



## Green-sourced polyphenols for ocular health: A sustainable NADES-based approach to dry eye disease treatment

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### ABSTRACT

Dry Eye Disease (DED) is a multifactorial ocular disorder driven by inflammation, oxidative stress, and microbial imbalance. In pursuit of a sustainable therapeutic alternative, this study developed a novel ophthalmic hydrogel (HAZ@gel) incorporating polyphenol-rich hazelnut cuticle (HC) extracts obtained via Natural Deep Eutectic Solvents (NADES), embedded into a Carbopol-based matrix. A wide panel of NADES combinations was screened to optimize extraction efficiency, resulting in acid-based NADESs delivering the highest total phenolic content with favorable physicochemical properties. The HAZ@gel formulation demonstrated excellent biocompatibility and sustained polyphenol release, along with significant antioxidant and anti-inflammatory activity in hyperosmotic-stressed human corneal epithelial cells, a validated in vitro DED model. The formulation also exerted antimicrobial effects against *Bacillus subtilis* and *Escherichia coli*, further supporting its multifunctional potential. This work exemplifies a circular economy strategy by valorizing agro-industrial waste through green extraction and eco-friendly formulation, proposing a biodegradable and efficacious ocular therapy aligned with sustainable pharmaceutical innovation.

### 1. Introduction

Dry Eye Disease (DED) is a multifactorial and increasingly prevalent condition of the ocular surface, characterized by tear film instability and symptoms such as burning, foreign body sensation, visual disturbances, and inflammation (Golden et al., 2025; Messmer, 2015). While more common in older adults, its incidence is rising among younger populations, likely due to increased screen

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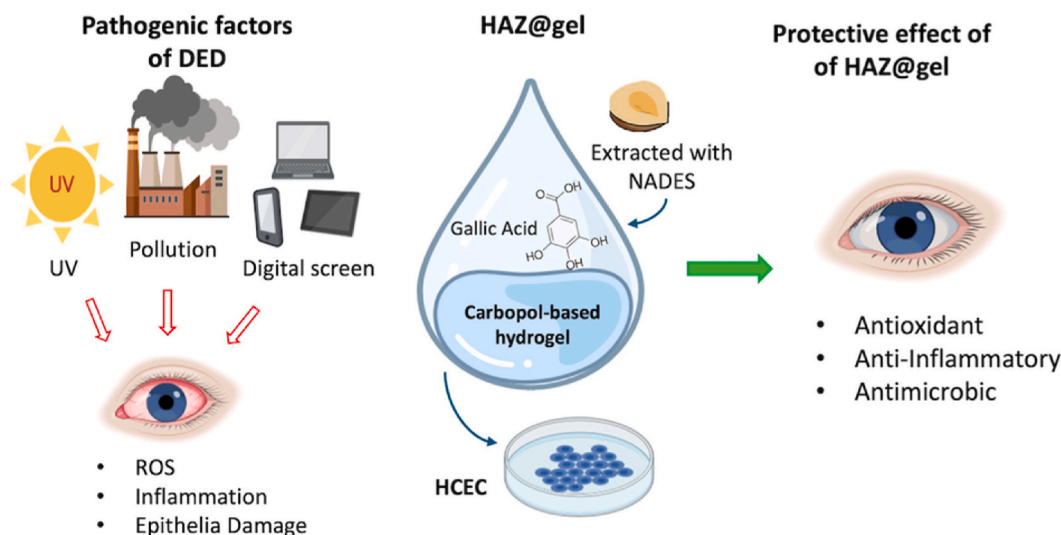


Fig. 1. HAZ@gel: a multifunctional, sustainable strategy for Dry Eye Disease.

exposure and environmental stressors (Kaur et al., 2022). DED not only impairs visual function and quality of life but also poses a growing public health challenge. The ocular surface is continuously exposed to environmental insults—including air pollutants, UV and ionizing radiation, and extreme climates—which can disrupt tear film homeostasis and trigger ocular surface damage (Alghamdi, 2025; Alves et al., 2023). These stressors promote oxidative stress by increasing reactive oxygen species (ROS), which activate pro-inflammatory pathways and damage epithelial cells (Böhm et al., 2023a; Ceravolo et al., 2021). ROS accumulation, a hallmark of DED, compromises lipid membranes, mucin production, and epithelial integrity, exacerbating tear film instability (Böhm et al., 2023a). Alongside oxidative and inflammatory mechanisms, alterations in the ocular microbiota have emerged as contributing factors to DED pathogenesis (Labetoulle et al., 2024; Schlegel et al., 2023). Dysbiosis can impair mucosal immunity and favor opportunistic pathogens like *S. aureus*, *P. aeruginosa*, and *E. coli*, which may worsen inflammation and delay healing (Ghita et al., 2023; Wang et al., 2022). These findings highlight the need for therapeutic strategies with both anti-inflammatory and antimicrobial activity. Treatment approaches vary according to disease severity. Mild DED is often managed with artificial tears, while moderate to severe cases may require topical corticosteroids, immunomodulators (e.g., cyclosporine) (Mohamed et al., 2022), systemic agents (Hwang and Shin, 2021), or biologically-derived treatments such as autologous serum eye drops (Vazirani et al., 2023). Recently, antioxidant compounds like vitamin B12, alpha-lipoic acid, and L-carnitine have shown promise in protecting the ocular surface by reducing ROS and inflammation (Jomova et al., 2023; X. Li et al., 2019; Shahidin et al., 2025). Despite these advances, current therapies have limitations. Long-term corticosteroid use is associated with adverse effects including intraocular pressure elevation and cataract formation (Cutolo et al., 2019; Lemp, 2008). Likewise, chronic use of preservative-containing eye drops (e.g., BAK) may lead to goblet cell loss and ocular surface toxicity (Goldstein et al., 2022). Therefore, preservative-free and safer long-term alternatives are critically needed.

Natural polyphenol-rich extracts from agro-industrial by-products have attracted attention for their antioxidant and anti-inflammatory properties. Hazelnut cuticles (HC), a by-product of roasting, are rich in bioactive compounds like gallic acid, catechins, and flavonoids, which have demonstrated protective effects on the ocular surface. Gallic acid, in particular, reduces ROS production, downregulates pro-inflammatory cytokines (e.g., IL-1 $\beta$ , TNF- $\alpha$ ), and preserves tear film integrity under stress conditions (Conte et al., 2025). To improve extraction efficiency and preserve bioactivity, Natural Deep Eutectic Solvents (NADES)—composed of natural metabolites like sugars, amino acids, and organic acids—offer an eco-friendly alternative to conventional solvents (García-Roldán et al., 2023). Acid-based NADESs, in particular, have demonstrated superior extraction efficiency and phenolic yield, attributed to their polarity and hydrogen-bonding capacity. Optimization of NADES composition and conditions enabled the development of tailored extracts with enhanced stability and bioactivity (Conte et al., 2025).

In this study, a novel ophthalmic formulation (HAZ@gel) was developed by incorporating NADES-extracted HC polyphenols into a Carbopol-based hydrogel. Carbopol is a mucoadhesive polymer that forms a viscoelastic gel on the ocular surface, enhancing bioavailability and retention time (Cassano et al., 2021; Moustafa et al., 2018). The HAZ@gel formulation was characterized and evaluated *in vitro* using a human corneal epithelial cell model of hyperosmotic stress, demonstrating antioxidant, anti-inflammatory, and antibacterial activities—including inhibition of *B. subtilis* and *E. coli*.

Overall, HAZ@gel represents a promising multifunctional treatment for DED, combining natural antioxidants, green extraction technologies, and sustainable formulation strategies. Its development not only addresses key pathological mechanisms of DED but also supports circular economy goals through the valorization of agro-industrial waste. A graphical overview of the pathogenic factors, extraction process, gel formulation and protective effects of HAZ@gel is shown in Fig. 1.

