

Article

Elderberry Hydrolate: Exploring Chemical Profile, Antioxidant Potency and Antigenotoxicity for Cosmetic Applications

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Abstract: Elderberry (*Sambucus nigra* L.) hydrolate, derived from domestic steam distillation, holds promise as a multifunctional ingredient for skincare and cosmetic applications. This study investigates the chemical composition and biological activities of elderberry hydrolate obtained through steam distillation. Despite the growing interest in elderberry hydrolate, there has been a lack of comprehensive studies elucidating its chemical composition and potential bioactive constituents. To address this gap, we conducted a detailed analysis of elderberry hydrolate's composition, antioxidant activity, and antigenotoxicity. Genotoxic evaluation and antioxidant assays (ABTS, DPPH) were conducted to assess its biological properties. We obtained elderberry hydrolate with a notable transfer of aromatic compounds through the steam distillation process, highlighting its efficacy and sustainability. The chemical characterization identified vital compounds, including phenylacetaldehyde, 2-acetyl-pyrrole, and an unidentified major component, collectively contributing to the hydrolate's aromatic and biological properties. The genotoxic evaluation using the Comet assay demonstrated the hydrolate's protective effects against DNA damage induced by hydrogen peroxide and streptonigrin. The optimal DNA protection was observed at 10% (*w/v*), attributed to the antioxidant activity of the identified compounds. The hydrolate exhibited significant antioxidant potential, demonstrating concentration-dependent responses and correlating higher concentrations with increased antioxidant activity. These findings underscore the multifaceted attributes of elderberry hydrolate, positioning it as a promising natural ingredient for skincare. This study supports elderberry hydrolate as a valuable natural and sustainable product development resource.

Keywords: elderberry hydrolate; steam distillation; antioxidants; cosmetics; chemical characterization



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1. Introduction

Due to the increased environmental awareness, there is growing interest in natural ingredients for use in cosmetic products. To make natural ingredients an exciting and economically sustainable source of bioactive compounds, selecting and optimizing suitable environmentally friendly extraction technologies that allow the recovery and sustainability of target analyses are indispensable.

Elderberry (*Sambucus nigra* L.) has been widely recognized for its historical and contemporary applications in traditional medicines, culinary arts, and functional foods due to its rich phytochemical content [1]. Among the diverse array of products derived from

elderberries, hydrolates have garnered increasing attention for their potential health benefits and multifunctional properties [2]. Hydrolates, also known as hydrosols, floral waters or distillates, are the aqueous co-products obtained during essential oil extraction [3]. These co-products have been valued for their aromatic profiles and for possessing a spectrum of bioactive compounds distinct from those found in the corresponding essential oils [4]. Elderberry hydrolate, in particular, holds promise as a valuable resource for various applications, including cosmetics and aromatherapy [5,6].

Despite the growing interest in elderberry hydrolate, there remains a dearth of comprehensive studies elucidating its chemical composition and potential bioactive constituents. Such investigations are essential to fully understanding the hydrolate's potential benefits and applications. Studies suggest that elderberry possesses significant antioxidant and antigenotoxic properties, yet detailed investigations are necessary to understand its potential benefits and applications entirely [7,8]. To address this gap, our study conducts an in-depth analysis of the chemical profile and biological activities of elderberry hydrolate obtained through steam distillation.

We employ a range of methodologies, including gas chromatography–mass spectrometry (GC-MS) for chemical characterization, antioxidant assays (ABTS, DPPH), and genotoxic evaluations using the Comet assay to assess its biological properties. By elucidating the antioxidant potency and antigenotoxicity of elderberry hydrolate, we aim to provide valuable insights into its potential applications, particularly in the cosmetics industry. Our findings contribute to the broader context of natural product research and development, highlighting elderberry hydrolate as a promising, sustainable, and multifunctional ingredient for skincare formulations. This comprehensive exploration aims to shed light on the versatile bioresource that elderberry hydrolate offers, emphasizing its significance as a sustainable and valuable asset for future product development.

2. Materials and Methods

2.1. Cells

Peripheral blood samples were collected from a healthy 35-year-old female volunteer. She was a non-smoker, did not consume alcohol, had no health issues, and had not taken any medication for six months prior to the study. This research was conducted with the approval of the ethical committee of the University of Trás-os-Montes and Alto Douro (Doc4-CE-UTAD-2023) and in accordance with the Declaration of Helsinki.

2.2. Chemicals

The streptonigrin (CAS 3930–19-6) was purchased from Santa Cruz Biotechnology Inc. of Santa Cruz, TX, USA. The Fpg (formamido-pyrimidine[fapy]-DNA glycosylase) was purchased from New England BioLabs (Ipswich, MA, USA). The ethyl acetate ACS (Reag. Ph. Eur./USP) and NaCl were purchased from Carlo Erba Reagents (Val-de-Reuil, France). The distilled water was purchased from MedicalShop (Ponte de Lima, Portugal). The distillation apparatus was purchased from Agrosprof (Braga, Portugal). The n-hexane GC-MS (SupraSolv), phenylacetaldehyde and all the other chemicals were purchased from Merck (Darmstadt, Germany).

2.3. Lysis Solution

The lysis solution was prepared by combining 2.5 M NaCl, 0.1 M disodium EDTA, and 0.01 M Tris base, and adjusting the pH to 10. Initially, the mixture, excluding Triton X-100, was dissolved in distilled water to just below the final volume, with precise amounts of each compound. The pH was then adjusted to 10 using a 10 M NaOH solution for 1 h at 4 °C. Finally, 1% Triton X-100 was added to the lysis solution before use.

2.4. Phosphate-Buffered Saline (PBS) Solution

A PBS solution was prepared by dissolving exact amounts of 2 mM KH_2PO_4 , 10 mM Na_2HPO_4 , 2.7 mM KCl, and 137 mM NaCl in distilled water, just below the final required

volume. The pH was then adjusted to 7.4 using a 1 M HCl solution. Finally, the remaining water was added to achieve the desired final volume of the PBS solution.

2.5. Enzyme Reaction Buffer for Fpg

The enzyme reaction buffer, comprising 0.04 M HEPES, 0.10 M KCl, 0.0005 M EDTA, and 0.2 mg/mL BSA, was formulated in H₂O. The pH of the buffer solution was adjusted to 8.0 by carefully adding a 6 M KOH solution.

2.6. Electrophoresis Solution

The electrophoresis solution was prepared by combining 0.3 M NaOH and 1 mM EDTA in a flask, then adding distilled water until the pH was below 13.

2.7. Enzyme Preparation

Commercially available, the lesion-specific enzyme for the Comet assay, Fpg, was sourced from bacteria and stored at -80°C in aliquots after the initial plasmid-engineered production. The thawed Fpg was diluted as per the supplier's guidelines with an Fpg buffer and maintained on ice until added to the gels during the experiments, ensuring proper enzyme functionality.

2.8. Elderberry Harvest and Preparation

In August 2022, elderberries were purchased from INOVTERRA (Vila Pouca de Salzedes, Portugal). The dried elderberries were kept in a hermetically closed glass container until further analysis [9]. The elderberries were ground into a powder using a coffee mill for each experiment.

2.9. Elderberry Steam Distillation

The appropriate ratio of plant material to water for steam distillation can vary depending on factors such as the type and quantity of the plant material being used and the desired outcome of the extraction. However, a standard recommendation in the literature is to use a ratio of 1:3 (1 part plant material to 3 parts water) to extract essential oils from plant materials (Table 1). It should be noted that the optimal ratio for steam distillation may also vary depending on the distillation apparatus used. In our experiments, a copper still (alembic) was used. The procedure is described in Table 1 [10].

Table 1. Elderberry steam distillation formulation and procedure.

