

Article

Dual Evaluation of *Malva* Extract in Eye-Drop Formulations: Antioxidant Efficacy and Physicochemical Properties Relevant to the Treatment of Dry Eye Disease

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Abstract

Background/Objectives: Dry eye disease (DED) is a multifactorial condition affecting the ocular surface. It is characterized by tear film instability, hyperosmolarity, inflammation, and oxidative stress. First-line treatment for DED relies on lubricating and hydrating eye drops, usually containing hyaluronic acid (HA), which supports tear film stability and epithelial healing. However, HA alone cannot correct oxidative stress, a key driver of cellular damage and inflammation in DED. Accordingly, this study aimed to evaluate the antioxidant capacity of *Malva sylvestris* tincture (MalvaT) and its physicochemical properties in experimental eye-drop formulations containing HA. **Methods:** The antioxidant activity of reconstituted MalvaT lyophilisate (Malva) was assessed in cell-free assays against several oxygen radicals and in cell-based assays using the human HaCat keratinocyte cell line. The refractive index was measured in eye-drop formulations containing 0.15% or 0.3% HA and 0.5% MalvaT. Surface tension was assessed in eye-drop formulations containing 0.15% HA and increasing concentrations (0.25–2.0%) of MalvaT. **Results:** Malva showed potent oxygen radical scavenging activity in both cell-free and cell-based assays, indicating its antioxidant capacity and the efficient cellular uptake of antioxidant components. The refractive indices of experimental eye-drop solutions containing HA and MalvaT were close to that of tear fluid (1.334). The surface tension of the experimental eye-drop formulations, while not impacted by 0.15% HA, was significantly reduced by increasing concentrations of MalvaT ($p < 0.0001$). At the concentration of 0.5% MalvaT, the mean surface tension was reduced from 68.17 mN/m (HA control) to 59.80 mN/m (HA + MalvaT), thereby bringing it closer to that of tear fluid. **Conclusions:** This pre-clinical study suggests that combining the antioxidant properties of Malva tincture with the lubricating and hydrating effects of HA in eye-drop formulations exhibiting optimal rheological characteristics may offer a promising therapeutic approach for managing DED.

Keywords: *Malva sylvestris*; mallow flower extract; malva extract; hyaluronic acid (HA); dry eye disease (DED); surface tension; refractive index; antioxidant activity; oxidative stress; reactive oxygen species (ROS)

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1. Introduction

Dry eye disease (DED) is a complex, multifactorial disorder of the ocular surface characterized by tear film instability, hyperosmolarity, chronic inflammation, and

oxidative stress [1–4]. These pathophysiological changes lead to discomfort, visual disturbance, and potential damage to the ocular surface, significantly impairing quality of life [1,2]. Epidemiological studies estimate that DED affects up to 34–50% of the global population, with prevalence increasing with age, screen use, and environmental factors [1,3,5]. In particular, exposure to high-energy blue light from prolonged screen use has been closely linked to increased digital eye strain and tear film instability, while excessive ultraviolet (UV) radiation from sunlight is known to contribute to ocular surface damage—both factors playing a recognized role in the onset and progression of DED [6–8].

Current management strategies for mild to severe DED primarily rely on lubricating eye drops to correct tear hyperosmolarity and improve tear film stability, while providing symptomatic relief [1,9,10]. Hyaluronic acid (HA) is one of the most widely used polymers in ophthalmic formulations, typically at concentrations ranging from 0.1% to 0.4%. Its widespread acceptance is due to its unique rheological profile, especially its viscoelasticity, water-retention capacity, and mucoadhesive properties, which enhance ocular surface hydration and lubrication, prolong tear film breakup time (a marker of tear film stability), and support epithelial healing [10–13]. However, standard lubricating eye drops, including those containing HA, lack intrinsic antioxidant activity and therefore cannot counteract oxidative stress—a major driver of cellular damage and inflammation in DED [3,4].

Oxidative stress and inflammation are central to the vicious cycle of DED, where tear hyperosmolarity due to loss of tear volume triggers epithelial stress responses, cytokine release, and reactive oxygen species (ROS) generation, further destabilizing the tear film and amplifying tissue damage [1,3,4]. To break this cycle, recent research has focused on incorporating natural substances with antioxidant and anti-inflammatory properties into eye-drop formulations [14]. Among these, *Malva sylvestris* (also known as common mallow) stands out as a traditional medicinal plant known for its soothing and anti-inflammatory effects [15]. It is rich in polyphenols and flavonoids that exhibit antioxidant and wound-healing activities [15–21]. A recent in vitro study has shown that Malva flower extracts reduce ROS levels, modulate pro-inflammatory cytokine secretion, and promote epithelial repair in models relevant to DED [21]. Preliminary clinical evidence from a recent open-label, multicenter, crossover pilot study in patients with moderate DED suggests that combining HA with Malva extract in eye drops may improve patient-reported outcomes compared to HA alone [22], indicating a promising therapeutic benefit of this HA-plus-Malva formulation.

Beyond bioactivity, physicochemical properties such as surface tension and refractive index are critical for ophthalmic formulations. Surface tension influences droplet formation, spreading on the ocular surface, and tear film stability, while the refractive index ensures optical clarity and compatibility with vision [23–26]. Despite their importance, systematic characterization of these physicochemical properties in eye-drop formulations remains limited [24,27,28].

Therefore, the aim of this study was to evaluate the antioxidant activity and physicochemical properties—specifically the surface tension and refractive indices—of Malva extract alone and in combination with HA in the context of eye-drop formulations for DED.

2. Materials and Methods

2.1. *Malva Sylvestris* Flower Extract

Malva sylvestris was organically grown and harvested for Weleda AG (GPS position: 48°48'37.2" N, 9°46'11.4" E). Botanical identification was carried out by qualified personnel in accordance with the testing specification, using both macroscopic and microscopic characteristics. Thin-layer chromatography (TLC) was performed following the European Pharmacopoeia (Ph. Eur.) method 2.2.27 to confirm that the chromatogram met the specified requirements.

Hydroethanolic liquid extracts of *M. sylvestris* flowers (Malvae flos sicc., ethanolic infusum Ø = D1 V.20, ethanol: 43% [m/m]) were prepared in accordance with the manufacturing guidelines described in Ph. Eur. method 1.2.13 (GHP 20). Briefly, whole dried flowers were mixed with ethanol 43% (m/m) and macerated in closed containers at room temperature for 15 min. The mixture was then boiled under reflux for 5 min, cooled to 40–45 °C, and left to macerate for an additional 12–36 h in a closed container. Following maceration, the mixture was pressed and then filtered to yield the tincture (hereafter referred to as “MalvaT”). MalvaT contains 43% (m/m) ethanol. Accordingly, 43% (m/m) ethanol in water served as a vehicle control in experiments testing MalvaT (i.e., refractive index and surface tension measurement experiments; see Sections 2.6 and 2.7, respectively).

The dry extract was obtained from the tincture (MalvaT) by concentration in a rotary evaporator (Rotavapor® R-300; Büchi Labortechnik AG, Flawil, Switzerland) at 40–45 °C under an absolute air pressure of 100–500 mbar for 2–3 h to remove ethanol. The concentrate was immediately frozen and subsequently freeze-dried using an ALPHA 1–4 LSC lyophilizer (Christ, Osterode am Harz, Germany). Residual moisture (3.2%) in the resulting dry extract (MalvaT lyophilisate) was determined with a Halogen Moisture Analyzer HB43 (Mettler Toledo, Greifensee, Switzerland) at 105 °C for 30 s.

For experimentation (antioxidant assays), MalvaT lyophilisate was dissolved using either 50% ethanol/50% purified water (m/m) (DPPH assay; see Section 2.4), 50% acetone/50% purified water (*v/v*) (ORAC assay; see Section 2.3), or DMSO (CAA assay; see Section 2.5), yielding a stock solution at 30 mg/mL. The suspension was vortexed for 10 min and then sonicated for 30 min with occasional vortexing. Samples were centrifuged for 10 min at 3000× *g*. The supernatant was harvested and used for experiments (hereafter referred to as “Malva”).