## 2. Materials and methods

### 2.1. Materials

Choline chloride (ChCl,  $\geq 99\%$  purity), lactic acid (LA,  $\geq 85\%$  purity), malic acid (MA,  $\geq 85\%$  purity), gallic acid, rutin hydrate, quercetin, cyanidin-3-O-glucoside, Trolox, ammonium acetate, iron(III) chloride ( $\text{FeCl}_3$ ), potassium persulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ), ethanol ( $< 99.5\%$  purity), sodium acetate, aluminum chloride, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), simulated tear fluid (STF), and all other reagents were obtained from Sigma-Aldrich (Milan, Italy). All HPLC analytical grade solvents were purchased from Fisher Scientific (Milan, Italy). Corneal epithelial cell basal medium containing growth supplements (ATCC; VA, USA), 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin (Euroclone).

### 2.2. Preparation and characterization of NADES

Choline chloride, betaine, L-proline and L-arginine were dried at  $60^\circ\text{C}$  for 24 h using a vacuum concentrator (Savant SPD131DDA SpeedVac129 Concentrator, Thermo Scientific, Milan, Italy) before use. The natural deep eutectic solvents (NADESs) were prepared by combining the hydrogen bond acceptors (HBA) with hydrogen bond donor (HBD) in selected molar ratios. The mixtures were heated between  $60^\circ\text{C}$  and  $80^\circ\text{C}$  in the presence of Milli-Q water, maintaining a water-to-NADES volume ratio of 33%. Stirring was applied throughout the heating process until the formation of a clear, uniform solution. The heating duration was approximately 30 min, ensuring full dissolution and interaction of the components. The resulting NADESs were then cooled to room temperature and stored in sealed containers to avoid moisture uptake. The successful formation of eutectic mixtures was confirmed by the absence of visible crystals and the appearance of a stable, viscous liquid under ambient conditions.

### 2.3. Viscosity measurement

The viscosities of the synthesized NADESs were evaluated using a rotational viscometer (Brookfield Dial Viscometer, AMETEK, Berwyn, PA, USA) fitted with a spindle suitable for fluids of low to moderate viscosity. Calibration was carried out with standard viscosity reference fluids to verify the accuracy of the instrument. For each test, 20 mL of NADES sample was placed into a clean, temperature-regulated container. Viscosity was assessed at  $25^\circ\text{C}$  and  $50^\circ\text{C}$ , simulating typical storage and application environments. The spindle speed was adjusted to stabilize torque readings, and measurements were recorded only after thermal equilibrium was reached. Each sample was measured three times, and the average viscosity in milliPascal-seconds (mPa·s) was recorded. To maintain data integrity, the spindle and sample container were thoroughly rinsed with deionized water and dried between measurements.

### 2.4. Extraction of polyphenols from roasted hazelnut cuticles

Solid-liquid extraction was carried out using a ratio of 0.1 g of freeze-dried roasted hazelnut cuticles per mL of prepared NADES. The samples were incubated at  $50^\circ\text{C}$  for 2 h under constant stirring. During incubation, extraction was assisted by intermittent probe sonication (BRANSON Sonifier 450, 20 kHz, 750 W) applied in 3 cycles of 5 min each, with 5-min pauses between cycles to prevent overheating. After incubation, the mixtures were centrifuged for 10 min at 2570 rcf using an OHAUS FC5718R centrifuge equipped with a 19/005 rotor. The supernatants were carefully decanted, filtered through Whatman No. 1 filter paper, and stored at  $4^\circ\text{C}$  until further LC-MS analyses (Conte et al., 2025).

### 2.5. Chemical antioxidant capacity of hNADES

To evaluate the chemical antioxidant activity of HAZ@gel, ABTS, DPPH, and FRAP methods were used as reported by Conte et al. (2025). The results were expressed as gallic acid equivalents (GA) per milliliter ( $\mu\text{mol/mL}$  GA).

### 2.6. Preparation of NADES-loaded hydrogels (HAZ@gel)

The Pickering Carbopol gels were prepared using a homogenization-based method. Briefly, the appropriate amount of Carbopol 940 was accurately weighed and slowly dispersed in purified distilled water under continuous gentle stirring to obtain gels with varying polymer concentrations (typically 0.5–1.5% w/v). The dispersion was allowed to hydrate and swell for at least 4–6 h at room temperature. After complete hydration, the pH of the gel was adjusted to 6.5–7.0 using a neutralizing agent (NaOH 0.1 M) to induce gelation and form a transparent, homogeneous gel matrix. To obtain the hNADES-loaded Carbopol gel (HAZ@gel), accurately weighed amounts of lyophilized hNADES (100 mM) were gradually added to the pre-neutralized Carbopol gel and mixed thoroughly using a mechanical homogenizer (Ultra-Turrax T25, IKA) at 6000 rpm for 5 min, followed by 3000 rpm for an additional 5 min to ensure uniform dispersion without excessive aeration. The final formulation was allowed to equilibrate at room temperature for 1 h before further use.

### 2.7. Gelation time

The gelation time ( $t_{\text{sol-gel}}$ ) of HAZ@gels was determined by modified test tube inversion method using simulated tear fluid (0.67 g

NaCl, 0.2 g NaHCO<sub>3</sub>, and 0.008 CaCl<sub>2</sub>·2H<sub>2</sub>O in 100 g of purified water, STF) as gelation solution following the methodology reported by De Luca et al. (De Luca et al., 2023).

## 2.8. In vitro NADES release and permeation studies

**Microstructures** The cumulative hNADES release was assessed in 5 mL of freshly prepared STF at two different osmolarity values, namely 312 and 500 mOsm/L. During the release test, the temperature was maintained at  $34.0 \pm 0.5$  °C under continuous magnetic stirring (200 rpm). At given time intervals, 0.5 ml of sample was withdrawn and replenished with fresh media. Samples were centrifuged for 45 min at 21.380×g (Corning LSE high speed microcentrifuge; Fisher Scientific Italia, Milan, Italy) and the amount of hNADES in the samples was determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS), as indicated by De Luca et al. (De Luca et al., 2023).

The permeation studies were performed using vertical Franz diffusion cells with a volume of 12 ml (contact area: 1.77 cm<sup>2</sup>, PermeGear Inc., Hellertown, PA, USA) as reported by Bao et al. (2018). Cellulose acetate membranes were placed on top of the receptor chambers of the cells following the addition of freshly prepared STF as release media. During the release test, the temperature was maintained at  $34.0 \pm 0.5$  °C. To simulate the small amount of tear secreted on the eye surface, 0.25 mL of the release medium were added to the top of the samples loaded into the donor chambers. At specified time intervals, aliquots (0.15 mL) of fresh media were used to replace the same volume withdrawn from the receptor chambers. Then, the supernatants were directly assayed by LC-MS/MS after centrifugation at 21.380×g (Corning LSE high speed microcentrifuge; Fisher Scientific Italia, Milan, Italy), 4 °C for 45 min.

### 2.8.1. Identification and quantification of gallic acid by LC-MS

Quantification of gallic acid was carried out using a Shimadzu LCMS-8060 triple quadrupole mass spectrometer coupled to a high-performance liquid chromatography (HPLC) system. Separation was performed on a Kinetex C18 column (100 × 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) maintained at 40 °C. The mobile phase consisted of acetonitrile and water containing 0.1% formic acid in an isocratic ratio of 30:70 (v/v), at a flow rate of 0.3 mL/min, with an injection volume of 25 μL. Mass spectrometric detection was performed using electrospray ionization (ESI) in negative ion mode, with the following parameters: ion source temperature at 300 °C, desolvation line temperature at 250 °C, and nebulizing gas flow at 3.0 L/min. Data acquisition was conducted in single ion monitoring (SIM) mode, targeting gallic acid at *m/z* 169.0, corresponding to the [M–H]<sup>–</sup> ion. Quantification was achieved using a calibration curve prepared from authentic gallic acid standards, with peak identification based on retention time and mass-to-charge ratio compared to the reference standard. Data were processed using LabSolutions software (Version 5.118).