Ingredient	g
Distilled Water	4050
Elderberries	1350
Procedure	
1.	Dried elderberries were placed in the distillation apparatus along with the distilled water.
2.	The apparatus was then connected to a water-cooled condenser and heated using a heating mantle.
3.	Once the water in the flask reached boiling point, the steam generated passed through the elderberries, extracting the essential oil components.
4.	The steam then condensed on the walls of the condenser and dripped into a collection vessel for 1 h.
5.	The collected liquid was a mixture of essential oil and water, separated using a funnel.
6.	The hydrolate was preserved in an airtight, light-resistance container, and exposure to direct sunlight and excessive heating was avoided.

2.10. Dried Herb-to-Hydrolate Ratio Calculation

In our study, the *dried herb-to-hydrolate ratio* was calculated as the ratio of the weight of the dried herb (elderberries) to the obtained hydrolate volume, expressed as a percentage:

$$\text{Dried herb-to-hydrolate ratio}(\%) = \frac{\text{Weight of Ingredient}(\text{g})}{\text{Volume of Hydrolate}(\text{mL})} \times 100$$

This metric quantitatively indicates how much of the elderberries' volatile components will be successfully transferred into the hydrolate.

2.11. Preparation of Hydrolate Extracts

Extraction of the hydrolate was carried out in glass containers and in triplicates using hexane and ethyl acetate as organic solvents. The ratio of hydrolate/hexane was 9:1 (*v/v*), with 9 mL of the hydrolate and 1 mL of the organic solvent, and the ratio of hydrolate/ethyl acetate was 8:2 (*v/v*), with 8 mL of the hydrolate and 2 mL of the organic solvent. The mixtures were shaken overnight at room temperature. Then, 0.5 mL of the upper (organic solvent) fraction was taken for the GC-MS analysis (Section 2.12). After the analysis, the relative standard deviations were calculated, and the results were averaged.

2.12. Profile of Volatile Substances Determined by Gas Chromatography Coupled with Mass Spectrometry (GC-MS)

The unprocessed hydrolate samples and hydrolate extracts were analyzed using a Shimadzu GC-MS system (GCMS-QP2010 Ultra, São Paulo, Brazil) equipped with an MS detector and an Rxi-5Sil MS capillary column (Restek, Pennsylvania, USA; 30 m × 0.25 mm, film thickness 0.25 µm). The injector and ion source temperatures were set to 250 °C and 200 °C, respectively. The column temperature was programmed to increase from 40 °C to 220 °C at a rate of 3 °C/min, holding the initial and final temperatures for 15 min each. Helium (99.99%) was used as the carrier gas at a flow rate of 1 mL/min. Hydrolate samples of 0.5 µL and 1.0 µL, and extract samples of 1.0 µL, were injected using an autosampler in split mode, with split ratios of 1:5 and 1:10 for the hydrolate samples, and 1:5 for the extract samples. The MS detection was performed in electron ionization mode with an ionization energy of 70 eV, and the MS transfer line temperature was set to 250 °C. The mass-to-charge (*m/z*) range was from 40 to 400, with a scanning frequency of 5 Hz.

The compound identification was based on the comparison of their mass spectra and the retention indices with those of synthetic compounds in the National Institute of Standards and Technology (NIST11) and the Flavors and Fragrances of Natural and Synthetic Compounds (FFNSC2) spectral libraries. The phenylacetaldehyde peak was confirmed using a reference standard. The linear retention indices were determined relative to a homologous series of n-alkanes (C6-C24). The components' relative concentrations were calculated from the GC peaks without correction factors.

2.13. Elderberry Hydrolate Treatment

For the Comet assay, four concentrations of elderberry were chosen based on previous results: 1%, 5%, 10%, and 15% (*w/v*) [8]. Two treatments were prepared: one with H₂O₂ and another with streptonigrin (SN). Two independent experiments, 10 days apart, were performed for each condition.

To apply the H₂O₂ treatment, 50 µL of H₂O₂ was mixed into 0.5 L of PBS. The experiment involved setting up ten Coplin jars: the first held only PBS, the second to the fifth contained different elderberry concentrations combined with PBS, the sixth held the H₂O₂-treated solution, and the remaining four contained various elderberry concentrations combined with the H₂O₂ treatment. Two slides were prepared for each condition. One slide in each set followed the standard protocol, while the second underwent enzymatic incubation before continuing. Figure 1 provides a schematic overview of these steps, depicting the sequential stages of the procedure.

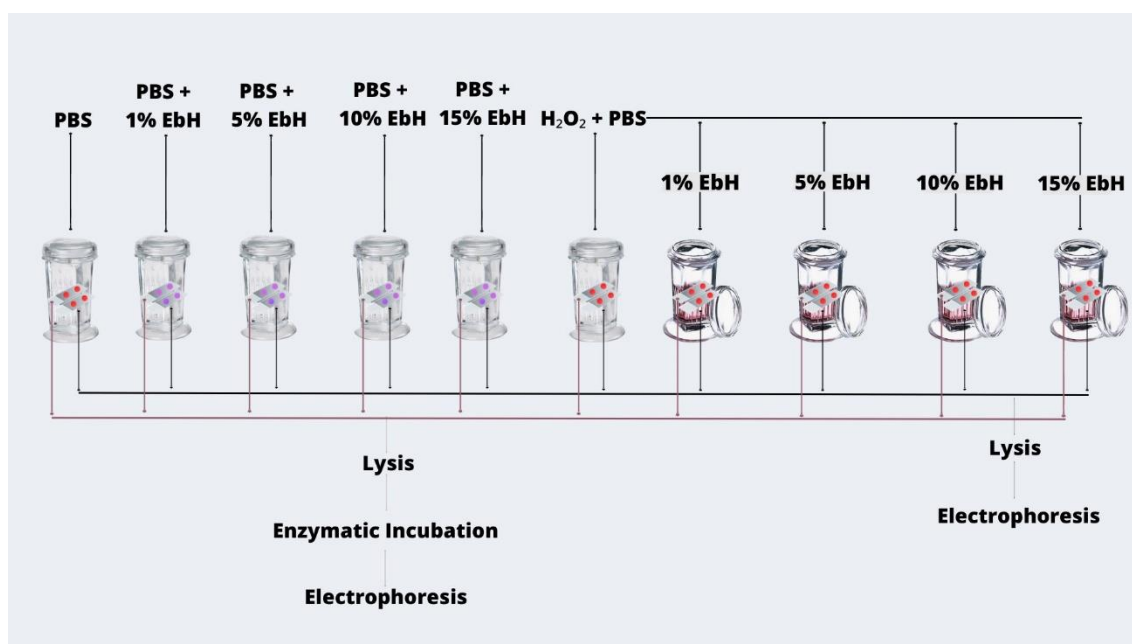


Figure 1. Schematic illustration of the experimental process.

The SN treatment involved dissolving SN in PBS to attain a final concentration of 20 μM within a 5 mL volume, following established methodologies [11]. A dual-slide method was employed for each condition, adhering to these parameters. One slide in each set followed the traditional pathway, involving standard protocol steps of lysis and electrophoresis. Simultaneously, its corresponding slide underwent an initial enzymatic incubation stage before proceeding to the subsequent steps of lysis and electrophoresis. The detailed workflow is visually represented in Figure 2.