In addition to Malva extracts (Malva and MalvaT), the Visiodoron Malva® eye-drop formulation (Weleda AG, Arlesheim, Switzerland) containing 0.15% hyaluronic acid (HA) and 0.5% Malva tincture in Eye Drop Buffer (ED Buffer; 7.77 g/L sodium chloride, 4.21 g/L tri-sodium citrate dihydrate, 0.027 g/L citric acid monohydrate in injection-grade purified water; pH 6.0–8.0; osmolality: 250–330 mOsm/kg) was tested as a control in some experiments (see Section 2.6). The concentration of Malva lyophilisate in Visiodoron Malva® is equivalent to 124 µg/mL. Considering an eye-drop volume of 33 µL and assuming that the volume of tear fluid (7 µL) plus that of conjunctival pocket (30 µL) is 37 µL [29,30], a 2.1-fold dilution of Visiodoron Malva® is expected upon topical application of one eye drop on the ocular surface, resulting in a local concentration of Malva of about 60 µg/mL.

Experimental eye-drop solutions were prepared using 0.15% or 0.3% HA (Contipro a.s., Dolní Dobrouč, Czech Republic) dissolved in ED Buffer, with or without various concentrations (0.5% or 0.25–2.0%) of MalvaT.

2.2. Composition and Semi-Quantitative Analysis of Malva Tincture (MalvaT)

The MalvaT composition was analyzed by the Austrian Drug Screening Institute GmbH (ADSI; Innsbruck, Austria) using ultra-high-performance liquid chromatography with quadrupole time-of-flight tandem mass spectrometry (UHPLC-UV-hr-qTOF/MS). MalvaT (50 µL) was diluted in 950 µL of an ethanol/water mixture (50/50, *v/v*) and measured as a technical duplicate. Chromatographic separation was achieved on the UHPLC ACQUITY Premier System (Waters Corporation, Milford, MA, USA) using an RRHD Zorbax C18 column (2.1 × 100 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA, USA). The mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B). The flow rate was set to 0.4 mL/min, the injection volume to 10 µL, the column temperature to 40 °C, and the autosampler temperature to 10 °C. The following linear gradient of the mobile phase B was applied: 0 min 5%, 5 min 25%, 14 min 60%, 20 min 100%, 23.5 min 100%, 24 min 5%, and 26 min 5%.

The liquid chromatography eluate was applied to the high-resolution Xevo G3 qTOF mass spectrometer (Waters Corporation, Milford, MA, USA). Ionization was performed using electrospray ionization (ESI) in positive and negative MSE modes over m/z 50–800, with a 6 V low-energy collision voltage and a 10–30 V high-energy collision ramp. The capillary voltages were 3.0 kV (ESI⁺) and 2.5 kV (ESI[−]), the cone voltage was 40 V (for both positive and negative modes), the source temperature was 100 °C, and the desolvation temperature was 250 °C. Nitrogen served as the collision and desolvation gas, with cone and desolvation gas flows of 100 and 1000 L/h, respectively. Mass accuracy was verified by sodium formate calibration and maintained through continuous lock-mass correction.

A semi-quantitative approach was applied by adding 100 µg/L amarogentin as an internal standard (IS) to the MalvaT sample. The peak areas of the detected compounds were normalized to the peak area of amarogentin. If a compound was detected in positive and in negative ionization mode, both relative values were reported. Notably, the values obtained in positive and negative ionization modes are not directly comparable, as amarogentin exhibits higher ionization efficiency in the positive mode, resulting in generally lower relative values for the detected compounds. Tentative peak assignments were made by calculating elemental compositions from high-accuracy mass data and confirming candidate structures through fragment-ion comparison against existing databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG) [31], MassBank [32], the Human Metabolome Database (HMDB) [33], and Natural Product Updates (NPU) [34].

2.3. Oxygen Radical Absorbance Capacity (ORAC) Cell-Free Assay

Oxygen radical absorbance capacity (ORAC)-based methods were used to measure the antioxidant capacity of Malva to neutralize biologically relevant reactive oxygen species (free radicals) [35–37]. The ORAC assay uses a fluorescent (e.g., fluorescein) or a fluorogenic (e.g., dihydrorhodamine-123 [DHR-123] and hydroethidine [HE]) molecule as an indicator of antioxidant activity. Oxidative damage to the indicator by free radicals results in either a loss (fluorescein) or an increase (fluorogenic probe) in fluorescence over time. Antioxidants can prevent oxidative damage by neutralizing free radicals, via hydrogen atom transfer (HAT; i.e., the dominant *in vivo* mechanism), thus preventing fluorescence loss/acquisition. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) or caffeic acid are usually used as a reference antioxidant [35–37]. Four ORAC assays were conducted by Brunswick Laboratories, Inc. (Southborough, USA) using various physiological (oxygen or nitrogen) radicals: peroxy (ROO•), hydroxyl (OH•), peroxynitrite (ONOO[−]), and superoxide (O₂•[−]) radicals, as previously described [36,37]. Reference compounds and Malva were prepared in 50% acetone/50% purified water (*v/v*). Accordingly, the vehicle control was 50% acetone/50% purified water (*v/v*).

For the peroxy radical assay, 2,2'-Azobis (2-amidino-propane) dihydrochloride (AAPH) was used as the source of peroxy radicals, which were generated by spontaneous decomposition of AAPH at 37 °C, and fluorescein was used as an indicator of antioxidant activity. Trolox (0.63–80 µg/mL; 2-fold dilution series) was used as a reference standard in parallel to Malva (3.7–472.5 µg/mL; 2-fold dilution series).

For the hydroxyl radical assay, fluorescein was used as an indicator of antioxidant activity, and the hydroxyl radical was generated from a reaction between cobalt and hydrogen peroxide (Fenton-type reaction). Caffeic acid (1.95–33.37 µg/mL; 1.5-fold dilution series) was used as a reference standard, in parallel to Malva (58.52–1000 µg/mL; 1.5-fold dilution series).

For the peroxynitrite assay, 3-morpholinosyndnonimine hydrochloride (SIN-1) was used as source of peroxynitrite, which is generated by spontaneous decomposition of SIN-1. The DHR-123 fluorogenic probe was used as an indicator molecule to detect an increase in fluorescence upon oxidative damage or a decrease in the presence of antioxidants

(neutralization of peroxynitrite). Trolox (0.16–20 µg/mL; 2-fold dilution series) was used as a reference standard in parallel to Malva (4.41–561 µg/mL; 2-fold dilution series).

For the superoxide assay, superoxide anions were generated by the catalyzation of xanthine oxidation by xanthine oxidase. The HE fluorogenic probe was used as an indicator of antioxidant activity. HE becomes highly fluorescent upon oxidation by superoxide anions. A decrease in fluorescence indicates antioxidant activity. Trolox (156.3–10,000 µg/mL; 2-fold dilution series) was used as a reference standard in parallel to Malva (117.2–7500 µg/mL; 2-fold dilution series).

ORAC values were calculated as previously described [36,37]. The percentage (%) of neutralization of oxidative damage by free radicals was calculated relative to the vehicle control (representing 0% inhibition or maximum oxidative stress) and the no-radical control (representing 100% inhibition or maximum antioxidant protection). Respective half-maximal inhibitory concentrations (IC₅₀) were calculated, and data were expressed as the mean (SD) % neutralization of the free radicals from duplicate measurements.