## 2.9. Assessment of anti-inflammatory and antioxidant capacity of HAZ@gel

### 2.9.1. Cells culture and in vitro dry eye model

Human Corneal Epithelial Cells (HCECs) were obtained from the American Type Culture Collection (PCS-700-010, ATCC; VA, USA) and cultured in corneal epithelial cell basal medium containing growth supplements according to the manufacturer's instructions (ATCC). The culture medium was further enriched with 10% fetal bovine serum (FBS), 4.5 mg/mL glucose, 2.0 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin. Cells were maintained in a humidified incubator at 37 °C, under an atmosphere of 5% CO<sub>2</sub> and 95% air. HCECs from the third to tenth passages that exhibited good morphology were used for experiments. The cell viability of HCECs on HAZ@gel was assessed following the international standard ISO 10993 by cell counting kit (CCK-8) according to the manufacturer's protocol (Microtech S.R.L., Italy). Briefly, the gel samples were immersed and incubated in corneal epithelial cell basal medium at 37 °C for 24 h to obtain the “conditioned medium” (CM). Following the adherence of HCECs, the culture medium was removed and replaced with CM for 24, 48, and 72 h. Then, 10 μL of CCK-8 solution was added to each well and the absorbance was measured at 450 nm. Cell viability was determined as a percentage compared to the untreated cells.

Cellular death by membrane damage was measured using Lactate Dehydrogenase (LDH) assay (Sigma-Aldrich) as for the manufacturer's instructions. Cells were treated as previously described and LDH released in the culture medium was measured at 490 nm. Triton X-100 solutions (1%) and culture media only were used as positive and negative controls, respectively.

For Dry Eye Cell Model, HCECs were treated with culture medium at different osmolarity ranging from 312 to 500 mOsm/L as reported by Shetty et al. (2020). The protective effects of hNADES were studied with the acute toxicity model by pre-treating cells with HAZ@gel for 24 h. A shorter exposure (4 h) was used to investigate effects on mRNA expression.

### 2.9.2. Intracellular antioxidant activities

To assess antioxidant enzyme activities, HCECs were treated as reported above, and resuspended in 0.5 mL PBS buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mM benzamidine. Measurements of Catalase (CAT) and Superoxide dismutases (SODs) activities were carried out on cell lysates after sonication. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) decomposition (at 240 nm) was followed to determine CAT activity (EC 1.11.1.6). Results were normalized to total protein content and expressed as percentage of control (100% of activity). SOD (EC 1.15.1.1) activity was measured using the standard assay mixture contained enzymatic sample as per manufacturer's instructions (Sigma-Aldrich). Samples were exposed 5 min to intense cool white light. One SOD unit was defined as the amount of enzyme necessary to inhibit 50% the reaction rate. Samples were measured at 560 nm in BioTek Cytation 3 Cell Imaging Multi-Mode Reader. Results were normalized to total protein content and expressed as percentage of control. Experiments were performed four times.

**Table 1**  
NADESPrimers used for qRT-PCR.

Gene	Accessionnumber	Forward (5'-3')	Reverse (5'-3')
<b>IL-1</b>	NM_000576.3	GGAGAATGACCTGAGCACCT	TGATCGTACAGGTGCATCGT
<b>IL-4</b>	NM_000589.4	GCAGTTCTACAGCCACCATG	GTCGAGCCGTTTCAGGAATC
<b>IL-6</b>	NM_000600.5	CGCCTTCGGTCCAGTTGCC	GCCAGTGCCTCTTTGCTGCTTT
<b>IL-10</b>	NM_000572.3	GGGGCTTCTTAACGTCTACA	AGTGGTTGGGAATGAGGTT
<b>TNF-<math>\alpha</math></b>	NM_000594.4	AACATCCAACCTTCCCAAACGC	TGGTCTCCAGATTCCAGATGTCAGG
<b>ACTB</b>	NM_001101.5	ACTCTTCAGCCTTCCTTCC	CGTACAGGTCTTTGCGGATG

### 2.9.3. Enzyme-Linked Immunosorbent Assay (ELISA)

To assess the levels of human interleukin-1 (IL-1), human interleukin-4 (IL-4), human interleukin-6 (IL-6) human interleukin-10 (IL-10) and tumor necrosis factor (TNF- $\alpha$ ) were measured in supernatants using Enzyme-Linked Immunosorbent Assay (ELISA) as reported by [Valentino et al. \(2024\)](#).

### 2.9.4. Determination of inflammatory factors expression

Total RNA was extracted from HCECs cell culture using TriFast (EuroClone, Italy), according to the manufacturer's protocol, and mRNA levels measured by RT-PCR amplification as reported by De Luca et al. ([De Luca et al., 2023](#)). Specific primers for IL-1, IL-4, IL-6, IL-10, and TNF- $\alpha$ , and  $\beta$ -Actin (ACTB) were used, with sequences provided in [Table 1](#). All reactions were run in triplicate, normalized to the housekeeping gene (ACTB), and the results expressed as mean  $\pm$  SD. The  $2^{-\Delta\Delta C_t}$  method was used to determine the relative quantification.

### 2.9.5. Antimicrobial screening and determination of Minimal Inhibitory Concentration (MICs)

The antimicrobial activity of hazelnut skin extracts was determined through the agar dilution method to obtain the minimum inhibitory concentrations (MIC) ([Portal et al., 2025](#)) and the disk diffusion method ([Kit et al., 2016](#)).

Bacterial cultures of *Escherichia coli* ATCC 25922 (Gram-negative) and *Bacillus subtilis* ATCC 6633 (Gram-positive) were prepared by logarithmic phase and direct colony suspension. The bacteria were pre-cultured overnight in Luria Bertani (LB) broth under constant stirring at 37 °C. Afterward, the culture density was determined spectrophotometrically (at 600 nm) and for the Disc Diffusion Method assay, 1 mL of each bacterial suspension at a concentration  $1 \times 10^5$  CFU/mL was spread on a solid growth medium in a Petri dish. Sterile paper disks (6 mm in diameter Dominique Dutschersas.), impregnated with 20  $\mu$ L of the several hazelnut CM (1:2, 1:5, 1:10, 1:25), were placed on the surface of agar plates. Disks impregnated with sterile distilled water was considered as negative controls, instead disk with the antibiotic (ampicillin, Sigma-Aldrich GmbH, Steinheim, Germany) was the positive control. The plates were incubated for 24 h at 37 °C, and the zones of inhibition around the disks were measured after incubation.

The MICs were determined through broth microdilution method. The overnight culture of each strain was adjusted at several bacteria of  $10^8$  cells/ml using 0.5 McFarland standard (CLSI Guidelines, 2022). An aliquot of the bacterial suspension (100  $\mu$ L) was added to a 96-well microplate containing equal volumes of serial dilutions of hazelnut CM, ranging from 0.15 to 25 mg/mL. LB and bacterial suspension were the positive control, while LB and extract at the same concentration of extracts tested was used as the negative control. All experiments were performed in triplicate ( $n = 3$ ) on a homogeneously mixed sample for each extract.

The microplate was incubated at 37 °C for 18 h and resazurin (6.75 mg/ml) was added to each well and incubated at 37 °C for 2 h. The absorbance was measured at 570 nm with a BioTekCytation 3 Cell Imaging Multi-Mode Reader.

The Inhibition was determined by the formula:

$$\text{Inhibition (\%)} = (\text{Abs of control} - \text{Abs of sample}) / (\text{Abs of control}) \times 100\%. \quad (1)$$

A change in color was observed and the lowest concentration prior to the color change was considered as MIC defined as the lowest concentration of extract that inhibited bacterial growth.

### 2.9.6. Statistical analyses

All biological assays were performed by GraphPad Prism® (version 6.0, software San Diego, CA, United States). All experiments were carried out at least three times and the data were reported as the mean (M)  $\pm$  standard deviation (SD). The statistical significance of differences between samples in the presence or absence of peptides was calculated through one-way analysis of variance One way-ANOVA with Tukey comparisons, with a significance level of  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Characterization and optimization of Natural Deep Eutectic Solvents (NADESs) for polyphenol extraction

Natural Deep Eutectic Solvents (NADESs) are emerging as viable green alternatives to traditional organic solvents for the extraction of bioactive compounds, owing to their sustainability, low toxicity, and high biodegradability ([Schuh et al., 2023](#)). Structurally, NADESs are formed through hydrogen bonding between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), often yielding solvents with desirable solvating capabilities and tuneable physical properties ([Wu et al., 2022](#)).