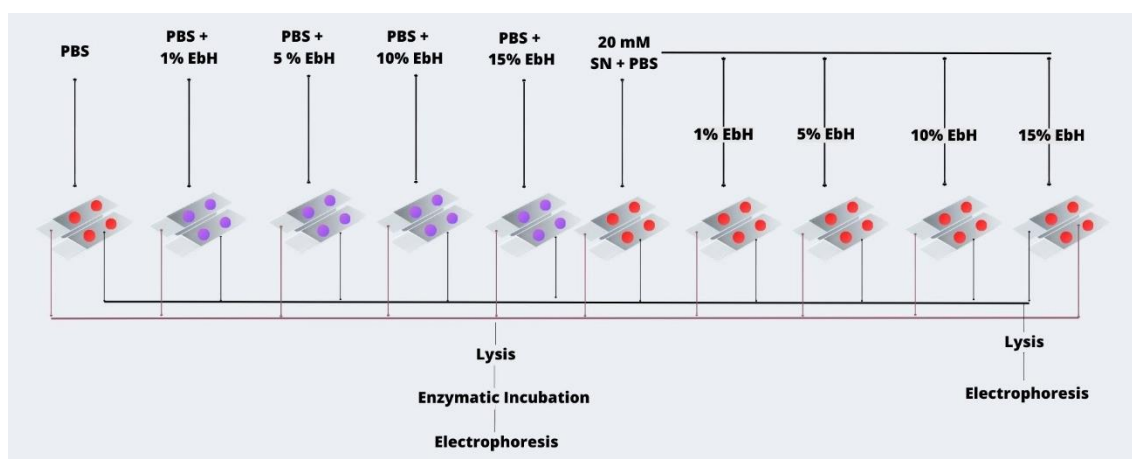


Figure 2. Illustration of the SN treatment process.

2.14. Genotoxic Evaluation

The genotoxic and antigenotoxic effects of elderberry were evaluated *in vivo* using the Comet assay on human peripheral blood mononuclear cells (PBMCs).

2.14.1. Comet Assay in Human PBMCs Using H_2O_2

The experimentation followed the methodology outlined in [11]. All the solutions and pre-coated slides containing 1% normal-melting-point agarose were prepared in advance. The blood samples were obtained via a finger prick, and 25 μL of each sample was mixed

with 0.8% low-melting-point agarose in PBS. Subsequently, two 70 μL drops of this mixture were placed onto pre-coated slides, each covered with a coverslip to evenly disperse the solution. This process was repeated for each concentration under examination. The slides were then refrigerated at 4 $^{\circ}\text{C}$ for 5 min to allow the agarose to solidify before the coverslips were removed. Next, the slides were subjected to various treatments in Coplin jars at 37 $^{\circ}\text{C}$ for 1 h. After treatment, the slides were placed in a cold, fresh lysis solution. The slides were arranged without gaps in the electrophoresis chamber and submerged in a cold denaturing and electrophoresis buffer for 20 min. Electrophoresis was performed in darkness at 4 $^{\circ}\text{C}$ with a current of 300 mA and a voltage of 25 V (equivalent to 0.8 V/cm) for 20 min. After electrophoresis, the slides were washed sequentially in PBS (10 min at 4 $^{\circ}\text{C}$) and distilled water (10 min at 4 $^{\circ}\text{C}$) before air-drying. Each gel was stained with 40 μL of DAPI (4',6-diamidino-2-phenylindole) (1 $\mu\text{g}/\text{mL}$ in dH_2O) and covered with a coverslip for examination under a fluorescence microscope (Leica DMLS, Massachusetts, USA) at 400 $\times$ magnification. Fifty cells per gel were observed, and the tail intensity of each cell was graded from 0 (no tail) to 4 (almost all the DNA in the tail) [12]. The final score, expressed in arbitrary units ranging from 0 to 400, was calculated using the genetic damage indicator (GDI) formula.

Genetic Damage Indicator (GDI)

$$= [(\% \text{ nucleoid class 0}) \times 0] + [(\% \text{ nucleoid class 1}) \times 1] \\ + [(\% \text{ nucleoid class 2}) \times 2] + [(\% \text{ nucleoid class 3}) \times 3] \\ + [(\% \text{ nucleoid class 4}) \times 4]$$

Additionally, another slide for each condition underwent enzymatic incubation. After washing with buffer B at 4 $^{\circ}\text{C}$, diluted Fpg enzyme (50 μL) was applied to each gel and incubated in a moisture box at 37 $^{\circ}\text{C}$ for 30 min. Electrophoresis and the subsequent steps were carried out as per the protocol. The use of agarose gel for embedding cells in the Comet assay serves to immobilize the cells and safeguard their structural integrity through subsequent assay steps. This methodology is commonly employed when analyzing adherent or monolayer-growing cells, facilitating their manipulation and handling [13]. The agarose gel provides a supportive matrix that encapsulates the cells, preventing dislodgment or disruption during subsequent assay steps like cell lysis and electrophoresis. Embedding cells in agarose ensures their uniform distribution on microscope slides, enabling even analysis [14].

Moreover, the porous nature of agarose facilitates the diffusion of lysis solution and other reagents while maintaining cell integrity. This effectively removes cellular proteins and contaminants during lysis, enhancing the DNA damage visualization and analysis [13,15]. In contrast, cells in suspension necessitate agarose embedding for Comet assay analysis. Cells in suspension would disperse during electrophoresis, hampering accurate assessment of the DNA damage levels. Embedding these cells in agarose immobilizes them, ensuring consistent and reliable DNA damage analysis in the subsequent steps of the Comet assay [16,17].

2.14.2. Comet Assay in Human PBMCs Using SN

The protocol remained consistent with previous procedures, yet the treatment varied. In this experiment, four concentrations of elderberry were dissolved in a mixture of SN and PBS. A 50 μL droplet of this solution was placed on the agarose gel and the blood sample, then covered with a coverslip. Subsequently, the slides were incubated at 37 $^{\circ}\text{C}$ for 1 h before being immersed in lysis solution and following the established protocol.

2.15. Hydrogen Peroxide (H_2O_2) Assay

The ability of the hydrolate to promote the dismutation of H_2O_2 was assessed using a modification of the method previously described [18]. This method is based on converting homovanillic acid (HVA) into its fluorescent dimer in the presence of H_2O_2 and horseradish peroxidase (HRP). Four different concentrations were used to assess the possible dismuta-

tion capacity of elderberry hydrolate (1%, 5% and 15% *w/v*). The elderberry hydrolate was incubated in phosphate buffer (50 mM, pH 7.4) in the presence of 20 mM H₂O₂, 0.1 mM HVA, and 6.4 mg/mL HRP. After 5 min, the reaction was stopped with 0.5 mL of cold glycine buffer (pH 12.0). The solution fluorescence was measured at 312 nm for excitation and 420 nm for emission wavelengths with a Varian Eclipse spectrofluorometer. The peroxide generation was calculated using a standard curve of H₂O₂, and the H₂O₂ levels were expressed as the nmol H₂O₂ dismutation/min.

2.16. Antioxidant Activity

2.16.1. ABTS Radical-Scavenging Activity

The ABTS radical-scavenging assay was assessed following a previously outlined method [19], with some modifications. The ABTS radical cation decolorization assay evaluated the elderberry hydrolate's free-radical-scavenging activity. To initiate the formation of the ABTS^{•+} cation radical, 7 mM ABTS was mixed with 2.5 mM potassium persulfate (1:1 *w/w*) in water and left to incubate in the dark at room temperature for 12–16 h before utilization. The resulting ABTS^{•+} solution was diluted with methanol until reaching an absorbance of 0.700 at 734 nm. After adding four different concentrations of the hydrolate (1%, 5% and 15%, *w/v*) to the diluted ABTS^{•+} solution, the absorbance was measured 30 min after thorough mixing. Each assay included an appropriate solvent blank, and all the measurements were performed in triplicate. The percent inhibition of absorbance at 734 nm was calculated using the formula:

$$\text{ABTS}^{\bullet+} \text{ scavenging effect(\%)} = \frac{(A_B - A_A)}{A_B} \times 100 \quad (2)$$

where A_B represents the absorbance of the ABTS radical mixed with methanol. A_A represents the absorbance of the ABTS radical mixed with the elderberry hydrolate or standard. The Trolox equivalent antioxidant capacity (TEAC) was calculated by preparing a standard Trolox curve of a standard Trolox solution.

