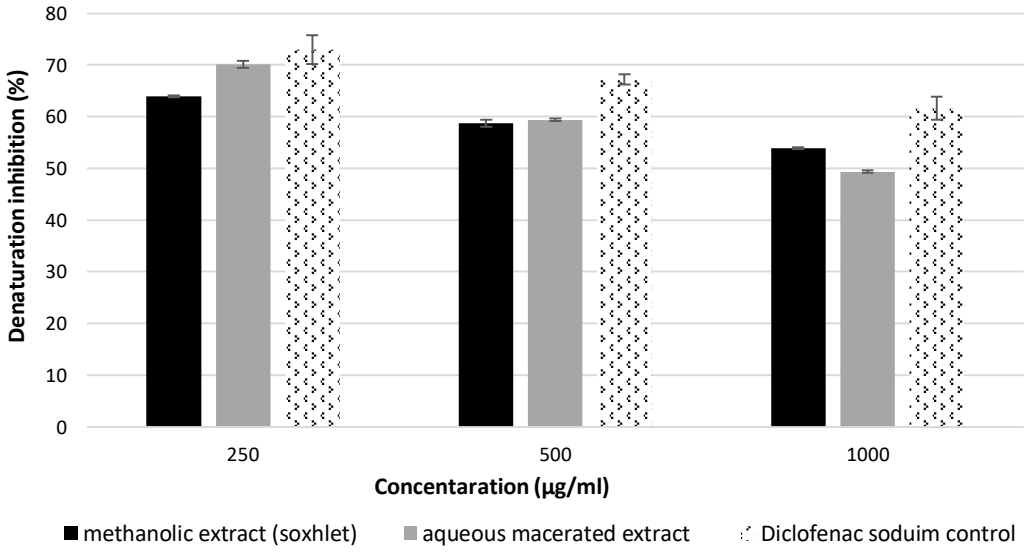

Figure 3. Egg Albumin Protein Denaturation Inhibition Activity of *A. unedo* Extracts.

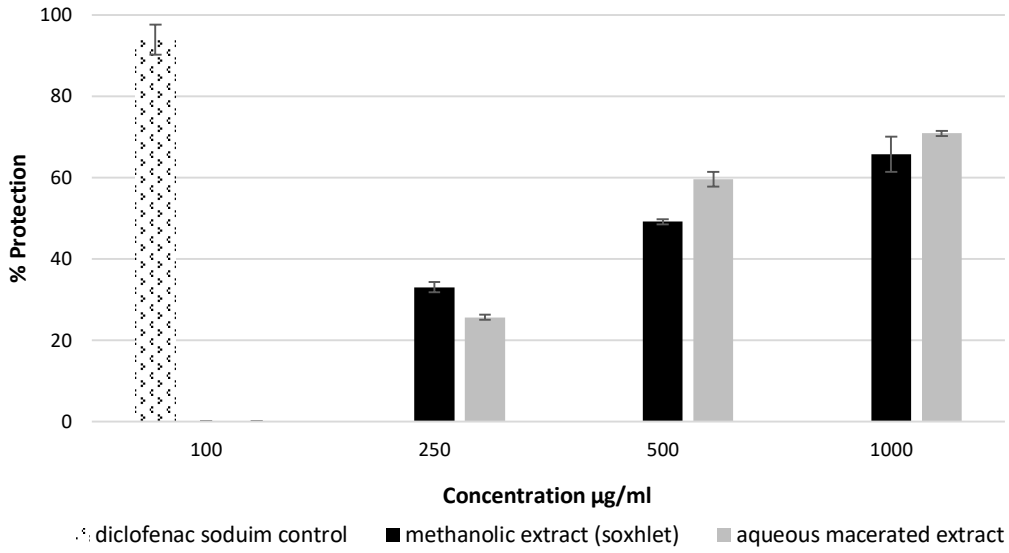

Figure 4. HRBC Membrane Stabilization Activity of *A. unedo* Extracts.

Inhibition of Albumin Denaturation

As shown in Figure 3, this inhibition of protein denaturation was not in a dose-dependent manner. The *A. unedo* AqEx exerted the highest inhibition activity of protein denaturation at concentrations of 250 and 500 µg/ml (70.064 ± 0.686 and 59.385 ± 0.228%, respectively) followed by the MetEx at concentrations of 250 and 500 µg/ml (63.915 ± 0.228 and 58.737 ± 0.686%, respectively). Although both extracts revealed high inhibition activities, none of them surpassed the standard diclofenac drug that had a high protein denaturation inhibition activity of (72.914 ± 2.977 and 67.162 ± 1.016% at concentrations 250 and 500 µg/ml, respectively). When comparing the activity of plant extract on albumin denaturation, it was observed that *A. unedo* extract was lower than that reported by Moualek et al.[13]

HRBC Membrane Stabilization

HRBC membrane stabilization assay results in Figure 4 indicated that the AqEx of *A. unedo* had the highest inhibition percentage at 500 and 1000 µg/ml (59.565 ± 1.844 and 70.867 ± 0.614, respectively) followed by the MetEx had an inhibitory activity of 49.130% ± 0.614 and 65.652% ± 4.304 at the same concentration. Both AqEx and MetEx exhibited lower stabilization activity when compared to the standard diclofenac sodium, which had an inhibition of 93.913% ± 3.689 at 100 µg/ml. These results provided evidence of the anti-inflammatory activity of the extract which showed a good protective effect of HRBC. Moreover, this result was in agreement with previous work obtained by Moualek et al.[13]

Protein denaturation was a well-documented cause of inflammation and rheumatoid arthritis. The ability of plant extract to inhibit the thermal denaturation of protein (egg albumin) was a reflection of its anti-inflammatory activity.[47] Also, Mariotto et al.[48] demonstrated that acute pulmonary inflammation was significantly attenuated by treatment with *A. unedo* extract. While the mechanisms of the anti-inflammatory effect of *A. unedo* extract seemed to be correlated with the reduction of IL-6 production, the successive activation of STAT3, the formation of other pro-inflammatory cytokines such as TNF- and IL-1, the increase of neutrophil recruitment, and the expression of iNOS and COX-2 proteins and their activity, which may ultimately lead to the decrease of tissue damage.[48] Stabilization of the lysosomal membrane was an essential factor in limiting inflammation by inhibiting the release of lysosomal contents by neutrophils, which are primarily responsible for inflammation and tissue damage.[31] The *in vitro* erythrocyte hemolysis test was commonly used to screen drugs for anti-inflammatory activity. Most anti-inflammatory drugs stabilized the plasma membrane of erythrocytes and thus inhibited heat- and hypotonia-induced hemolysis.[49]

Conclusion

The present study showed that both aqueous and methanolic extracts of *A. unedo* fruits contained various phytochemicals. Furthermore, *A. unedo* methanolic extract possessed the highest total phenolic, flavonoid, and tannin content. Interestingly, all *A. unedo* extracts exhibited promising antioxidant and anti-inflammatory properties. Results indicated that both extracts can be utilized as future antioxidants and anti-inflammatory ethnomedicines.

Authors' Contributions

Conception and design of the study by FS; Acquisition of data and doing the laboratory phase by FL and HB; Analysis

and interpretation of data by FL, HB, and FS. The final manuscript was read and approved by all the authors.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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