**Table 2**

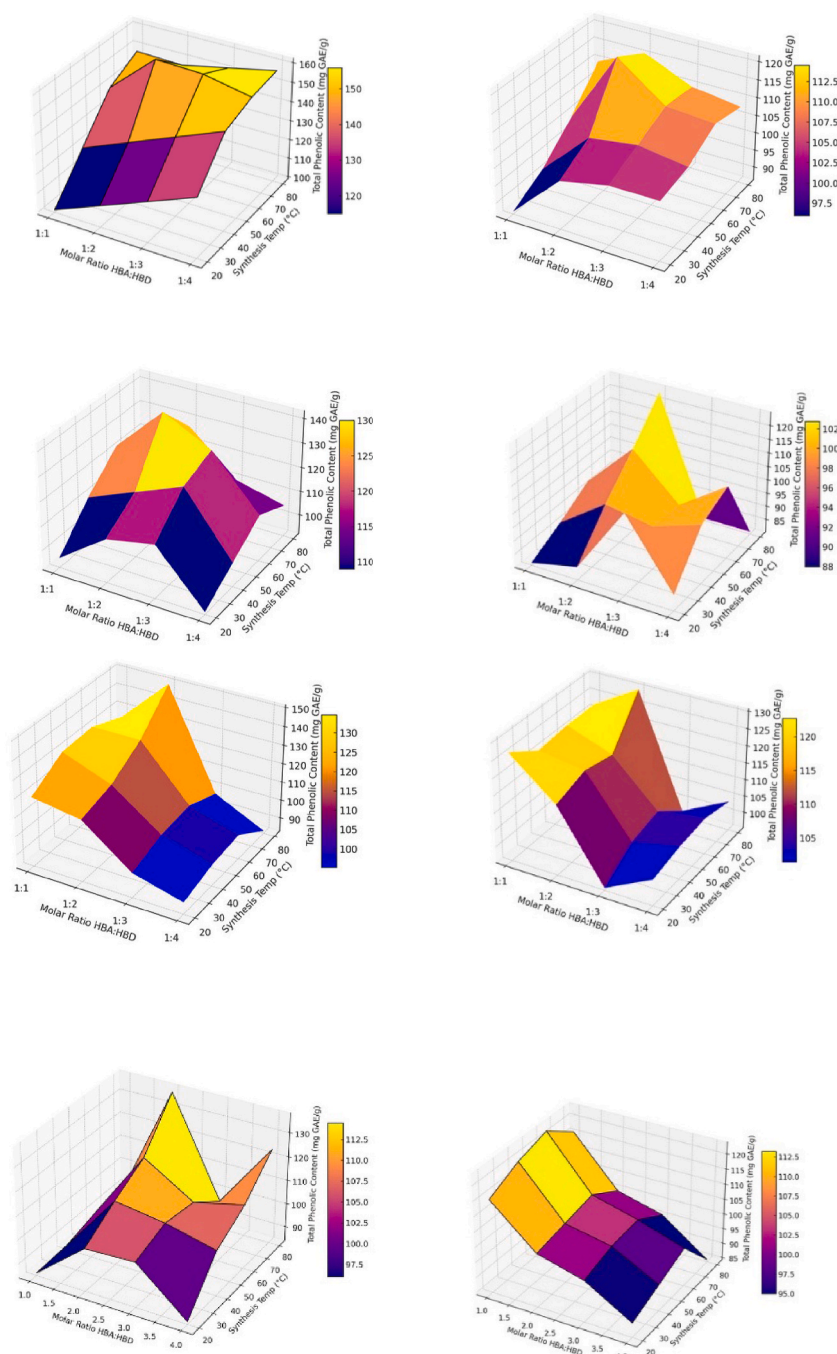
NADES formulations using different combinations of HBAs and HBDs.

Sample	HBA:HBD	Molar Ratio	SynthesisTemp (°C)	PhysicalCharacteristics	Viscosity at 25 °C (mPa·s)	Viscosity at 50 °C (mPa·s)	Total Phenolic Content (mg GAE/g)
S1	ChCl:Lactic Acid	1:2	60	Homogeneous, transparent, viscous	1.56	0.98	160.88
S2	ChCl:Malic Acid	1:3	80	Homogeneous, viscous, lessstable	2.65	0.99	147.56
S3	ChCl:Citric Acid	1:2	60	Viscous, stable	2.91	1.34	132.22
S4	ChCl:Glucose	1:2	60	Stable, moderatelyviscous	3.45	1.95	120.45
S5	ChCl:Sucrose	1:2	80	Stable, thick	4.12	2.65	118.77
S6	Betaine:Lactic Acid	1:2	60	Clear, moderatelyviscous	1.88	1.21	141.77
S7	Betaine:Malic Acid	1:2	60	Slightlyviscous	2.21	1.42	134.12
S8	Betaine:Citric Acid	1:2	80	Stable, clear	2.74	1.88	127.33
S9	Betaine: Glucose	1:2	80	Clear, viscous	3.12	2.17	123.67
S10	Betaine: Sucrose	1:2	80	Stable, high viscosity	3.72	2.87	117.89
S11	Proline:Lactic Acid	1:2	60	Moderate viscosity	2.05	1.22	149.44
S12	Proline:Malic Acid	1:2	80	Stable, slightlyviscous	2.11	1.29	135.88
S13	Proline:Citric Acid	1:2	60	Stable, low fluidity	2.67	1.61	122.18
S14	Proline: Glucose	1:2	80	Moderate fluidity	3.02	1.98	119.55
S15	Proline: Sucrose	1:2	80	Moderate fluidity	2.73	1.68	129.33
S16	Arginine: Lactic Acid	1:2	60	Viscous, stable	2.44	1.39	134.67
S17	Arginine: Malic Acid	1:2	60	Viscous, stable	2.44	1.39	129.75
S18	Arginine: Citric Acid	1:2	60	Moderate viscosity	2.33	1.51	136.12
S19	Arginine: Glucose	1:1	60	Veryviscous	4.55	3.22	123.45
S20	Arginine: Sucrose	1:2	60	Thick and clear	3.85	2.77	116.45

In this study, various NADES formulations were synthesized using different combinations of HBAs and HBDs to assess their efficiency in extracting polyphenols from plant-based matrices. As HBDs, both acid-based compounds (lactic acid, malic acid, citric acid) and sugar-based compounds (glucose and sucrose) were tested. For HBAs, besides the commonly used choline chloride (Conte et al., 2025), alternative natural compounds including betaine, L-proline and L-arginine were investigated. These HBAs—recognized for their capacity to form deep eutectic mixtures with a variety of hydrogen bond donors—were used to formulate different NADESs. The mixtures were prepared at varying molar ratios (1:1, 1:2, and 1:3) and temperatures of synthesis (from 60 °C to 80 °C) to assess how composition and processing conditions influence their physical properties, viscosity, and total phenolic content (TPC). Table 2 reports the composition based on their extraction performance and physicochemical stability.