2.16.2. DPPH Radical-Scavenging Assay

The DPPH free-radical-scavenging capacity of the samples from the elderberry hydrolate was assessed following a previously outlined method [20], with slight adjustments, utilizing the stable DPPH radical, which exhibits an absorption peak at 515 nm. A solution of the DPPH radical was prepared by dissolving 2.4 mg of DPPH in 100 mL of methanol. To 3.995 mL of methanolic DPPH, a test solution (1%, 5% and 15% *w/v*) was added. The mixture was vigorously shaken and left at room temperature in darkness for 30 min. The absorbance of the reaction mixture was then measured at 515 nm using a spectrophotometer. Additionally, the absorbance of the DPPH radical without any hydrolate (i.e., the blank) was measured. All the experiments were conducted in triplicate. The ability to scavenge the DPPH radical was determined using the following equation:

$$\text{DPPH Scavenged(\%)} = \frac{(A_B - A_A)}{A_B} \times 100$$

where A_B represents the absorbance of the blank at $t = 0$ min, and A_A represents the absorbance of the hydrolate at $t = 30$ min. The Trolox equivalent antioxidant capacity (TEAC) was calculated by preparing a standard Trolox curve of a standard Trolox solution.

2.17. Statistical Analysis

The data analysis was conducted using IBM SPSS Statistics software, version 20 (Chicago, IL, USA). The statistical evaluation included an analysis of variance (ANOVA), followed by post hoc testing with Tukey's method. Statistical significance was determined at p -values less than 0.05.

3. Results

3.1. Elderberry Steam Distillation

In this study, 3330 mL of hydrolate was obtained. The *dried herb-to-hydrolate ratio* was determined to be 40.54%. This metric provides valuable insights into the efficiency of the steam distillation process, indicating the degree of enrichment of a hydrolate with volatile compounds. The higher the mass of the dried herb, the higher the content of volatile compounds in the hydrolate.

The pH of the obtained hydrolate was measured using a pH meter (MP511, Benchtop, Apera Instruments, Columbus, OH, USA), and the pH value was found to be 3.35.

3.2. Chemical Characterization

Direct hydrolate analysis, i.e., analysis of the unprocessed hydrolate, revealed the presence of three compounds, phenylacetaldehyde, 2-acetyl-pyrrole and an unknown compound (Table 2), accounting for 26.7%, 13.7% and 59.7% of the total composition according to their relative peak intensities, respectively. Phenylacetaldehyde was then quantified using a standard calibration curve. Using an injection volume of 1 μ L, a concentration of 0.063 ± 0.083 mg/mL was determined for the split 1:5 and a concentration of 0.068 ± 0.047 mg/mL for the split 1:10. Using a lower injection volume (0.5 μ L), better repeatability was achieved, particularly in the split 1:5; the concentration of phenylacetaldehyde was 0.096 ± 0.049 mg/mL.

Table 2. Chemical characterization.

Unprocessed Samples	Area %	
	26.7%	Phenylacetaldehyde
	13.7%	2-acetyl-pyrrole
	59.7%	MS: 43 (100) 44 (72) 144 (60)
Phenylacetaldehyde quantification		
	0.5 μ L, split 1:5	0.096 ± 0.049 mg/mL
	0.5 μ L, split 1:10	0.160 ± 0.074 mg/mL
	1 μ L, split 1:5	0.063 ± 0.083 mg/mL
	1 μ L, split 1:10	0.068 ± 0.047 mg/mL
Hexane Extracts	Area %	
	3.1%	n-hexanal
	4.5%	Furfural
	82.6%	Phenylacetaldehyde
	2.2%	(E)-beta-damascenone
Phenylacetaldehyde quantification		
	1 μ L, split 1:5	0.025 ± 0.001 mg/mL
Ethyl acetate extracts	Phenylacetaldehyde quantification	
	1 μ L, split 1:5	0.069 ± 0.003 mg/mL

In the hydrolate extracts with ethyl acetate, only phenylacetaldehyde was detected in an average concentration of 0.069 ± 0.003 mg/mL, which corresponds to the concentration of 0.009 mg/mL in the hydrolate. Four compounds were detected in the hexane extracts, n-hexanal, furfural, phenylacetaldehyde and (E)-beta-damascenone, accounting for 3.1%, 4.5%, 82.6% and 2.2% of the total composition according to their relative peak intensity, respectively. All four compounds have already been reported as volatile constituents of elderberries [21]. The average concentration of phenylacetaldehyde was 0.025 ± 0.001 mg/mL,

which corresponds to a concentration of 0.003 mg/mL in the hydrolate. The repeatability of hexane and ethyl acetate extractions was good.

3.3. Comet Assay: H_2O_2 -Challenge

In this study, the assessment of the DNA damage in PBMCs was conducted using the Comet assay to investigate the impact of the elderberry hydrolate concentrations and H_2O_2 challenges. The results revealed a noteworthy elevation in DNA damage in the 'C+' group exposed solely to H_2O_2 , signifying heightened genotoxic effects (Figure 3). Additionally, a potential concentration-dependent relationship between the elderberry hydrolate treatments and DNA damage emerged, with higher concentrations displaying trends of increased damage compared to lower concentrations. Additionally, the H_2O_2 -challenged groups that underwent elderberry hydrolate treatment showcased a notable reduction in GDI, positioning them favorably against the 'C+' group. Analyzing the GDI data, it becomes evident that the optimal outcome emerged with the 1% treatment, showcasing the most minor DNA damage among all the tested concentrations.

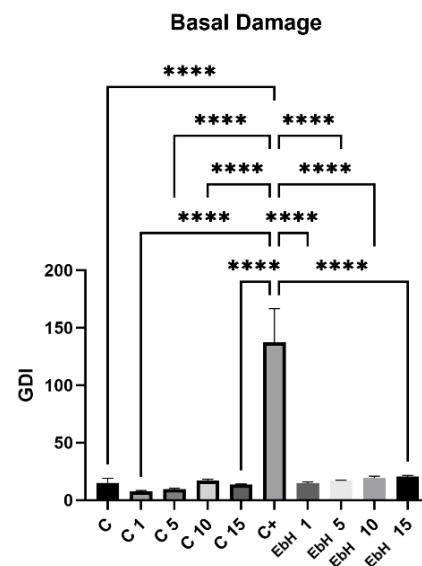


Figure 3. Assessment of the genetic damage indicator (GDI) in human PBMCs. The mean values of the DNA damage, quantified as arbitrary units using the *in vivo* Comet assay, were determined in both the unchallenged and H_2O_2 -challenged groups. The 'C' designation corresponds to the control group treated exclusively with PBS. The designations 'C1', 'C5', 'C10' and 'C15' correspond to PBS treatment and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). 'C+' signifies the group subjected solely to H_2O_2 treatment. The tested groups are distinguished by abbreviations denoting the constituent ingredient (EbH: elderberry hydrolate) and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). The quadruple asterisk stands for significant differences between the control treatment and all EbH treatments, but also between 'C+' and all control without the SN treatment. The error bars represent standard errors.

In the 'Basal Damage + Oxidative Damage' category, the 'EbH1' group exhibits the lowest mean DNA damage among the elderberry hydrolate-treated group. The control groups ('C', 'C1', 'C5', 'C10', 'C15') displayed diverse levels of DNA damage. Once again, the group exclusively treated with H_2O_2 ('C+') demonstrated significantly higher mean DNA damage, indicating its pronounced genotoxic effects (Figure 4, Basal Damage + Oxidative Damage).