2.4. DPPH Free Radical Scavenging Assay

The cell-free DPPH assay was used to measure the ability of Malva to reduce the stable artificial free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) into 2,2-diphenyl-1-picrylhydrazine (DPPH-H) via single-electron transfer (SET). This reaction is measured by the change in color from violet (DPPH; absorbing at ~530 nm) to yellow (DPPH-H). The decrease in absorbance at 530 nm is proportional to the antioxidant activity of the sample [35]. Malva was prepared in 50% ethanol/50% purified water (m/m) and Trolox reference standard in 100% DMSO.

DPPH assays were performed by Eurofins Panlabs Discovery Services (Taiwan Ltd., New Taipei City, Taiwan). Malva (3–300 µg/mL in 0.5% ethanol final concentration; ~3-fold dilution series), Trolox reference standard (Sigma-Aldrich, now Merck, Darmstadt, Germany; 0.25–25 µg/mL in 1% DMSO final concentration; ~3-fold dilution series) and respective vehicle controls (0.5% ethanol or 1% DMSO) were incubated with DPPH (Sigma-Aldrich, now Merck, Darmstadt, Germany; 250 µM in 0.5% ethanol) for 5 min at 25 °C in a final volume of 100 µL in a 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA). Scavenger activity was determined using the spectrophotometer Infinite M200 Pro (Tecan, Männedorf, Switzerland) by quenching the DPPH radical at a wavelength of 530 nm.

The % inhibition of the free radical DPPH relative to the vehicle control (0% inhibition or maximum oxidative stress) and the no-radical control (100% inhibition or maximum antioxidant protection) was calculated for Malva and Trolox. Respective half-maximal inhibitory concentrations (IC₅₀) were calculated, and data were expressed as the mean (SD) % inhibition of DPPH from duplicate measurements.

2.5. Cellular Antioxidant Activity (CAA) Assay

The CAA assay was used to assess the antioxidant potential of Malva in adherent living cells. The CAA assay measures the fluorescence produced by oxidation of a non-fluorescent dye pre-absorbed into cells after the addition of a radical generator that produces peroxy radicals inside the cells. The addition of antioxidants neutralizes radicals and therefore fluorescence formation, allowing the quantification of intracellular antioxidant activity [38].

The CAA assay was performed by Institut Kurz GmbH (Cologne, Germany), as previously described [38] and according to a modified version of the method described by Wolfe and Liu [39], using the immortalized human keratinocyte cell line HaCat (obtained by Institut Kurz from Izler, Brescia, Italy; cat. no. BSCL168) [40]. Briefly, HaCat cells were cultured to confluence, then pre-incubated in replicate wells ($n = 4$) for one hour with the

cell-permeable dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 25 μ M) and either the vehicle control (1% DMSO), the reference standard (5 μ M and 50 μ M quercetin in 1% DMSO) or increasing concentrations of the Malva test sample (25–300 μ g/mL in 0.08–1% DMSO). Cells were washed with sterile PBS to remove excess non-absorbed compounds and the free radical initiator 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH; 600 μ M) was added to the wells. The kinetics of oxidation of the intracellular dye (i.e., the fluorescence intensity of the resulting 2',7'-Dichlorofluorescein [DCF] product, in relative light units [RLU]) was monitored every 5 min for one hour with the FLUOstar Optima microplate fluorometer (BMG Labtech, Ortenberg, Germany; excitation: 485 nm, emission: 540 nm). The area under the fluorescence curve (AUC) vs. time was integrated using the FLUOstar Optima Data Analysis software version 1.3 (BMG Labtech, Ortenberg, Germany). The AUC values of the Malva and quercetin samples were normalized to those of the vehicle control to determine the CAA units, according to the formula

$$\text{CAA Units} = 100 - (\text{AUC}_{\text{sample}}/\text{AUC}_{\text{vehicle}}) \times 100$$

Data were expressed as mean (SD) CAA units of quadruplicate measurements.

2.6. Refractive Index Measurement Method

The refractive index of a given medium, as described in Ph. Eur. method 2.2.6., represents the ratio of the phase velocity of light in a vacuum to its velocity in that medium. It measures how much light refracts when it passes from one medium into another. In an eye-drop formulation, the refractive index should be close to that of natural tear fluid to ensure visual comfort and clarity. The refractive indices of the Visiodoron Malva product and MalvaT (0.5% final; prepared from Malva tincture at 43% [m/m] ethanol) containing 0.15% or 0.3% HA in ED Buffer were measured using the ATR-F refractometer (Schmidt & Haensch GmbH, Berlin, Germany). Samples were placed on the refractometer pre-heated to 34 °C, which corresponds to ocular surface temperature. Measurements ($n = 3$ per sample) were made at a wavelength of 589 nm. The refractometer was equilibrated with distilled water until the refractive index had stabilized for at least one minute. The measuring surface of the refractometer was thoroughly cleaned between each measurement. Data were expressed as the mean (SD) refractive index of triplicate measurements.

2.7. Surface Tension Measurement

To evaluate the surface moistening capacity of MalvaT, surface tension measurements were conducted. The surface tension of liquids is defined as the free surface enthalpy per unit of surface area, given in millinewtons per meter (mN/m). The principle of the test method is based on the measurement of the maximum force which is necessary to change the surface area of a sample. Accordingly, a reduction in surface tension indicates a potential reduction in tear film breakup and an increase in surface moistening capacity.

The surface tension of the liquid samples was determined at a constant temperature by applying the ring tensiometer method, as described in DIN EN 14370 [41]. Surface tension was measured using the commercial Force Tensiometer K11 (Krüss GmbH, Hamburg, Germany) with glass vessels that had an inner diameter of 47 mm and a platinum-iridium wire ($d = 0.4$ mm; circumference 60 mm) as a measuring ring. Between each measurement, all parts of the device were cleaned with double-distilled water and subsequently rinsed with 2-propanol. The solvent was removed by evaporation at room temperature. Finally, the inner surface of the vessel was flamed out with a gas burner. The measuring ring was rinsed with water and 2-propanol and heated up to red heat with a gas burner. For each measurement vessel, the overall operability and cleanliness of the test equipment was verified by measuring the surface tension of double-distilled water, which was cross-checked with the literature-reported value of 72.75 mN/m at 20 °C [42].

Upon verification of the test equipment, the surface tension of the test sample was measured in the same vessel. Surface tension was measured on the ED Buffer control, 0.15% HA in ED Buffer, 0.15% HA + various concentrations (0.25–2.0%) of MalvaT (in ED Buffer; prepared from Malva tincture at 43% [m/m] ethanol) or of the respective ethanol control (in ED Buffer; prepared from a control solution of 43% ethanol in water), as indicated in the respective figure legends. Each sample was placed in a temperature-controlled measurement vessel and equilibrated at 25 °C for 30 min. Ten consecutive surface tension measurements were performed via the tensiometer automatic measuring procedure. Raw values were corrected according to Harkins–Jordan, as described in DIN EN 14370 [41], by the instrument software K11, firmware version 1.0. The mean value of the displayed surface tensions was calculated and reported as the test result. Each experiment was performed in triplicate vessels, and data were expressed as the mean (SD) surface tension of triplicate measurements in mN/m.

2.8. Statistical Analysis

Curves and graphs were prepared using GraphPad Prism version 10.4.2 (GraphPad Software Inc., San Diego, CA, USA). All values are presented as the mean $\pm$ standard deviation (SD). Data are representative of at least two independent experiments, each including duplicate to quadruplicate measurements. Half-maximal inhibitory concentrations (IC_{50}) of Malva and reference standards in the cell-free ORAC and DPPH assays were calculated in GraphPad Prism using a four-parameter logistic (4PL) model (nonlinear regression using a sigmoidal dose–response). Statistical analysis of group comparisons (vs. the control, as indicated in the respective figure legends) was performed using a one-way ANOVA followed by Dunnett’s multiple comparison test. Statistical analysis of the effect of increasing concentrations of Malva on surface tension (vs. the respective ethanol controls) was conducted using a two-way ANOVA followed by Šídák-adjusted post hoc pairwise comparisons. A p -value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Composition of Malva Extract

The composition of the Malva tincture (MalvaT) was analyzed using ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-UV-hr-qTOF/MS). Semi-quantitative profiling was carried out by normalizing the chromatographic peak areas to that of the internal standard amarogentin, following electrospray ionization in both positive (ESI⁺) and negative (ESI[−]) modes. The resulting total ion chromatograms (TICs) for each ionization mode are presented in Figure 1, and the corresponding tentatively identified compounds are listed in Table 1.