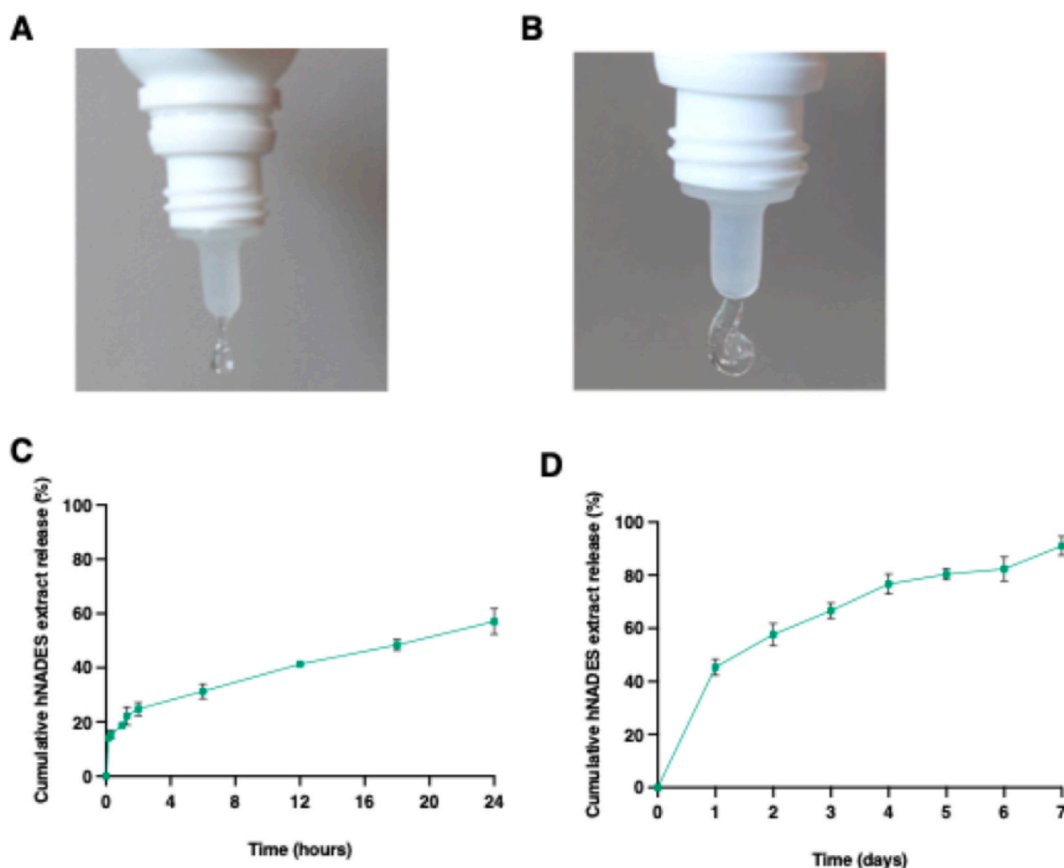
The screening trials revealed distinct differences between acid- and sugar-based NADESs. Consistently, acid-based formulations, particularly those incorporating lactic, malic, or citric acid, demonstrated better solubility for phenolic compounds. This behaviour is likely attributed to their higher polarity and stronger hydrogen bonding capabilities, which enhance solvent–solute interactions and ultimately improve extraction efficiency (Zhang et al., 2021). In contrast, sugar-based NADESs, such as those formed with glucose or sucrose, often exhibited excessive viscosity and poor homogeneity. These characteristics hinder mass transfer during extraction, resulting in reduced total phenolic content (TPC) as shown in Fig. 2 – Examples of extraction efficiency of NADES containing acid- or sugar-based HBDs, as influenced by the HBA:HBD molar ratio and synthesis temperature (Zhang et al., 2021).

Viscosity was identified as a critical parameter influencing extraction performance (Makarov and Kolker, 2025). NADESs synthesized at a 1:1 M ratio between HBA and HBD tended to be overly viscous and insufficiently homogeneous, which negatively affected their capacity to extract phenolic compounds. The 1:2 ratio generally offered the best compromise between stability and fluidity, particularly when the synthesis was carried out at 60 °C. This ratio allowed for efficient diffusion through plant matrices and facilitated higher TPC values. While a 1:3 ratio could reduce viscosity further, it often compromised the homogeneity of the resulting mixture, thereby limiting its practical applicability. Interestingly, within the tested temperature range (60–80 °C), synthesis temperature had no significant influence on phenolic content, suggesting that the choice of components and their ratios is more determinant than thermal processing.



**Fig. 2.** Extraction efficiency of NADES comprising acid- or sugar-based HBDs, as influenced by the HBA:HBD molar ratio and synthesis temperature. The figure illustrates representative examples of NADES formulations using various HBAs paired with either acid or sugar-based HBDs. Specifically: (a) Choline:Lactic Acid (S1), (b) Choline:Glucose (S4), (c) Betaine:Lactic Acid (S6), (d) Betaine:Glucose (S9), (e) Proline:Lactic Acid (S11), (f) Proline:Sucrose (S15), (g) Arginine:Citric Acid (S18), and (h) Arginine:Glucose (S19).

The nature of the HBA was also found to significantly affect the physical characteristics and extraction efficiency of the resulting NADES. Choline chloride (ChCl) proved to be the most versatile and widely compatible HBA, especially when combined with organic acids such as lactic acid, where it delivered excellent stability, low viscosity, and the highest TPC. However, other naturally occurring HBAs showed promising results as well. Betaine, for instance, formed stable and moderately viscous mixtures with both acid and sugar-based donors, offering good performance and biocompatibility. Proline-based NADESs showed compatibility with acids like lactic and malic acid, achieving comparable TPC values to ChCl systems while maintaining acceptable viscosity. In contrast, arginine led to



**Fig. 3.** Cumulative GA release profile from HAZ@gel at 34 °C in (A) Isosmolar medium (ISO, 312 mOsM) and (B) Hyperosmolar medium (HYPER, 400 mOsM); (C – D). GA permeation profile obtained from HAZ@gel in the above conditions. Data were expressed as mean  $\pm$  SD,  $n = 3$ .

highly stable but significantly more viscous systems, especially when paired with disaccharides like sucrose, potentially limiting its use unless combined with viscosity-reducing strategies.

Among all the tested formulations, considering both physicochemical properties, low toxicity, and TPC value, the ChCl:LA was the NADES chosen for preparation of hNADES for HAZ@gel.