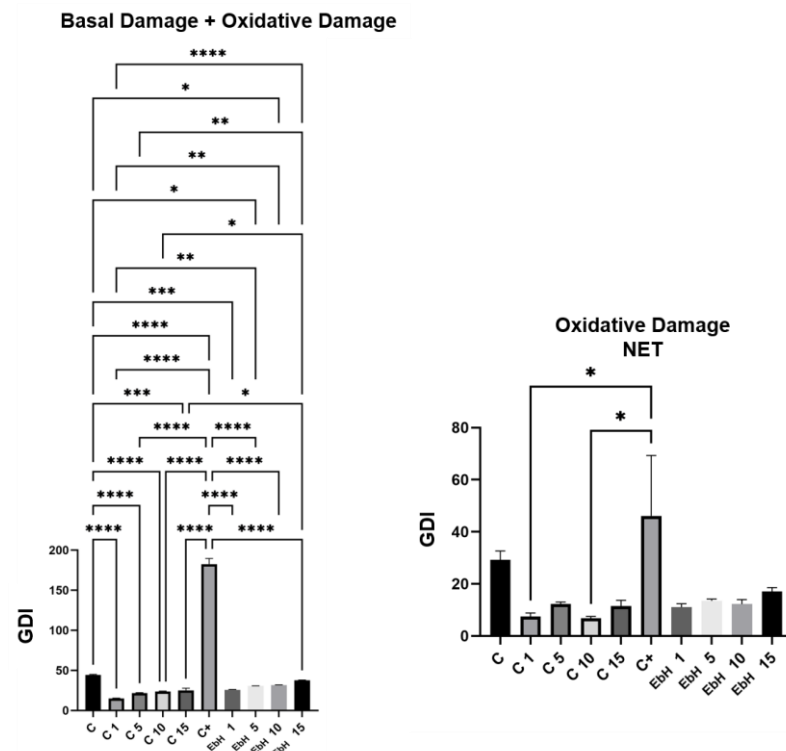


Figure 4. Assessment of the oxidative purine DNA damage. The enhanced Comet assay in human PBMCs post-elderberry hydrolate treatment quantified the mean oxidative purine DNA damage values. The ‘C’ designation corresponds to the control group treated exclusively with PBS. The designations ‘C1’, ‘C5’, ‘C10’ and ‘C15’ correspond to PBS treatment and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). ‘C+’ signifies the group subjected solely to H_2O_2 treatment. The tested groups are distinguished by abbreviations denoting the constituent ingredient (EbH: elderberry hydrolate) and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). The error bars represent standard errors. ‘Basal Damage + Oxidative Damage’ represents overall damage using Fpg, and ‘Oxidative Damage NET’ corresponds to the net Fpg-sensitive sites. Regarding ‘Basal Damage+Oxidative Damage’, the single asterisk stands for significant differences between the control treatment with PBS and EbH treatment of 5% and 10%, between ‘C10’ and ‘Eb15’ and between ‘C15’ and ‘EbH15’. The double asterisk stands for significant differences between ‘C1’ and treatment with EbH at 5%, 10% and 15%. The triple asterisk stands for significant differences between control treatment with PBS and EbH treatment of 15% and with ‘C15’. The quadruple asterisk stands for significant differences between control treatment with PBS and control treatment with 1%, 5% and 10% Ebh and also with ‘C+’; significant differences also between ‘C1’ and ‘C+’ and ‘EbH15’, between ‘C5’ and ‘C+’, between ‘C10’ and ‘C+’, between ‘C15’ and ‘C+’ and between ‘C+’ and all treatments of EbH and SN. Regarding the ‘Oxidative Damage NET’, the single asterisk stands for significant differences between ‘C1’ and ‘C+’ and between ‘C10’ and ‘C+’. Error bars illustrate the standard error.

Furthermore, in the ‘Oxidative Damage NET’ category, the ‘EbH1’ group exhibits the lowest mean DNA damage among the elderberry hydrolate-treated groups. Similar to previous categories, the group exclusively exposed to H_2O_2 (‘C+’) showed notably higher mean DNA damage, emphasizing its heightened genotoxic impact. Analyzing the GDI data with Fpg (Figure 4, Oxidative Damage NET), it becomes evident that the optimal outcome emerged with the 1% treatment, showcasing the most minor DNA damage among all the tested concentrations.

The effects of the different concentrations of elderberry hydrolate on the DNA damage were examined under basal and basal plus oxidative stress conditions (Table 3). Under basal damage conditions, the control group (‘C’) showed a GDI of 15.00 and a % DNA

in the tail of 3.75. The elderberry hydrolate treatment groups exhibited increased DNA damage with higher concentrations. ‘EbH 1’ had the lowest GDI of 14.75 and % DNA in the tail of 3.69. In the combined ‘Basal Damage + Oxidative Damage’ condition, the control group showed a GDI of 44.25 and % DNA in the tail of 11.06. The elderberry hydrolate treatment groups also showed increased DNA damage with higher concentrations. The lowest DNA damage was observed in ‘EbH 1’, with a GDI of 25.75 and % DNA in the tail of 6.44. These results indicate that elderberry hydrolate induces a dose-dependent increase in DNA damage under basal and oxidative stress conditions, with the highest concentration (‘EbH 15’) causing the most significant damage.

Table 3. Antigenotoxic effects of elderberry hydrolate on DNA damage in human peripheral blood mononuclear cells with H₂O₂ insult.

Treatment	GDI	% DNA in Tail
Basal Damage		
C	15.00	3.75
C 1	7.50	1.88
C 5	9.50	2.38
C 10	17.00	4.25
C 15	13.50	3.38
C+	137.41	34.35
EbH 1	14.75	3.69
EbH 5	17.00	4.25
EbH 10	19.25	4.81
EbH 15	20.50	5.13
Basal and Oxidative Damage		
C	44.25	11.06
C 1	15.00	3.75
C 5	21.75	5.44
C 10	23.75	5.94
C 15	25.25	6.31
C+	182.50	45.63
EbH 1	25.75	6.44
EbH 5	30.50	7.67
EbH 10	31.50	7.88
EbH 15	37.50	9.38

3.4. Comet Assay: SN Challenge

The investigation into the DNA damage in PBMCs revealed distinctive outcomes across the varied treatment groups. Within the ‘Basal Damage’ category, where the elderberry concentrations were tested, the group treated with a 1% elderberry concentration (‘EbH1’) consistently exhibited the lowest mean DNA damage, suggesting a potential protective effect against genotoxic stress. Conversely, the control groups (‘C’, ‘C1’, ‘C10’, and ‘C15’) displayed varying levels of DNA damage (Figure 5). Analyzing the GDI data, it becomes evident that the optimal outcome emerged with the 1% treatment, showcasing the most minor DNA damage among all the tested concentrations.

The extended analysis of the DNA damage in PBMCs using various treatments revealed nuanced outcomes across the distinct experimental groups. In the ‘Basal Damage + Oxidative Damage’ category, the ‘EbH5’ subgroup exhibited the lowest observed DNA damage (Figure 6, Basal Damage + Oxidative Damage). Following closely, the ‘EbH1’ subgroup demonstrated relatively lower DNA damage. Similarly, in the ‘Oxidative Damage NET’ subgroup, the ‘EbH10’ subgroup illustrated the least observed DNA damage (Figure 6, Oxidative Damage NET). Turning attention to the GDI data with Fpg (Figure 6, Oxidative Damage NET), it becomes evident that the most favorable outcome was achieved with the 10% treatment, as evidenced by the lowest recorded level of DNA damage.

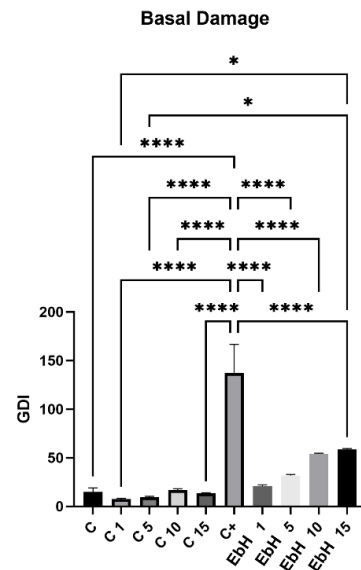


Figure 5. Assessment of the genetic damage indicator (GDI) in human PBMCs. The mean values of the DNA damage, quantified as arbitrary units using the in vivo Comet assay, were determined in both the unchallenged and SN-challenged groups. The ‘C’ designation corresponds to the control group treated exclusively with PBS. The designations ‘C1’, ‘C5’, ‘C10’ and ‘C15’ correspond to PBS treatment and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). ‘C+’ signifies the group subjected solely to SN treatment. The tested groups are distinguished by abbreviations denoting the constituent ingredient (EbH: elderberry hydrolate) and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). The single asterisk stands for significant differences between ‘EbH15’ and ‘C1’ and ‘C5’. The quadruple as asterisk stands for significant differences between all control groups and ‘C+’ and between ‘C+’ and all treated groups. The error bars represent standard errors.