The UHPLC-UV-hr-qTOF/MS analysis of the MalvaT extract revealed a chemically diverse profile dominated by organic acids, phenolic acids, coumarins, flavonoids, and fatty acid derivatives. Early-eluting peaks corresponded mainly to small polar metabolites such as amino acids and organic acids (e.g., L-valine, malic acid, and citric acid), while mid-retention times featured phenolic constituents, including caffeic and coumaric acids, as well as several coumarin derivatives. A range of flavonoid glycosides—including orientin-rhamnoside, kaempferol and apigenin glucosides—were detected with lower relative intensities. Later-eluting peaks were primarily long-chain hydroxylated and unsaturated fatty acids, with notable contributions from trihydroxyoctadecenoic and octadecatrienoic acids, as well as linoleic and oleic acids. Overall, the chromatographic profile indicates a complex mixture enriched in phenolic and lipidic compounds, with a few metabolites (such as caffeic acid, trihydroxyoctadecenoic acid, and linoleic acid) showing comparatively higher normalized peak areas (Figure 1 and Table 1).

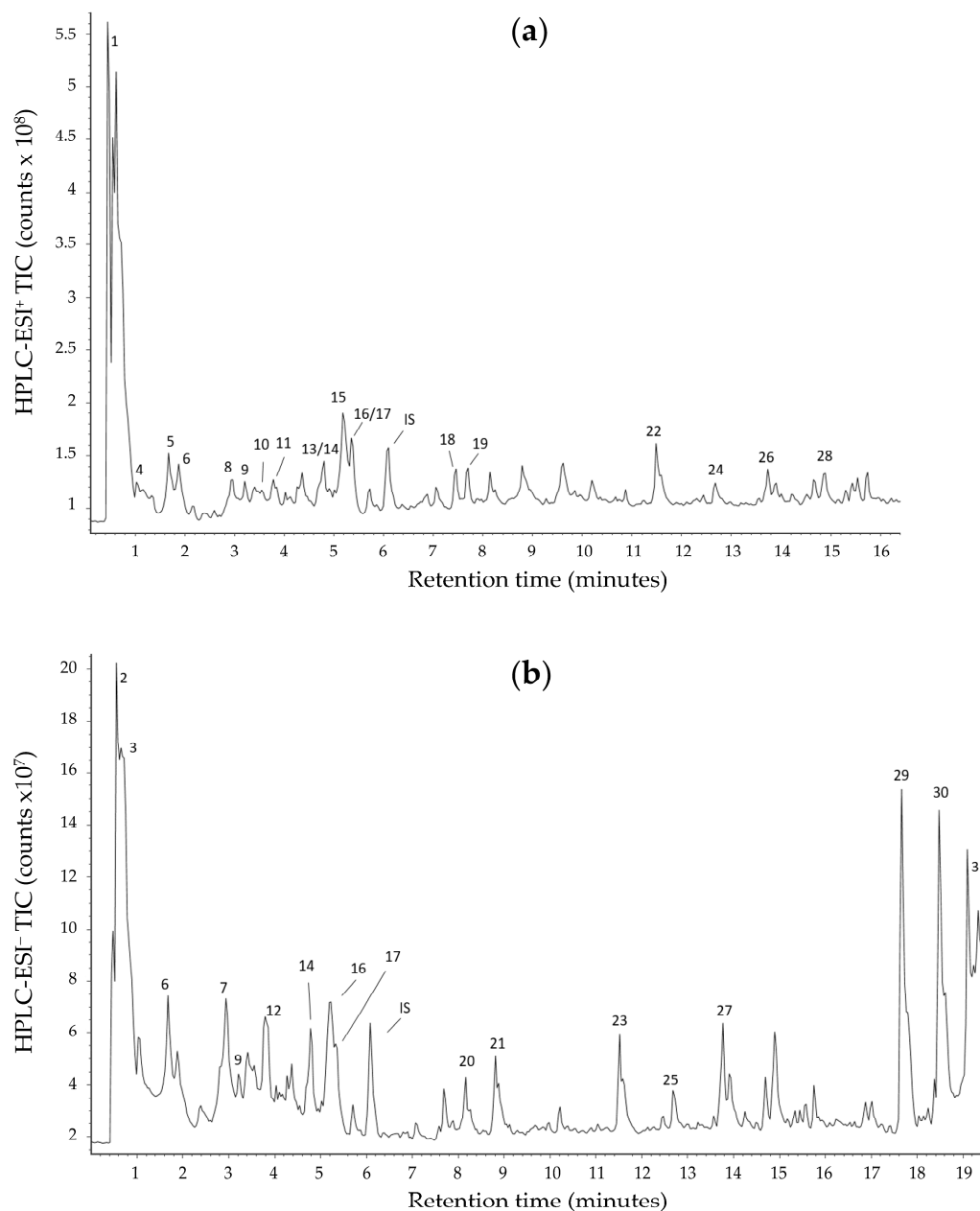


Figure 1. Total ion chromatograms (TICs) of the Malva tincture (MalvaT) acquired using electrospray ionization in positive (ESI⁺) (a) and negative (ESI⁻) (b) modes. The TIC signal (counts) represents the summed intensities of all ions detected at each time point during HPLC separation (shown as retention time in minutes). Peak assignments corresponding to the detected compounds are displayed in Table 1.

Table 1. Semi-quantitative analysis of compounds identified in the MalvaT extract by UHPLC-UV-hr-qTOF/MS.

Peak Number	Retention Time (min)	Normalized Peak Area *	[M – H] [–]	[M + H] ⁺	Tentatively Identified Compounds
1	0.6	1.41		118.085	L-valine
2	0.7	3.80	133.014		Malic acid
3	0.7	4.10	191.02		Citric acid
4	1.3	1.35		166.0854	Phenylalanine
5	1.7	0.07		195.028	Trihydroxycoumarin
6	1.9	2.36 (–)/0.09 (+)	206.046	208.06	3-Amino-4,7-dihydroxymethylcoumarin
7	2.9	5.44	179.035		Caffeic acid
8	3.0	0.51		163.038	Hydroxycoumarin
9	3.2	0.49 (–)/0.22 (+)	197.046	199.059	Ethyl gallate
10	3.6	0.13		595.166	Orientin-rhamnoside
11	3.8	0.12		473.105	Tetrahydroxyflavone glucopyranoside
12	3.9	3.47	163.04		Coumaric acid
13	4.7	0.01		465.135	Hesperetin glucoside
14	4.8	0.39 (–)/0.87 (+)	593.149	595.165	Kaempferol-glucoside-rhamnoside
15	5.2	0.13		271.059	Trihydroxyflavone
16	5.3	0.30 (–)/3.13 (+)	431.097	433.113	Apigenin glucoside/genistein glucoside
17	5.3	0.75 (–)/0.44 (+)	447.093	449.108	Kaempferol glucoside/Luteolin glucoside
18	7.7	0.48		271.059	Trihydroxyflavone
19	7.9	0.03		287.053	Tetrahydroxyisoflavone
20	8.2	0.58	327.217		Trihydroxyoctadecadienoic acid
21	8.8	2.27	329.233		Trihydroxyoctadecenoic acid
22	11.5	0.30		277.2157	Stearidonic acid
23	11.6	1.70	311.222		Dihydroxyoctadecadienoic acid
24	12.7	0.07		297.242	Hydroxyoctadecadienoic acid
25	12.7	0.73	313.236		Octadecanedioic acid
26	13.8	0.18		277.216	Octadetetraenoic acid
27	13.8	2.59	293.212		Hydroxyoctadecatrienoic acid
28	14.9	0.00 (1)		279.213	Linolenelaidic acid
29	17.7	4.48	277.217		Octadecatrienoic acid
30	18.5	5.81	279.233		Linoleic acid
31	19.3	1.29	281.249		Oleic acid

* Peak areas of the detected compounds were normalized to that of the internal standard amarogentin; for compounds detected in both negative (ESI[–]) and positive (ESI⁺) ionization mode, both relative values are reported (indicated by (–) and (+) respectively). Abbreviations: [M – H][–], deprotonated molecule detected in negative mode; [M + H]⁺, protonated molecule detected in positive mode; UHPLC-UV-hr-qTOF/MS, ultra-high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry.