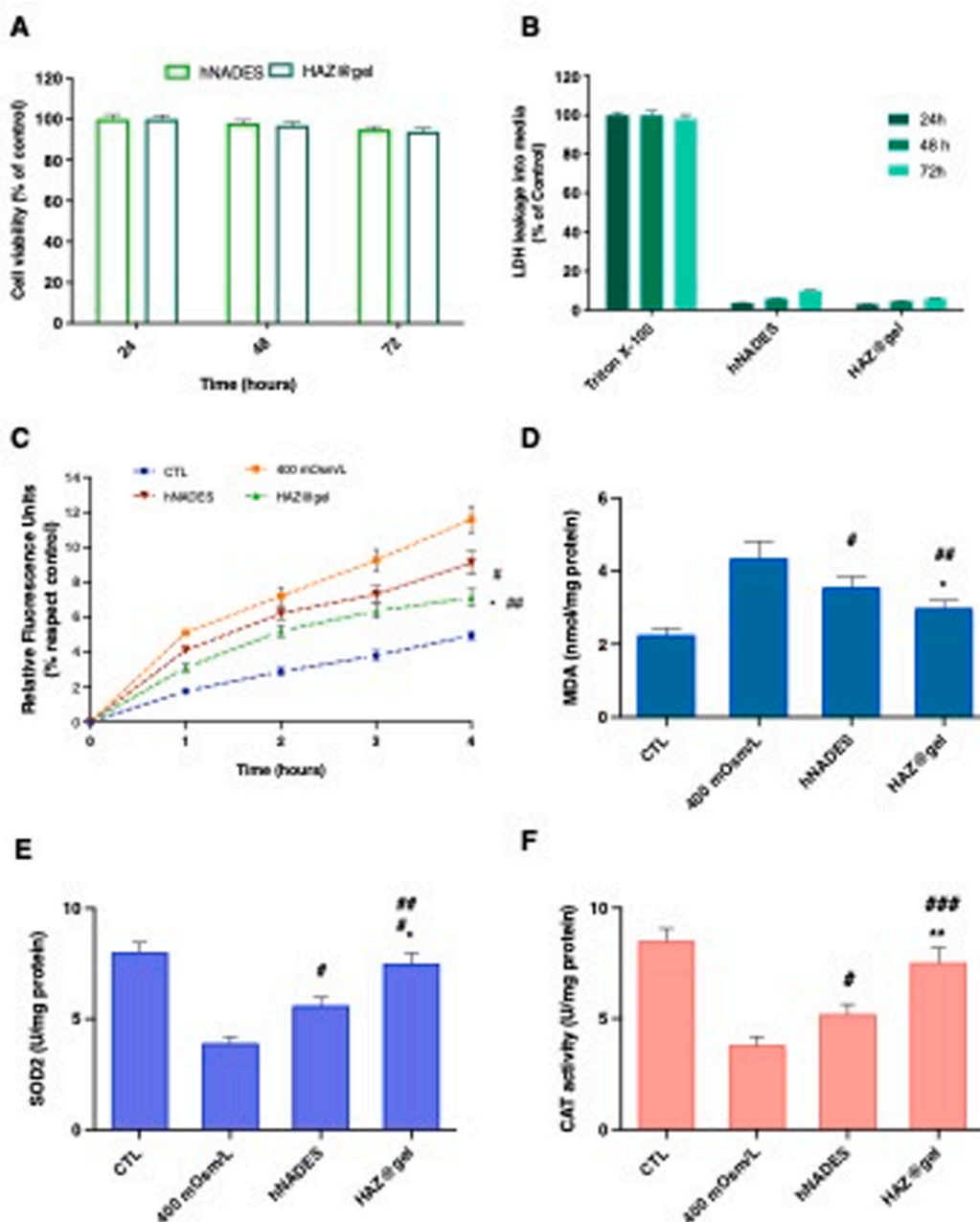
### 3.2. hNADES release from HAZ@gel

The Tear hyperosmolarity is a central pathological mechanism in dry eye disease (DED). To simulate *in vivo* conditions, the release profile of gallic acid (GA)—the most abundant polyphenol identified in the HAZ extract (Conte et al., 2025)—was evaluated from HAZ@gel in both isosmolar (ISO, 312 mOsM) and hyperosmolar (HYPER, 400 mOsM) media at 34 °C, mimicking the ocular surface environment. As shown in Fig. 3 (3A and 3B), approximately 12% of GA was released from HAZ@gel within the first 30 min under ISO conditions, with cumulative release reaching around 60% after 12 h. In contrast, the release from HAZ alone (i.e., without gel incorporation) was significantly faster, with nearly 25% of GA released at 30 min and over 80% within 12 h. The slower release from HAZ@gel is likely due to the Carbopol gel matrix, which forms a dense polymer network that physically restricts the diffusion of bioactives like GA. This matrix-mediated sustained release behavior may provide prolonged therapeutic activity, reducing the need for frequent re-application—an advantage over the burst release profile observed with free HAZ. To assess the potential for corneal permeation, a Franz diffusion cell study was conducted using a cellulose membrane to simulate the human corneal barrier. After 6 h, the cumulative permeation of GA into the receptor compartment reached approximately 62% for HAZ alone and 45% for HAZ@gel (Fig. 3C and D). The relatively lower permeation of HAZ@gel further supports the controlled release behavior imparted by the gel structure. Similar findings were reported by Alruwaili et al., where embedding nanoparticles in gel reduced permeation rate due to the hydrogel's barrier effect (Alruwaili et al., 2020).

### 3.3. HAZ@gel protection of corneal cells from oxidative damage

The accumulation of reactive oxygen species (ROS) represents one of the main causes of oxidative damage in diseases such as Dry Eye Disease (DED), contributing to cellular damage, inflammation, and tear film instability (Böhm et al., 2023a). Under physiological

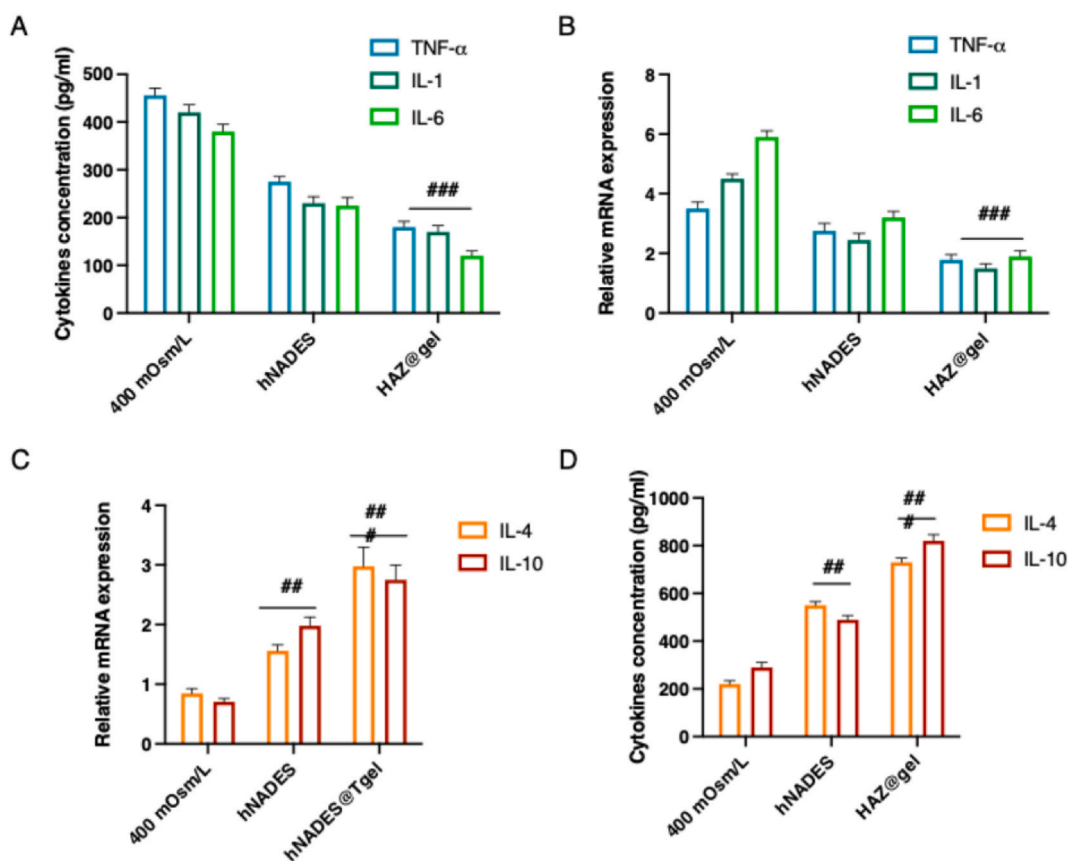




**Fig. 4.** Cytotoxicity was determined in HCEC cells after 24, 48, and 72 h of incubation with concentrations of hNADES and HAZ@gel using: (A) the Cell Counting Kit-8 (CCK-8) assay, and (B) the Lactate Dehydrogenase (LDH) assay. Effect of HAZ@gel on ROS production. HCECs were cultured under hyperosmotic stress (400 mOsm) in presence of hNADES or HAZ@gel for 24h. (C). Intracellular ROS release determined by oxidized H2DCFDA (DCF). (D). Quantification of Malondialdehyde used as a marker of lipid peroxidation. (E) Superoxide dismutase (SOD2) and (F) Catalase (CAT) activities measured by assay kit. Results are expressed as the means of three independent experiments  $\pm$  S.D. (n = 3). Statistical analysis was performed by One-way ANOVA, \*\* $p < 0.01$  versus hNADES 100  $\mu$ M; # $p < 0.01$  and ## $p < 0.01$  versus 400 mOsm-treated cells.

conditions, ROS are naturally produced by mitochondrial metabolism and are effectively balanced by antioxidant defense mechanisms (Zorov et al., 2014). However, prolonged exposure to environmental factors such as atmospheric oxygen, air pollution, low humidity, or wind can disrupt this balance, leading to pathological ROS overproduction on the ocular surface (Böhm et al., 2023a). The subsequent oxidative damage affects cellular lipids, proteins, and nucleic acids, exacerbating epithelial injury and inflammation (Chandimali et al., 2025).

The first prerequisite for a novel ophthalmic formulation is to demonstrate excellent biocompatibility and minimal cytotoxicity. To this end, human corneal epithelial cells (HCECs) were incubated in presence of different concentrations of HAZ@gel conditioned



**Fig. 5.** Effect of hNADES and HAZ@gel on pro- and anti-inflammatory cytokines in hyperosmotic-stressed HCECs. HCECs were exposed to hyperosmotic stress (400 mOsm/L) and treated with either free hNADES extract or the hydrogel formulation (HAZ@gel). (A) ELISA quantification of pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in cell supernatants. (B) qRT-PCR analysis of relative mRNA expression levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. (C) qRT-PCR analysis of relative mRNA expression levels of anti-inflammatory cytokines IL-4 and IL-10. (D) ELISA quantification of IL-4 and IL-10 levels in cell supernatants. Data are presented as mean  $\pm$  SD of three independent experiments ( $n = 3$ ). Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test. ## $p < 0.01$ , ### $p < 0.001$  vs. hyperosmotic condition (400 mOsm/L group).

medium (CM; 1:1, 1:2, and 1:5 v/v, polyphenolic content 16 mg/mL) for 24, 48 and 72 h. As expected, more than 90 % of HCECs remained viable after 72 h of incubation, even at the higher tested concentrations (Fig. 4A). Furthermore, the release of lactate dehydrogenase (LDH) — a marker of membrane integrity — remained below 10%, confirming negligible cytotoxicity of the gel (Fig. 4B). By contrast, treatment with Triton X-100, used as a positive control for membrane disruption, significantly decreased cell viability. These data strongly support the excellent safety profile of HAZ@gel and its potential suitability for ophthalmic application.