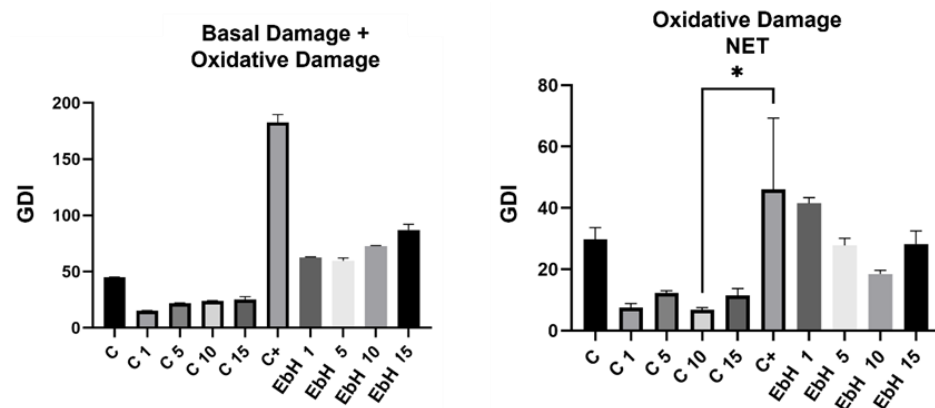


Figure 6. Assessment of the oxidative purine DNA damage following elderberry hydrolate treatment. The figure presents the mean oxidative purine DNA damage values quantified through the advanced Comet assay in human PBMCs post-elderberry hydrolate treatment. The ‘C’ designation corresponds to the control group treated exclusively with PBS. The designations ‘C1’, ‘C5’, ‘C10’ and ‘C15’ correspond to PBS treatment and the respective elderberry concentration (1:1%, 5:5%, 10:10%, and 15:15%). ‘C+’ signifies the group subjected solely to SN treatment. The tested groups are distinguished by abbreviations denoting the constituent ingredient (EbH: elderberry hydrolate) and the respective elderberry hydrolate concentration (1:1%, 5:5%, 10:10%, and 15:15%). The error bars represent standard errors. The ‘Basal Damage + Oxidative Damage’ label indicates overall damage assessment using Fpg, while ‘Oxidative Damage NET’ represents the count of the net Fpg-sensitive sites. The single asterisk stands for significant differences between ‘C10’ and ‘C+’. The error bars illustrate standard error.

The effects of varying concentrations of elderberry hydrolate on the DNA damage were investigated under ‘Basal Damage’ and ‘Basal Damage + Oxidative Damage’ stress conditions. Under the basal damage conditions, the control group (‘C’) exhibited a GDI of 15.00 and % DNA in the tail of 3.75. Among the elderberry hydrolate treatments, the lowest DNA damage was observed at ‘EbH 1’, with a GDI of 21.00 and % DNA in the tail of 5.25. Higher concentrations resulted in increased DNA damage, with ‘EbH 15’ showing the highest GDI of 58.75 and % DNA in the tail of 14.69 (Table 4).

Table 4. Antigenotoxic effects of elderberry hydrolate on DNA damage in human peripheral blood mononuclear cells with SN insult.

Treatment	GDI	% DNA in Tail
Basal Damage		
C	15.00	3.75
C 1	7.50	1.88
C 5	9.50	2.38
C 10	17.00	4.25
C 15	13.50	3.38
C+	137.41	34.35
EbH 1	21.00	5.25
EbH 5	31.75	7.94
EbH 10	54.00	13.50
EbH 15	58.75	14.69
Basal and Oxidative Damage		
C	44.25	11.06
C 1	15.00	3.75
C 5	21.75	5.44
C 10	23.75	5.94
C 15	25.25	6.31
C+	182.50	45.63
EbH 1	62.50	15.63
EbH 5	59.50	14.88
EbH 10	72.50	18.13
EbH 15	87.00	21.75

In the combined ‘Basal Damage + Oxidative Damage’ condition, the control group showed a GDI of 44.25 and % DNA in the tail of 11.06. The lowest DNA damage among the elderberry hydrolate treatments was again observed at ‘EbH 1’, with a GDI of 62.50 and % DNA in the tail of 15.63. Similar to the basal condition, higher concentrations led to greater DNA damage, with ‘EbH 15’ displaying the highest GDI of 87.00 and % DNA in the tail of 21.75.

These results indicate that, although elderberry hydrolate treatment at the lowest concentration (‘EbH 1’) exhibits the least DNA damage among the tested concentrations, it still results in higher DNA damage than the control group under basal and oxidative stress conditions.

3.5. Hydrogen Peroxide Scavenging Activity

We evaluated the ability of elderberry hydrolate to dismutate H_2O_2 to understand whether the protective effect observed in terms of genotoxicity resulted from the fact that elderberry hydrolate had a direct effect on H_2O_2 . However, we found that the hydrolate did not promote the dismutation of H_2O_2 . However, this result allows us to rule out a possible direct interaction of hydrolate with H_2O_2 , converting it into less harmful forms. Therefore, the observed effect of hydrolate could neutralize free radicals formed by the action of H_2O_2 in cells or through the activation of DNA repair systems present in cells, helping to correct damage caused by H_2O_2 before it becomes permanent.

Therefore, the observed protective effect of H_2O_2 hydrolate can be attributed to combining these mechanisms, which reduces the DNA damage caused by hydrogen peroxide.

3.6. Antioxidant Activity

The investigation into the antioxidant potential of elderberry hydrolate yielded significant insights (Figures 7 and 8). The analysis revealed a concentration-dependent relationship between elderberry hydrolate and the antioxidant activity of ABTS, as measured by Trolox equivalents (Figure 7). With increasing concentrations of elderberry hydrolate (ranging from 0% to 15%), there was a proportional rise in the Trolox equivalent values, demonstrating a corresponding escalation in the antioxidant capacity.

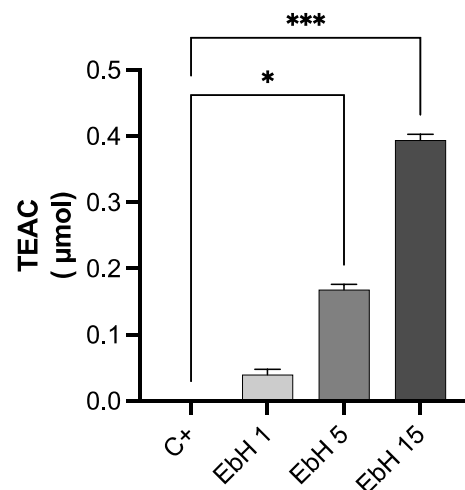


Figure 7. Relationship between the elderberry hydrolate concentration and the antioxidant activity by ABTS inhibition in μmol Trolox. The tested groups are distinguished by abbreviations denoting the constituent ingredient (EbH: elderberry hydrolate) and the respective elderberry hydrolate concentration (1:1%, 5:5% and 15:15%). The single asterisk stands for significant differences between 'C+' and 'EbH5'. The triple asterisk stands for significant differences between 'C+' and 'EbH15'.