3.2. Antioxidant Properties of Malva Extract

3.2.1. Antioxidant Activity of Malva in Cell-Free Assays

The antioxidant activity of Malva (prepared from MalvaT lyophilisate) was monitored by measuring its radical scavenging activity against biologically relevant reactive oxygen and nitrogen radicals (peroxyl, hydroxyl, peroxynitrite and superoxide) using oxygen radical absorbance capacity (ORAC) assays (Figure 2a) and against the artificial DPPH radical (DPPH assay; Figure 2b). The vitamin E derivative Trolox and caffeic acid were used as positive controls (reference standards). Dose–response experiments revealed

radical scavenging activity for Malva, with IC_{50} values ranging from 29.83 to 2931 $\mu\text{g/mL}$ (vs. 1.13–472 $\mu\text{g/mL}$ for the reference standard) depending on the radical tested (Figure 2). IC_{50} values of Malva were in a range close to that of the reference standard in the peroxyl (3.9 times the reference standard) and superoxide (6.2 times the reference standard) ORAC assays (Figure 2a), in an intermediate range in the hydroxyl (22.9-fold) and peroxynitrite (26.4-fold) ORAC assays (Figure 2a), and in a wider range (70.9-fold) in the DPPH assay (Figure 2b).

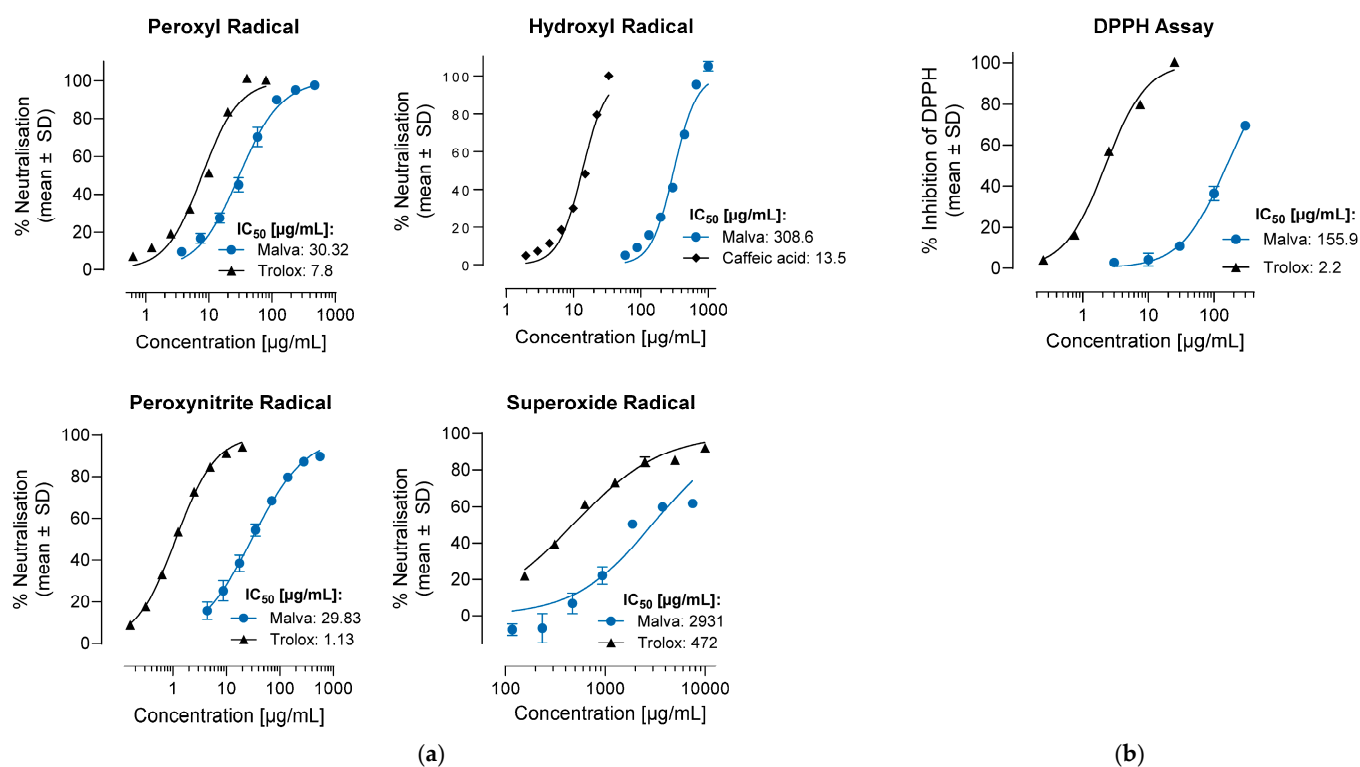


Figure 2. Antioxidant activity of Malva in cell-free assays. **(a)** Oxygen radical absorbance capacity (ORAC) assays were conducted as described in Materials and Methods (see Section 2.3) against various free radicals: peroxyl ($\text{ROO}\cdot$), hydroxyl ($\text{OH}\cdot$), peroxynitrite (ONOO^-), and superoxide ($\text{O}_2\cdot^-$) radicals. Trolox or caffeic acid was used as reference standard, as indicated. **(b)** DPPH assays with Malva and Trolox as reference standard were performed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, as described in Section 2.4. Data are expressed as the mean (SD) % neutralization (or inhibition) of free radicals from duplicate measurements. Respective half-maximal inhibitory concentrations (IC_{50}) are indicated within each graph. In **(a)** and **(b)**, data are from one representative experiment out of two independent experiments. Abbreviations: IC_{50} , half-maximal inhibitory concentration; Malva, Malva extract prepared from Malva tincture (MalvaT) lyophilisate; SD, standard deviation.

3.2.2. Antioxidant Activity of Malva in Cell-Based Assay

The antioxidant potential of Malva was also evaluated in a more physiological cell-based assay. Cellular antioxidant activity (CAA) assays were conducted in the human keratinocyte cell line HaCat against peroxyl radicals, using increasing concentrations of Malva (25–300 $\mu\text{g/mL}$) and quercetin (5 μM and 50 μM). Quercetin is a validated reference standard in CAA assays, and the concentration of 50 μM is expected to result in a strong antioxidant activity in HaCat cells [38,39,43]. Malva exhibited a concentration-dependent inhibition of intracellular oxidative stress, reaching 74.6% and 81.4% of the antioxidant activity of the reference standard (50 μM quercetin) at 200 $\mu\text{g/mL}$ and 300 $\mu\text{g/mL}$ Malva, respectively (Figure 3). At these concentrations, Malva's antioxidant activity was

statistically significantly higher than that of the 5 μM quercetin condition ($p < 0.0001$) and statistically comparable to that exerted by 50 μM quercetin (Figure 3).