To mimic the oxidative stress characteristic of DED, HCECs were exposed to hyperosmotic stress (400 mOsm/L), a well-validated *in vitro* dry eye model (De Luca et al., 2023). As shown in Fig. 3C, this condition caused a 2.4-fold increase in intracellular ROS levels compared to untreated cells, as measured by the oxidation-sensitive dye 2',7'-dichlorodihydrofluorescein (DCF). Pre-treatment with hNADES alone (30  $\mu$ M) slightly reduced ROS accumulation ( $\sim 20\%$ ,  $p < 0.05$  vs. hyperosmotic group). However, the ROS levels were still significantly higher than those observed in the control group. The HAZ@gel showed superior ROS scavenging activity compared with free extract, with an intracellular ROS level in gel-treated group similar to the levels observed in the control group (no significant difference to the control group).

Lipid peroxidation, a key consequence of ROS accumulation, was evaluated by quantifying malondialdehyde (MDA) production. As shown in Fig. 3D, hyperosmotic stress increased MDA levels by 1.9-fold, indicating oxidative damage to cellular membranes. HAZ@gel treatment induced a  $\sim 40\%$  reduction of MDA, restoring values close to baseline, whereas hNADES alone caused a moderate 20% decrease. These findings confirm that the formulation exerts strong membrane-protective antioxidant activity, mitigating ROS-induced peroxidative damage.

The antioxidant mechanism was further investigated by measuring the activity of endogenous antioxidant enzymes, including superoxide dismutase (SOD2) and catalase (CAT). These enzymes constitute the initial defensive mechanism in ocular tissues against oxidative damage, transforming superoxide radicals into hydrogen peroxide, which is then further decomposed into water and oxygen (Hong et al., 2024). Under hyperosmotic conditions, the activities of both SOD2 and CAT were significantly suppressed by 47% and 52%, respectively, compared to isotonic controls (Fig. 4E and F). Pre-treatment with HAZ@gel partially reversed this decline, restoring

SOD2 and CAT activities by 32% and 39%, respectively, indicating an effective preservation of mitochondrial antioxidant capacity.

Several clinical and experimental studies have documented the reduction of SOD, CAT, and glutathione peroxidase (GPx) activities in patients with ocular surface disorders, including DED (Bu et al., 2024). Gallic acid (GA), the most abundant polyphenol found in hazelnut cuticle extracts, has been widely reported to exert protective antioxidant effects by enhancing endogenous defense systems. For instance, Jiang et al. demonstrated that GA significantly upregulates the activities of SOD, CAT, and GPx on retinal ganglion cells under oxidative stress (Jiang et al., 2024). Similarly, Gao et al. reported that GA treatment improved cell viability and reduced lipid peroxidation markers, such as MDA, in oxidative damage models (Gao et al., 2019). The enhanced antioxidant effect observed with HAZ@gel may therefore be attributed not only to the direct ROS-scavenging capacity of GA, but also to its ability to stimulate the enzymatic antioxidant defense network.

Of note, the combination with NADES facilitates the solubilization and stabilization of phenolic compounds, improving their bioavailability and cellular uptake. NADES themselves have also shown mild antioxidant effects, further contributing to the overall bioactivity of the formulation (Hikmawanti et al., 2021). Taken together, these results strongly support the multifaceted antioxidant potential of HAZ@gel, evidenced by decreased ROS and MDA levels, restoration of antioxidant enzyme activities, and robust cytocompatibility.

### 3.4. HAZ@gel Suppression of inflammatory response under hyperosmotic stress

The chronic inflammation observed in DED is closely linked to oxidative stress and the upregulation of pro-inflammatory cytokines such as IL-6, IL-8, and TNF- $\alpha$ , which contribute to ocular surface damage and perpetuate disease symptoms (Bu et al., 2024). Hyperosmotic stress activates intracellular signaling cascades, including NF- $\kappa$ B and MAPK pathways, leading to the overexpression of these inflammatory mediators in corneal epithelial cells (L. Wang et al., 2011). Recent studies in both animal and cell-based DED models suggest hyperosmotic stress as a principal pathogenic factor responsible for producing inflammatory signaling molecules, including interleukin, tumor necrosis factor, and matrix metalloproteinases. (Baudouin et al., 2013; Corrales et al., 2008). The induced-inflammatory state leads to further cell damage, such as apoptosis of conjunctival, corneal, and lacrimal gland epithelial cells, initiating a cycle of events that perpetuate DED condition. In this context, molecules that can interrupt this vicious cycle, such as gallic acid are of great relevance.

To evaluate the anti-inflammatory potential of HAZ@gel under DED-like conditions, HCECs were exposed to hyperosmotic stress (400 mOsm/L) and treated with either free hNADES extract or hNADES incorporated into the gel. The levels of pro-inflammatory (TNF- $\alpha$ , IL-1, and IL-6) and anti-inflammatory (IL-4 and IL-10) cytokines were quantified by both ELISA and qRT-PCR.

As shown in Fig. 5A, hyperosmotic stress significantly increased the secretion of TNF- $\alpha$ , IL-1, and IL-6 compared to baseline, confirming the inflammatory response characteristic of DED. Treatment with hNADES extract moderately reduced cytokine levels, whereas HAZ@gel led to a pronounced reduction in cytokine secretion. Notably, TNF- $\alpha$ , IL-1, and IL-6 levels were decreased by approximately 40–50% in HAZ@gel-treated cells compared to the untreated hyperosmotic group ( $p < 0.001$ ). Consistently, Fig. 5B demonstrates that mRNA expression of these cytokines was significantly suppressed in HAZ@gel-treated cells with a reduction of more than 2-fold with respect to hyperosmotic-stressed controls, indicating that the formulation not only blocks cytokine release but also modulates the inflammatory signaling at transcriptionally level.

In addition to suppressing inflammatory mediators, the immunomodulatory effect of HAZ@gel was reflected by its ability to enhance anti-inflammatory cytokines. As shown in Fig. 5C, the mRNA levels of IL-4 and IL-10 were significantly elevated following treatment, with the HAZ@gel group showing a marked increase compared to both the hyperosmotic group and the free hNADES treatment ( $p < 0.01$ ). Similarly, ELISA data reported in Fig. 5D confirmed the increased of IL-4 and IL-10 secretion in HAZ@gel-treated HCECs, reaching levels nearly 2-fold higher than in hyperosmotic conditions. Since IL-4 and IL-10 are known to counteract pro-inflammatory responses and promote epithelial healing, the increase in secretion levels suggests that the formulation was able not only to inhibit harmful inflammatory cascades but also to actively restore immunological balance on the ocular surface.

The anti-inflammatory effects observed with HAZ@gel are strongly supported by emerging evidence on plant-derived polyphenols and innovative delivery systems in ocular disease models. Polyphenols such as gallic acid and catechins—abundantly present in hazelnut cuticle extracts—have been shown to inhibit key inflammatory pathways, including NF- $\kappa$ B and MAPK signaling, and reduce the expression of cytokines such as IL-6, IL-8, and TNF- $\alpha$  in ocular surface cells (Lakshmi et al., 2020; K. Li et al., 2023). These findings are consistent with our results, where HAZ@gel significantly reduced the expression and release of pro-inflammatory cytokines under hyperosmotic stress conditions.