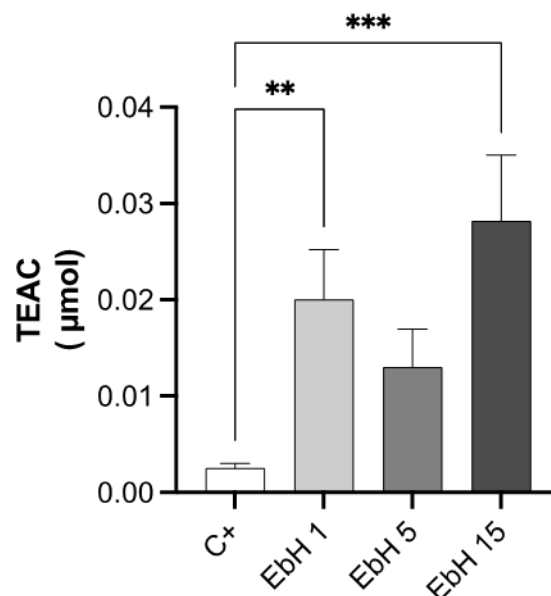


Figure 8. Relationship between the elderberry hydrolate concentration and the antioxidant activity by DPPH inhibition in μmol Trolox. The tested groups are distinguished by abbreviations denoting the constituent ingredient (EbH: elderberry hydrolate) and the respective elderberry hydrolate concentration (1:1%, 5:5% and 15:15%). The double asterisk stands for significant differences between 'C+' and 'EbH1'. The triple asterisk stands for significant differences between 'C+' and 'EbH15'.

The impact of the varying concentrations of hydrolate on the antioxidant activity was also investigated using the DPPH assay. The Trolox equivalent values were determined at concentrations of 0%, 1%, 5%, and 15% hydrolate. As expected, elderberry hydrolate shows antioxidant activity because, during the extraction process, some of the antioxidant compounds (e.g., furfural, phenylacetaldehyde and (*E*)-beta-damascenone) are released from the berry and dissolved in the water. Surprisingly, the results revealed a non-linear relationship between the hydrolate concentration and the antioxidant activity. At 1% concentration, the Trolox equivalent value significantly increased compared to the baseline, suggesting a positive influence of hydrolate on the antioxidant properties. However, at 5%, a decrease in the Trolox equivalent values was observed, indicating a complex concentration-dependent effect. Strikingly, the highest antioxidant activity was noted at a 15% hydrolate concentration, underscoring the presence of an optimal concentration for maximum efficacy.

4. Discussion

4.1. Elderberry Steam Distillation

Based on the calculated *dried herb-to-hydrolate ratio*, the results highlight an efficient transfer of aromatic, volatile compounds from elderberries, showcasing the proficiency of the extraction process and, ultimately, potential for therapeutic or cosmetic applications, also emphasizing the importance of steam distillation as a sustainable extraction technique. However, it has to be taken into account that variations in the *dried herb-to-hydrolate ratio* may arise from factors like the plant material characteristics, distillation parameters, and handling during collection and separation, while these may significantly affect the distillation efficiency. The observed pH level affects the potential applications and emphasizes the need for precise quality control. Adjusting the pH for specific applications may enhance elderberry hydrolate's utility in various industries.

This study explores elderberry's potential for use in natural cosmetics, investigating steam distillation as an accessible method for obtaining elderberry hydrolate. The findings contribute to understanding elderberry's benefits for skin health, emphasizing steam distillation's sustainability for aromatic and therapeutic extraction. Moreover, to the best of our knowledge, this is the first study on the composition and in vitro biological properties of elderberry hydrolate.

This study also contributes to a better understanding of the characterization of the volatile constituents of elderberry hydrolate. In the analysis of hydrolates, liquid–liquid extraction is usually used to extract the volatile compounds into an organic solvent due to limitations of GC-MS as a tool for analyzing aqueous samples [22]. Hexane is most commonly used as an organic solvent [23], although it is not an optimal extraction solvent for the purpose of identifying the composition of an hydrolate, as it is a highly non-polar solvent ($\log P = 3.90$). Therefore, we additionally used extraction with ethyl acetate, which is more polar ($\log P = 0.73$) than hexane but still does not mix with water. Considering that a hydrolate is an aqueous solution of volatile compounds and that polar volatile compounds are present in higher contents than less polar ones, we expected to detect more compounds in the ethyl acetate extract. However, this was not the case; we could only detect phenylacetaldehyde in the ethyl acetate extract, while (*E*)-beta-damascenone, n-hexanal and furfural were also detected in the hexane extract, but in trace amounts.

Generally, the total number of compounds detected in an hydrolate depends on the method chosen to analyze the hydrolate, with fewer compounds expected to be detected when the hydrolate is applied directly to the GC-MS, i.e., without pretreatment or preparation of the sample, than when it is previously extracted with an organic phase such as hexane or ethyl acetate. Considering this, we conclude that both direct GC-MS analysis of unprocessed hydrolate as well as the analysis of extracts are necessary to obtain the most detailed information possible about the composition of an hydrolate.

Elderberry has been widely recognized for its diverse array of bioactive compounds, including antioxidants, vitamins, and essential fatty acids [5]. The antioxidant properties

of elderberry are of particular interest, as they hold the potential to counteract oxidative stress and prevent premature aging. This aligns with previous studies that have suggested elderberry's capacity to protect the skin from free radical damage [8]. These findings also underline elderberry's anti-inflammatory and antimicrobial properties, which contribute to its ability to soothe irritated skin and defend against potential infections. The antigenotoxic properties reported in elderberry are of significant interest, suggesting a connection between its usage and anti-aging benefits. Altogether, elderberry's multifaceted attributes position it as a promising natural ingredient for enhancing skin health and addressing various skin concerns. We believe that the results of our study expand the possibilities of elderberry application by demonstrating the tested biological effects of elderberry hydrolate, which indicate potential cosmetic or therapeutic properties.

Steam distillation's significance in extracting essential oils and hydrolates from plant materials, rooted in traditional medicine and aromatherapy, minimizes waste and supports eco-friendly practices. Elderberry hydrolate production aligns with industry demand for natural and sustainable alternatives.

Crafting cosmetics through steam distillation allows customization for individual skin types and preferences, aligning with the trend of personalized skincare regimens. A notable observation occurred during the study as the essential oil presence was visually perceived during steam distillation, but none was collected. This prompted an investigation into the factors influencing this, such as variabilities in the essential oil content, volatility, and potential alternative compounds imparting aromatic characteristics. Errors or suboptimal parameters in the distillation process may have contributed, highlighting the need for meticulous reassessment to gain deeper insights into the steam distillation dynamics and implications for extracting valuable aromatic compounds.

4.2. Genotoxic Evaluation

The observed protective effects of elderberry hydrolate against genotoxic stress, as revealed through the Comet assay with H_2O_2 and SN challenges, align with the known properties of elderberry, particularly its rich phytochemical composition [24].

The elevation in DNA damage seen in the 'C+' group exposed solely to H_2O_2 underscores the pronounced genotoxic impact of hydrogen peroxide. In contrast, the concentration-dependent relationship observed in the elderberry hydrolate-treated groups suggests a nuanced interplay between the hydrolate concentrations and DNA protection. This aligns with the determined antioxidant properties of elderberry hydrolate, as antioxidants are known to scavenge reactive oxygen species, reducing oxidative stress-induced damage to cellular components, including DNA.

The optimal outcome observed with the 10% elderberry hydrolate treatment in the SN condition and the 1% treatment with the H_2O_2 condition suggests a potential concentration-specific protective effect. This finding may be attributed to the optimal balance of bioactive compounds at this concentration, as higher concentrations displayed trends of increased DNA damage. The 1% and 10% concentrations, with their minimal DNA damage and a notable reduction in genotoxicity, highlight the potential of elderberry hydrolate to mitigate oxidative stress. Also, the hydrolate likely enhances the cellular antioxidant defense systems, including the upregulation of antioxidant enzymes or direct scavenging of free radicals.

Furthermore, the diverse outcomes observed with the different hydrolate concentrations and the challenges underscore the complexity of elderberry hydrolate's effects on DNA damage. The nuances in the results may be linked to variations in the concentrations of specific phytochemicals within elderberry hydrolate, each contributing differently to its overall protective capacity.