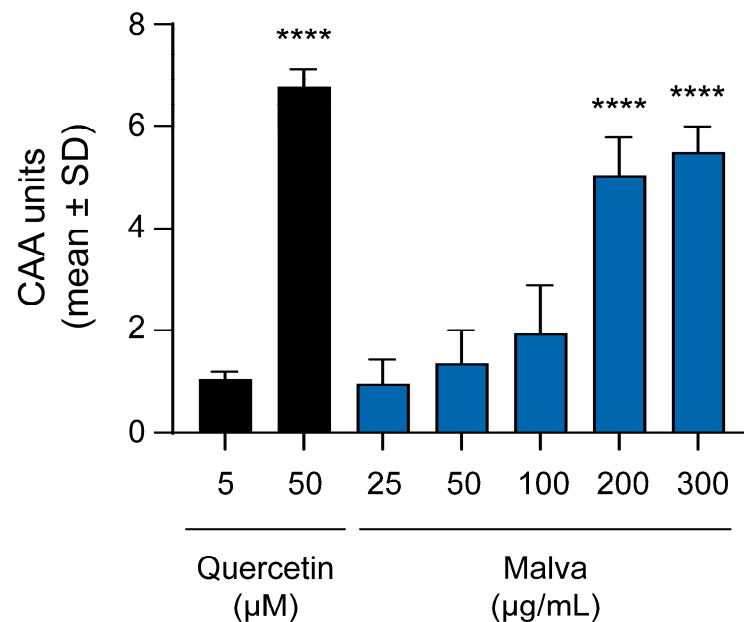


Figure 3. Cellular antioxidant activity (CAA) assay. HaCat cells were treated with vehicle control (1% DMSO), 5 μM or 50 μM quercetin (reference standard in 1% DMSO), or 25–300 $\mu\text{g/mL}$ Malva (in up to 1% DMSO), as described in Materials and Methods (see Section 2.5). Cellular antioxidant activity data were normalized to the vehicle control and expressed as mean (SD) CAA units. Data are from one representative experiment out of three independent experiments. Differences in antioxidant activity relative to the 5 μM quercetin condition (low concentration of the reference standard) were tested using a one-way ANOVA with Dunnett's post-test (**** $p < 0.0001$). Abbreviations: Malva, Malva extract prepared from Malva tincture (MalvaT) lyophilisate; SD, standard deviation.

3.3. Physical Properties of Malva Extract

3.3.1. Refractive Index Measurement

The refractive index is very important in eye-drop formulations. It should lie close to that of tear fluid to ensure visual comfort and clarity and to minimize visual distortion and blurred vision (due to changes in how light bends through the tear film) [24,44]. The mean refractive index of the human cornea ranges from 1.369 to 1.400, depending on its depth, while that of the overlying tear film ranges from 1.337 for the aqueous layer (and harvested tears) to 1.482 for the outermost lipid layer [23,25].

We verified the refractive index of experimental eye-drop preparations containing 0.5% Malva tincture (MalvaT) and either 0.15% or 0.3% HA. The eye-drop buffer (ED Buffer) used for these preparations contained sodium chloride (7.77 g/L), tri-sodium citrate dihydrate (4.21 g/L), and citric acid monohydrate (0.027 g/L) in injection-grade purified water (pH 6.0–8.0; osmolality: 250–330 mOsm/kg). This is the same buffer composition as that in the Visiodoron Malva (Weleda AG, Arlesheim, Switzerland) eye-drop formulation, which contains 0.5% Malva tincture and 0.15% HA [45]. Visiodoron Malva was tested as a positive control, in parallel to the experimental eye-drop preparations. The mean refractive indices of all three preparations were very similar, around 1.334 (ranging from 1.3337 to 1.3341) (Figure 4).

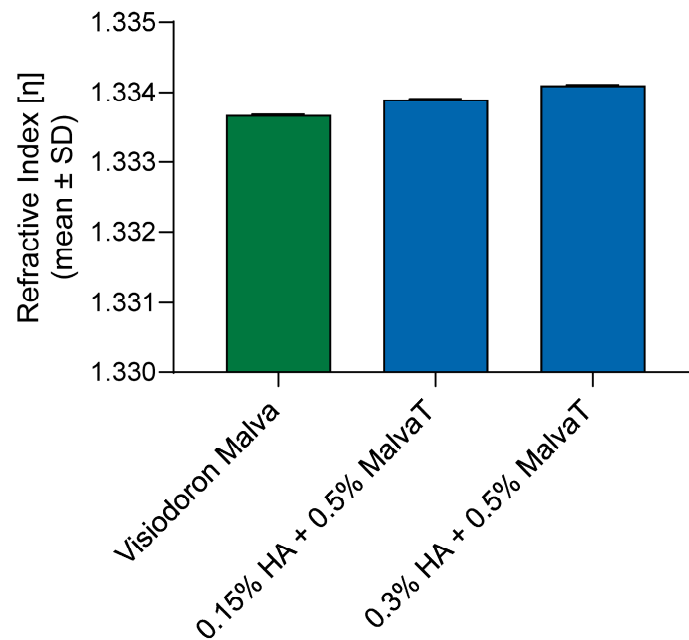


Figure 4. Refractive indices of Visiodoron Malva® and of Malva tincture (MalvaT) in combination with hyaluronic acid (HA). The refractive index of the Visiodoron Malva product (green bar) and of MalvaT (0.5% final; prepared from Malva tincture at 43% [m/m] ethanol) containing 0.15% or 0.3% HA in ED Buffer (see Section 2.1) (blue bars) was determined at 34 °C, as described in Section 2.6. Data are expressed as the mean (SD) refractive index of triplicate measurements. Data are from one representative experiment out of two independent experiments. In comparison, the mean refractive index of tear fluid reported in the literature is 1.337 [23,25]. Abbreviations: HA, hyaluronic acid; MalvaT, Malva tincture; SD, standard deviation.

3.3.2. Effect of Malva on Surface Tension in Eye-Drop Solutions

Surface tension is critical for eye-drop formulations because it directly affects how the drops interact with the ocular surface. Eye drops should have a surface tension close to that of natural tears to ensure optimal spreading, comfort, and efficacy. In particular, optimal surface tension is essential for proper wetting of the cornea, for the maintenance of tear film integrity, and for uniform drug distribution over the ocular surface. It is also important to control drop formation and size by designing the eye-drop dispenser to allow proper delivery and dosage and to improve comfort [24,26]. Normal tear film and intact tears have a surface tension of approximately 43–46 mN/m [26].

We measured the surface tension of the experimental eye-drop preparations containing 0.15% HA in ED Buffer vs. ED Buffer alone (see Section 3.3.1). The presence of 0.15% HA had no significant impact on the surface tension of the eye drop preparation, with a mean surface tension of 62.10 vs. 62.93 mN/m (Figure 5a) and 68.90 vs. 68.73 mN/m (Figure 5b) in HA vs. ED Buffer conditions respectively. We then tested the possible impact of Malva tincture on the surface tension of the 0.15% HA-containing eye-drop preparation. In a preliminary experiment, the presence of 0.5% MalvaT resulted in a statistically significant reduction in surface tension compared to the buffer control (Figure 5a; 23.0% reduction, from 62.93 to 48.47 mN/m; $p = 0.0054$). The specificity of MalvaT's effect was confirmed by showing a concentration-dependent reduction in surface tension, from 63.10 mN/m at 0.25% MalvaT to 41.73 mN/m at 2.0% MalvaT ($p < 0.0001$) in 0.15% HA-containing buffer, compared to 68.90 mN/m for 0.15% HA alone (Figure 5b). At the concentration of 0.5% MalvaT (in 0.15% HA-containing buffer), surface tension was 59.80 mN/m, which represents a 12.3% reduction in surface tension compared to the 0.15% HA-containing ethanol control (68.17 mN/m; $p < 0.0001$) (Figure 5b).