Overall, the observed anti-inflammatory efficacy underscores the relevance of using plant-derived polyphenol-rich extracts in combination with NADES and advanced delivery matrices. The dual action of HAZ@gel—suppressing pro-inflammatory and enhancing anti-inflammatory cytokines—underlines its potential as a multi-targeted, biocompatible therapy for DED. This immunoregulatory profile may be attributed to the synergistic action of HC-derived polyphenols and the bioadhesive, sustained-release properties of the Carbopol hydrogel system.

### 3.5. Evaluation of antimicrobial activity and determination of Minimal Inhibitory Concentrations (MICs)

Recent studies highlighted that alterations in the ocular bacterial flora have been implicated in the etiology of dry eye disease (Qi et al., 2021). Topical or systemic antibiotics with anti-inflammatory activity such as Azithromycin have been proven to be effective in addressing underlying bacterial infections that contribute to dry eye symptoms. However, the increase of antibiotic resistance observed in dry eye individuals has led to numerous works on the research and application of natural substances as sources of

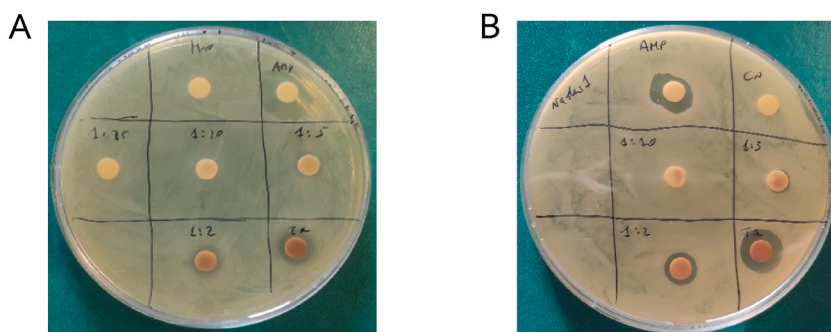


Fig. 6. Variations of Inhibition halo diameter of hNADES extracts against (A) *B. subtilis* and (B) *E. coli*.

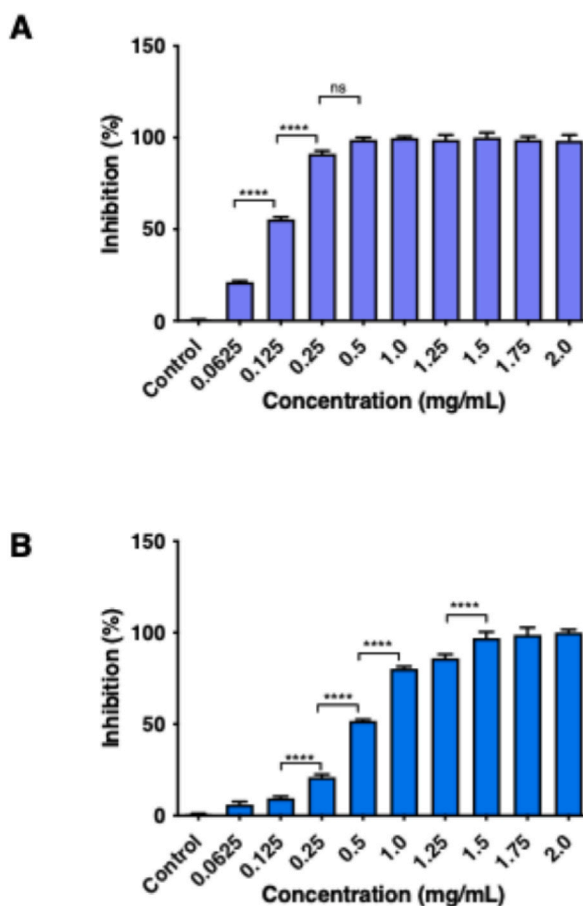


Fig. 7. Evaluation of the Minimal Inhibitory Concentrations (MIC) of hazelnut cuticle extracts with hNADES against: (A) *B. subtilis* and (B) *E. coli* tested in the range of 0.0625–2.0 mg/mL concentration. The data are expressed as means of three independent experiments  $\pm$  S.D. ( $n = 3$ , mean  $\pm$  SD). Statistical analysis was performed by One-way ANOVA,  $*p \leq 0.01$ ,  $**p \leq 0.005$  and  $***p \leq 0.0001$ .

antimicrobial compounds (Kotakonda, 2024). In this context, the antimicrobial properties of HAZ@gel in terms of its inhibitory activity against Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria was determined.

Using the disc diffusion method, the antimicrobial effect of 1:25, 1:10, 1:5, 1:2 and undiluted CM were tested after 24 h of incubation. As shown in Fig. 6A, undiluted CM yield inhibition zone of 23 mm for *B. subtilis*, with no significant difference with respect to the 20 mm zone produced by the ampicillin used as positive control. The 1:2 dilution resulted in 15 mm inhibition zones, indicating a concentration-dependent decrease in antimicrobial activity. This trend was confirmed by results obtained with further dilutions, demonstrating diminished efficacy at lower concentrations. In the case of *E. coli* (Fig. 6B), undiluted CM produced 20 mm inhibition zone, whereas the ampicillin exhibited a larger zone of 28 mm. As for *B. subtilis*, further dilution produced smaller inhibition zones,

reflecting reduced antimicrobial activity with decreasing concentrations. The lower susceptibility of *E. coli* with respect to *B. subtilis* is consistent with the presence of outer membrane in Gram-negative bacteria that acts as a barrier to many antimicrobial agents, including phenolic compounds, thereby reducing their efficacy (De Rossi et al., 2025; Maher and Hassan, 2023).

Minimum inhibitory concentration (MIC) assays corroborated these findings. In particular, the MIC values for *E. coli* was 1.5 mg/mL with a percentage of inhibition of 86 % at 1:2 dilution, while for *B. subtilis* it was significantly lower, reaching 0.5 mg/mL (with a 92 % inhibition at 0.25 mg/mL of extract) (Fig. 7). These results confirm the increased sensitivity of Gram-positive bacteria to hazelnut cuticle extracts. The superior antimicrobial activity observed with NADES-based extractions may be attributed to the solvents' ability to efficiently extract and stabilize phenolic compounds. hNADES have been shown to enhance the extraction of phenolic compounds from various plant sources, leading to improved antimicrobial efficacy (Jurić et al., 2021). Compared to conventional and microwave-assisted extraction methods, hNADES extracts demonstrated improved antimicrobial efficacy against *E. coli* and comparable or better performance against Gram-positive strains. This highlights the potential of NADES not only as green solvents but also as enhancers of biological activity (Lazović et al., 2024).

Consequently, hazelnut cuticle extracts obtained using NADES represent a promising class of natural antimicrobial agents, with potential applications in food safety and preservation, particularly in the context of increasing demand for clean-label and additive-free food products.

#### 4. Conclusions

This study presents a novel and sustainable ophthalmic formulation—HAZ@gel—designed to address the complex pathophysiology of Dry Eye Disease. By leveraging Natural Deep Eutectic Solvents (NADES) for green extraction of bioactive polyphenols from hazelnut cuticle waste, the approach not only enhances the therapeutic efficacy of the formulation but also contributes to waste valorization and eco-innovation. The hydrogel demonstrated prolonged release, potent antioxidant and anti-inflammatory effects, and antibacterial activity, offering a biocompatible and preservative-free platform with potential for long-term ocular application. These results highlight HAZ@gel as a promising candidate for DED treatment and underscore the relevance of integrating circular economy principles and green chemistry in the development of next-generation ophthalmic therapies.

#### CRedit authorship contribution statement

**Fabrizia Sepe:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Ezia Costanzo:** Investigation, Formal analysis. **Viviana Chiappini:** Investigation, Formal analysis. **Loredana Marcolongo:** Writing – review & editing, Supervision, Formal analysis. **Sabrina Margarucci:** Formal analysis, Data curation. **Orsolina Petillo:** Formal analysis, Data curation. **Giuseppe Ruggiero:** Methodology, Data curation. **Diego Circolo:** Methodology, Data curation. **Raffaele Conte:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Anna Valentino:** Writing – review & editing, Supervision, Conceptualization. **Gianfranco Peluso:** Supervision, Project administration, Funding acquisition. **Anna Calarco:** Supervision, Project administration, Funding acquisition, Conceptualization.

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#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Data availability

Data will be made available on request.



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