The presence of phenylacetaldehyde, 2-acetyl-pyrrole, and the unidentified compound in elderberry hydrolate suggests potential bioactivity that could correlate with the observed genotoxic effects. Phenylacetaldehyde, predominant (26.7%) in the hydrolate as determined when using the unprocessed sample, as well as in both ethyl acetate (100%; it was the only

detected compound) and hexane (82.6%) extracts, is a known fragrant compound that has demonstrated various biological activities, including antimicrobial and antioxidant properties [25–27]. These properties might contribute to the antigenotoxic effects observed in the Comet assay, as antioxidants are known to mitigate oxidative stress-induced DNA damage.

The unidentified compound, which constitutes a significant portion of the hydrolate (59.7%; unprocessed hydrolate), warrants further investigation to elucidate its structure and potential biological activity. The genotoxic evaluation, which showed a reduction in DNA damage, indicates that the combined effect of these compounds may confer protective properties against genotoxic agents.

Moreover, 2-acetyl-pyrrole, another identified compound, has been studied for its potential biological activities, including its antioxidant properties [28,29]. This compound might also play a role in the antigenotoxic effects observed, although its exact mechanism of action in this context remains to be further explored.

n-Hexanal (3.3%) and furfural (4.5%), detected in hexane extracts at lower concentrations, also possess known biological activities, including antioxidant properties [30,31]. Their presence, although minor, might synergistically enhance the overall antigenotoxic effect of the hydrolate.

(E)-beta-Damascenone, a compound present in hexane extracts at 2.2%, is another contributor to the biological activity of the hydrolate. Its antioxidant properties further support the hydrolate's ability to protect against DNA damage [32].

Furthermore, the 2-acetyl-pyrrole (13.7%; unprocessed hydrolate) in elderberry hydrolate may enhance its ability to protect against DNA damage. The antioxidant properties of 2-acetyl-pyrrole could mitigate the effects of oxidative stress induced by genotoxic agents like SN and H₂O₂ [29]. This protective effect would be consistent with the observed reduction in DNA damage in the Comet assay.

The chemical characterization of elderberry hydrolate identifies essential compounds that likely contribute to its biological activity. The genotoxic evaluation supports the hypothesis that these compounds, particularly phenylacetaldehyde and possibly the unidentified compound, have antigenotoxic potential. Further studies are required to fully understand the mechanisms by which these compounds exert their protective effects against DNA damage.

These findings underscore the potential of elderberry hydrolate as a valuable resource for applications requiring antioxidant and antigenotoxic properties, such as in cosmetics. Further studies are needed to fully elucidate the mechanisms by which these compounds exert their protective effects and identify the unidentified compound's structure and activity.

4.3. Hydrogen Peroxide Scavenging Activity

The absence of detectable H₂O₂-scavenging activity in the elderberry hydrolate is due to the absence of chemical compounds capable of catalyzing the breakdown of H₂O₂ into H₂O and O₂, as well as due to the inactivation of enzymes like catalase and other peroxidases during the steam distillation process, which holds significant implications for understanding the potential protective role of elderberry hydrolate against DNA damage. Catalase is a crucial enzyme responsible for catalyzing the breakdown of H₂O₂ into water and oxygen, thereby preventing the accumulation of this reactive oxygen species (ROS) and mitigating oxidative stress-induced damage [33].

The steam distillation process, involving elevated temperatures, may have resulted in the denaturation or inactivation of peroxidases present in the elderberries. This inactivation could explain the lack of catalase activity observed in the subsequent assay, where the elderberry hydrolate was tested for its ability to dismutate H₂O₂ [34].

In light of these findings, the absence of catalase activity in elderberry hydrolate suggests that other antioxidant mechanisms may contribute to its potential protective effects against DNA damage. The observed concentration-dependent reduction in DNA damage, particularly under oxidative stress conditions, implies that elderberry hydrolate may harbor bioactive compounds capable of scavenging free radicals and mitigating

genotoxic effects. These protective effects on DNA may be attributed to the presence of other antioxidants, such as polyphenols or flavonoids, which could play a role in counteracting oxidative damage.

The implications of elderberry hydrolate as a protective agent against DNA damage, even without antioxidant enzymes, highlight its bioactive components' complex and multifaceted nature. Further investigations into the specific antioxidant compounds present in elderberry hydrolate, their mechanisms of action, and their potential synergistic effects will contribute to a more comprehensive understanding of elderberry's role in mitigating oxidative stress and preserving DNA integrity. These findings have promising implications for developing natural products with DNA-protective properties suitable for skin care applications and other industries.

4.4. Antioxidant Activity

The results obtained with the two methods for evaluating antioxidant activity (ABTS and DPPH) strongly suggest that elderberry hydrolate has considerable antioxidant potential, with higher concentrations correlating with more significant antioxidant activity [35]. These data underscore the promising role of elderberry hydrolate as a source of natural antioxidants, holding potential implications for various industries seeking natural antioxidant-rich compounds for product development and health applications.

These findings shed light on the nuanced relationship between hydrolate's qualitative and quantitative composition and antioxidant activity, providing valuable insights for further exploration of the underlying mechanisms and potential applications in cosmetics, functional foods or pharmaceuticals. It should be noted that the antioxidant properties of the compounds present in hydrolate may explain the protective effect observed in the DNA damage induced by H_2O_2 and SN. However, it cannot be ruled out that some of the compounds may stimulate the DNA-repairing systems present in the cell.

4.5. Limitations

While this study provides valuable insights into the potential benefits of elderberry hydrolate for skincare and other applications, several limitations warrant consideration. Firstly, the study's sample size, which relied on data obtained from a single healthy volunteer, may limit the generalizability of the findings to a broader population. Additionally, using elderberries sourced from a specific location and at a specific time may introduce variability due to geographical differences and seasonal variations in plant composition. Confounding variables such as diet, lifestyle factors, and environmental exposures were not controlled for, potentially influencing the observed outcomes. Addressing these limitations through larger sample sizes, controlled experimental designs, and consideration of potential confounders could enhance the validity and generalizability of future research in this area.

5. Conclusions

This study sheds light on the potential of elderberry as a natural ingredient in skincare and beauty products. The exploration of elderberry steam distillation highlights its diverse benefits for skin health and emphasizes the accessibility and sustainability of using this method. As the demand for natural cosmetics and sustainable practices continues to rise, these findings contribute to the growing body of research that supports the integration of nature's resources into contemporary skincare regimens. By bridging tradition and modern science, elderberry exemplifies the harmonious synergy between nature and personal well-being. This study highlights the multifaceted attributes of elderberry hydrolate, positioning it as a promising natural ingredient for enhancing skin health and addressing various skin concerns. The presence of bioactive compounds such as phenylacetaldehyde and 2-acetyl-pyrrole, along with the potential activity of the unidentified compound, underscores the hydrolate's potential in cosmetics and other applications requiring antioxidant and antigenotoxic properties. Further research is needed to fully elucidate the mechanisms

by which these compounds exert their protective effects and to identify the unidentified compound's structure and activity. These findings contribute to the growing body of knowledge on elderberry's benefits, supporting its use as a valuable natural and sustainable product development resource.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of the University of Trás-os-Montes and Alto Douro (Doc4-CE-UTAD-2023, 27 January 2023).

Informed Consent Statement: Informed consent was obtained from all the subjects involved in the study.

Data Availability Statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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Conflicts of Interest: The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflicts of interest.

Abbreviations

Abbreviation	Full Term
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
C	Control group treated with PBS
C 1, C 5, C 10, C 15	Control groups treated with PBS and the respective elderberry hydrolate concentrations (1%, 5%, 10%, 15%)
C +	Group subjected solely to SN treatment
DPPH	2,2-diphenyl-1-picrylhydrazyl
EbH	Elderberry hydrolate
Fpg	formamido-pyrimidine[fapy]-DNA glycosylase
GDI	Genetic damage indicator
HRP	Horseradish peroxidase
HVA	Homovanillic acid
H ₂ O ₂	Hydrogen peroxide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
SN	Streptonigrin
TEAC	Trolox equivalent antioxidant capacity

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