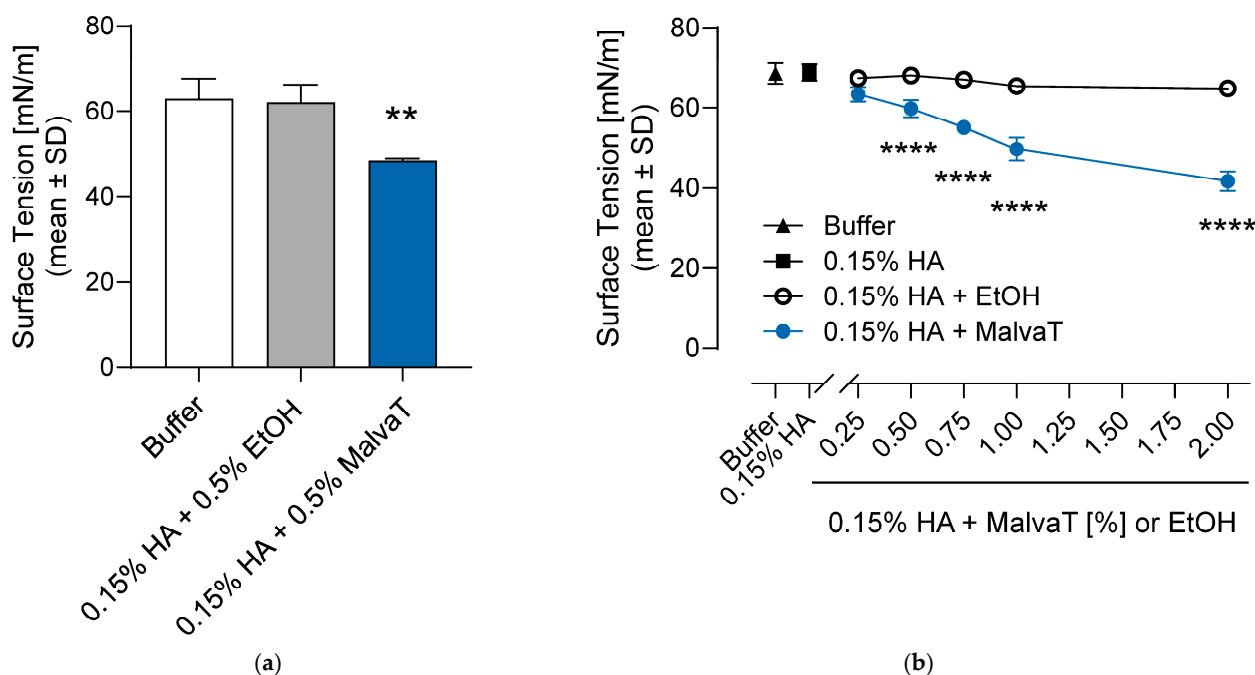


Figure 5. Impact of Malva tincture (MalvaT) combined with hyaluronic acid (HA) in Eye Drop Buffer on surface tension. Surface tension was measured as described in Section 2.7, under the following conditions. **(a)** Surface tension in Eye Drop Buffer (ED Buffer; see Section 2.1) (white bar), ED Buffer supplemented with 0.15% HA + 0.5% ethanol (from a control solution of 43% ethanol in water) (grey bar), and ED Buffer supplemented with 0.15% HA + 0.5% MalvaT (from Malva tincture at 43% [m/m] ethanol) (blue bar). **(b)** Surface tension in ED Buffer (black triangle), ED Buffer supplemented with 0.15% HA (black square), and ED Buffer supplemented with 0.15% HA and with either 0.25–2.0% MalvaT (from Malva tincture at 43% [m/m] ethanol; blue curve) or 0.25–2.0% of a hydroethanolic control solution with 43% ethanol (black curve). In other words, ED Buffer was present in all conditions, and 0.15% HA was present in all conditions except the ED Buffer-only (“Buffer”) condition. In **(a)** and **(b)**, % MalvaT is given as x% (m/m), and data are expressed as mean (SD) surface tension (in mN/m) from triplicate measurements. In **(a)** and **(b)**, data are from one representative experiment out of two independent experiments. In **(a)**, differences in surface tension in HA alone and HA + MalvaT conditions vs. ED Buffer control were tested using a one-way ANOVA with Dunnett’s post-test (** $p < 0.01$). In **(b)**, differences in surface tension in HA + MalvaT (blue curve) vs. HA + EtOH (black curve) for each MalvaT concentration (%) were tested using a two-way ANOVA with Šídák’s post-test (**** $p < 0.0001$). Abbreviations: EtOH, ethanol; HA, hyaluronic acid; MalvaT, Malva tincture; SD, standard deviation.

4. Discussion

This pre-clinical study evaluated the antioxidant properties of Malva flower extract and the physicochemical properties of experimental eye-drop formulations containing HA (0.15% or 0.3%) and Malva extract.

We demonstrated a clear radical scavenging activity of Malva against a variety of radicals (peroxyl, hydroxyl, peroxynitrite, superoxide, and DPPH radicals), with IC_{50} values in cell-free assays in the range of 4 to 70 times higher than that of the reference standard (Trolox or caffeic acid), depending on the radical tested. More precisely, Malva showed antioxidant activity in a range close to that of the reference standards against the peroxyl, superoxide, hydroxyl and peroxynitrite physiological radicals (IC_{50} values 4 to 26 times higher than that of the reference standard) and a weaker antioxidant activity against the DPPH artificial radical (IC_{50} ~70 times higher than that of Trolox). The observation that

Malva showed a stronger radical scavenging activity against biologically relevant reactive oxygen and nitrogen radicals (peroxyl, superoxide, hydroxyl and peroxynitrite) that mimic actual oxidative stress conditions occurring in vivo, including in the context of DED, might reflect its strong antioxidant potential. Moreover, the fact that Malva herbal extract, which is composed of a mixture of substances of different chemical natures and concentrations, shows antioxidant activity in a similar range to that of the pure reference standard (Trolox or caffeic acid) further supports the antioxidant potential of Malva. Malva's antioxidant activity was also demonstrated in a cell-based assay, which—besides confirming their antioxidant potential in a more physiological model—indicated the efficient cellular uptake of Malva's antioxidant substances [15–18,21].

Previous studies demonstrated the antioxidant activity of herbal extracts from Malva leaves and/or flowers, notably using the DPPH assay [16–18,21]. Our DPPH data obtained using Malva flower extract are thus in line with those previously reported. In addition, the results of our cell-free and cell-based antioxidant assays are in agreement with those recently published by Areesanan et al., which also demonstrated a concentration-dependent reduction in the ROS level in the immortalized human corneal epithelial cell line HCE-T using the same Malva flower extract as the one used in our study, with significant effects observed in a similar concentration range to the one reported here [21].

Finally, our HPLC analysis of Malva tincture reinforces the results of both the cell-free and cell-based antioxidant assays by revealing the presence of numerous constituents with well-established antioxidant activity, including phenolic acids (caffeic acid, coumaric acid, and ethyl gallate), multiple coumarin derivatives (e.g., hydroxycoumarin, trihydroxycoumarin, and amino-dihydroxymethylcoumarin), and several flavonoid glycosides (e.g., apigenin, kaempferol, hesperetin, and orientin-rhamnoside derivatives) [15,16,19,46–52]. Further studies are warranted to characterize Malva's compounds with beneficial antioxidant activity in the context of DED.

In our study, HA, which is a constituent of the experimental eye-drop formulations that we evaluated, was not tested for its potential antioxidant activity. Indeed, HA has been already extensively investigated, notably in the context of DED treatment. When used alone in ophthalmic formulations, HA primarily acts as a hydrating, lubricating and viscoelastic agent without exhibiting intrinsic antioxidant activity [10–13]. Antioxidant effects of eye-drop formulations are observed only when HA is combined with specific antioxidant molecules, such as trehalose or vitamin B12 [3,4]. By demonstrating the antioxidant properties of Malva flower extract, our study therefore supports the relevance of combining HA with Malva extract in eye-drop formulations intended for the management of DED. This is the case notably for the CE-marked medical devices Visiodoron Malva (0.15% HA, 0.5% Malva) and Visiodoron Malva Intense (0.3% HA, 0.5% Malva) (Weleda AG). Moreover, the HA concentrations tested in our study—and those contained in the Visiodoron Malva medical devices—(i.e., 0.15% or 0.3%) are in a similar range to those usually applied in eye-drop formulations for the treatment of DED (0.1–0.4%) [11,12], further validating the relevance of their composition for ophthalmic formulations. Finally, in terms of dose–response translation, considering Malva concentration in Visiodoron Malva, the topical application of one eye drop of the product would result in a local Malva concentration of about 60 µg/mL (see the calculation in Section 2.1) and therefore in the (lower) range of Malva concentrations exhibiting antioxidant potential in the CAA assay. We may speculate that repeated daily eye-drop applications could create a local ocular surface environment that promotes HA-mediated hydration and Malva-mediated reduction of oxidative stress. Future clinical studies should investigate this possibility. Interestingly, a recent pilot study including 20 patients with moderate DED and comparing Visiodoron Malva to HA (0.15%) alone identified a favorable perception of Visiodoron Malva by ophthalmologists and patients in a subjective assessment analysis [22]. Whether

this favorable assessment was related to the lubricating and/or antioxidant properties of the product remains to be shown. Besides the evaluation of antioxidant capabilities of eye-drop formulations, the characterization of their physicochemical properties, including refractive index and surface tension, is essential [23–26]. A proper refractive index is critical for visual clarity. We demonstrated that the refractive index of experimental eye-drop preparations containing Malva tincture and HA, but also that of the Visiodoron Malva medical device tested as a control, was 1.334, which is in a similar range to that of normal tear fluid (1.337) [23,25]. Our study therefore validates these preparations with respect to their refractive index characteristics.

The surface tension of eye-drop formulations is also critical to ensure correct drop formation, spreading on the ocular surface, and tear film stability. We found no effect of HA (0.15%) on surface tension in our experimental eye-drop formulations. This result is in line with that reported by Gasztych et al., which showed a stable surface tension (67.08–71.73 mN/m) for 0.1–1% HA solutions [28]. Also, the surface tension of HA (0.15%) measured in our study (around 62.10–68.9 mN/m) was comparable to that reported by Gasztych et al. at a similar HA dosage (i.e., 67.09 mN/m for 0.1% HA and 71.43 mN/m for 0.25% HA) [28].

On the other hand, we demonstrated a concentration-dependent reduction in surface tension exerted by Malva tincture. To the best of our knowledge, this is the first report of an effect of Malva extract in eye-drop solutions in terms of modulating surface tension. Surface tension in our experimental eye-drop formulation containing 0.5% MalvaT (in ED Buffer containing 0.15% HA) was reduced to about 48.47–59.8 mN/m (from 62.10 to 68.9 mN/m for 0.15% HA alone in ED buffer). The measured surface tension of the Malva-plus-HA formulation is in the range of that of existing ophthalmic formulations for DED (reported in the literature to be between 54.1 and 70.9 mN/m) [53]. In addition, preliminary experiments performed on the Visiodoron Malva Intense eye-drop product (containing 0.3% HA and 0.5% Malva) indicated a surface tension of 53.4 mN/m (unpublished data) and thus in the range of that of the experimental eye-drop formulations reported in this study.

The ability of Malva tincture to reduce the surface tension of an eye-drop preparation is relevant because this might allow the relatively elevated surface tension associated with HA solutions (see above) to be lowered to a value closer to that of tear fluid (43–46 mN/m) [26], thus potentially facilitating fluid distribution over the ocular surface. In that regard, it is noteworthy that in their recent pilot clinical study comparing Visiodoron Malva (0.15% HA, 0.5% Malva tincture; Weleda AG, Arlesheim, Switzerland) to a 0.15% HA solution of equivalent quality (BLUyal UD, PHARMA STULLN GmbH, Stulln, Germany), Basile et al. mentioned that Visiodoron Malva's surface tension was lower than that of HA alone [22], which is in agreement with our reported experimental data comparing HA-plus-Malva combinations to HA alone. Further studies are needed to better characterize the mechanism behind the reduction in surface tension associated with Malva extract and to identify which components are responsible for this effect. Interestingly, our HPLC analysis of MalvaT identified a range of long-chain fatty acids and oxidized fatty acid derivatives—such as linoleic acid, oleic acid, octadecatrienoic acid, and several hydroxylated C18 lipids—that have amphiphilic or surfactant-like behavior [54–58]. Others have identified saponins—known for their surfactant properties [59]—as components of Malva [60]. However, saponins were not among the compounds identified in MalvaT by UHPLC-UV-hrQTOF/MS. Additional studies are needed to further investigate the potential surfactant property of Malva and its possible effect on surface tension in the context of DED.

A recent open-label, multicenter clinical cross-over pilot study comparing Visiodoron Malva to HA alone in patients with moderate DED showed that both products were well tolerated and that Visiodoron Malva was not inferior to HA alone in terms of tear

film breakup time (TBUT), improvement in tear film stability and ocular surface integrity, and ocular surface disease index (OSDI) scoring by patients [22]. Subjective assessment by ophthalmologists and patients was in favor of Visiodoron Malva, showing statistically significant differences in patient satisfaction and preferences and in ophthalmologist- and patient-based efficacy assessment. The reason for the perceived superiority of Visiodoron Malva is unclear. The authors proposed that a reduction in surface tension mediated by Malva extract might explain this difference [22,61]. Our present pre-clinical data tend to support their proposition. Clearly, further clinical evidence is needed to better characterize the benefit of combining Malva extract with HA for the management of DED.

Our study presents several strengths and limitations. Its strengths include the detailed HPLC analysis of the chemical composition of Malva extract, the thorough cell-free and cell-based antioxidant assays against several free radicals and the characterization of the physicochemical properties (refractive index and surface tension) of Malva-containing eye-drop formulations. A major limitation of our study is the use of the immortalized human keratinocyte cell line HaCat for the cell-based antioxidant assay. The use of a corneal epithelial cell line, such as HCE-T, would have been more relevant to DED and would have allowed us to confirm the data recently published by Areesanan et al. that was obtained using HCE-T cells [21].

5. Conclusions

This pre-clinical study demonstrated that Malva flower extract exhibits significant antioxidant activity and that experimental eye-drop formulations containing Malva extract plus HA present physicochemical properties (notably refractive indices and surface tension) compatible with those required for eye drops intended for the management of DED. These antioxidant properties of Malva are particularly meaningful in the context of DED, where environmental exposures such as high-energy blue light from prolonged screen use and UV radiation are major contributors to ocular-surface oxidative stress and therefore to DED onset and progression. Further clinical studies are warranted to confirm the utility of combining Malva extract with HA for the ocular surface treatment of patients with DED.

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Conflicts of Interest: J.R., M.-R.P.-B., M.M.-v.E. and G.K. are employees of Weleda AG. The funder was involved in the design of the study; in the collection, analysis and interpretation of data; in the decision to publish the results; and in the writing of the manuscript.

Abbreviations

The following abbreviations are used in this manuscript:

CAA	Cellular antioxidant activity (cell-based assay)
DED	Dry eye disease
DPPH	1,1-diphenyl-2-picryl-hydrazyl (radical used for cell-free antioxidant assay)
HA	Hyaluronic acid
Malva	Malva flower extract solution prepared from MalvaT lyophilisate
MalvaT	Malva flower tincture
ORAC	Oxygen radical absorbance capacity (cell-free assay)
OSDI	Ocular surface disease index
ROS	Reactive oxygen species
TBUT	Tear film breakup time

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