Universidad de Valladolid

Escuela de Doctorado Universidad de Valladolid

PROGRAMA DE DOCTORADO EN CIENCIAS DE LA VISIÓN

TESIS DOCTORAL:

VALORIZATION OF THE OLIVE BY-PRODUCTS PHENOLIC COMPOUNDS AND THEIR EVALUATION AS THERAPEUTIC AGENTS FOR OCULAR SURFACE INFLAMMATORY DISEASES

Presentada por Nikolaos Katsinas para optar
al grado de
Doctor por la Universidad de Valladolid

Dirigida por:
Dra. Amalia Enríquez de Salamanca y Aladro
Dra. Soraya Rodríguez Rojo

Universidad de Valladolid

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Impreso 1T

AUTORIZACIÓN DE LOS DIRECTORES DE TESIS

(Art. 7.2 de la Normativa para la presentación y defensa de la Tesis Doctoral en la UVa)

Dª. AMALIA ENRÍQUEZ-DE-SALAMANCA ALADRO, con D.N.I 05408578J, investigadora principal del Instituto de Oftalmobiología Aplicada (IOBA) de la Universidad de Valladolid, con dirección a efecto de notificaciones en Edificio IOBA, Paseo de Belén 17, 47011 Valladolid, y e-mail amalia@ioba.med.uva.es

Dª SORAYA RODRÍGUEZ ROJO con D.N.I. 09796094A investigadora postdoctoral de la Universidad de Valladolid, del Instituto de Bioeconomía de la Universidad de Valladolid y con dirección a efectos de notificaciones en Departamento de Ingeniería Química y Tecnología del Medio Ambiente, Escuela de Ingenierías Industriales, Sede Doctor Mergelina, y e-mail soraya.rodriguez@uva.es

como Directoras de la Tesis Doctoral titulada:

"VALORISATION OF THE OLIVE BY-PRODUCTS PHENOLIC COMPOUNDS AND THEIR EVALUATION AS THERAPEUTIC AGENTS FOR OCULAR SURFACE INFLAMMATORY DISEASES"

realizada por D. NIKOLAOS KATSINAS alumno del Programa de Doctorado de CIENCIAS DE LA VISIÓN **autorizan** su presentación, considerando que cumple todos los requisitos necesarios para presentarlo como una tesis y que es **APTA** para su defensa.

Dª. AMALIA ENRÍQUEZ-DE-SALAMANCA ALADRO [1]:

☒ Declara que cumple los requisitos para poder ejercer la dirección de la tesis doctoral que establecen el RD 99/2011 (modificado por el RD 195/2016) y el Acuerdo del Comité de Dirección de la Escuela de Doctorado de la Universidad de Valladolid del 17 de febrero de 2014, es decir, que posee "al menos un período de actividad investigadora reconocido de acuerdo con las previsiones del RD 1086/1989 de 28 de agosto"

Dª SORAYA RODRÍGUEZ ROJO

☒ Declara que cumple los requisitos para poder ejercer la dirección de la tesis doctoral que establecen el RD 99/2011 (modificado por el RD 195/2016) y el Acuerdo del Comité de Dirección de la Escuela de Doctorado de la Universidad de Valladolid del 17 de febrero de 2014, es decir, que posee "al menos un período de actividad investigadora reconocido de acuerdo con las previsiones del RD 1086/1989 de 28 de agosto"[2].

Valladolid, a fecha de firma electrónica

Las Directoras de la Tesis,

ENRIQUEZ DE SALAMANCA ALADRO AMALIA - 05408578J

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Fecha: 2021.12.02 14:46:55 +01'00'

Firmado por RODRIGUEZ ROJO, SORAYA (FIRMA) el día 03/12/2021 con un certificado emitido por AC DNIE 006

Fdo.: Dra. Amalia Enríquez de Salamanca Aladro

Fdo.: Dra. Soraya Rodríguez-Rojo

SR/SRA. PRESIDENTE/A DE LA COMISIÓN DE DOCTORADO

[1] *Esta declaración deben realizarla todos los codirectores de la tesis, en el caso de que haya más de un director.*

[2] *En el caso de que la figura contractual no sea evaluable por tramos o se trate de un profesor de una universidad extranjera, deberá haber aportado a la Comisión académica del Programa de doctorado un CV u otro documento acreditativo de poseer méritos equivalentes a un tramo de actividad investigadora.*

University Hospital Cologne
Division of Dry Eye and ocular GVHD
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University of Valladolid
to whom it may concern

Cologne, 12.07.2021

Letter of Confirmation for Nikolaos Katsinas

I hereby confirm, that Nikolaos Katsinas successfully accomplished his secondment at the University of Cologne (Germany) from April 12th until July 12th 2021. During this time he was supervised by Dr. Uta Gehlsen from the Division of Dry-Eye and ocular GVHD.

It was a great pleasure to work with Mr. Katsinas, who did an incredible job in our lab. We wish him all the best for his further scientific and professional career.

Sincerely,

Dr. Uta Gehlsen

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Oeiras (Portugal), July 21st 2020

To whom it may concern

I the undersigned, Naiara Fernández, Principal Scientist of Nutraceuticals & Bioactives Process Technology Lab (Food and Health Division) at iBET, confirm that Nikolaos Katsinas, student of the University of Valladolid (Spain), has performed at research stay at "Nutraceuticals and Bioactive Process Technology" laboratory from 10th January 2020 to 15th April of 2020.

He has satisfactorily carried out valuable research work at our laboratory, in the frame of the project IT-DED^3 (H2020-MSCA-ITN-ETN-2017).

Due to the COVID-19 pandemic, the period from 13th March to 15th April was carried out virtually, according to an agreed plan of activities.

Signature:

Dr. Naiara Fernández

To my family, José, and friends

Acknowledgments

A doctoral dissertation cannot be achieved without the guidance, support, and supervision of a group of people. Experienced researchers, experts in their field, together with technicians and scientists of all positions, make it possible for the dissertation to be embodied through their scientific, practical, and psychological support. This dissertation is dedicated to all these people, without the support of whom it could not have been accomplished.

Firstly, I would like to thank the directors of this thesis, Dr. Amalia Enríquez de Salamanca y Aladro and Dr. Soraya Rodríguez Rojo for the amazing opportunity of working with them these 3 years, their guidance throughout this trip, and their supervision since the beginning. Thank you, Amalia, for being present from the very first moment, for all the planning that tremendously helped me, for teaching me time and project management, for never leaving me scientifically alone, and above all for trusting a pharmacological topic to a "natural chemistry" pharmacist. On a personal level, thank you for supporting me whenever needed, for trying to make me feel at home in a different country, for your corrections in Spanish, and for your kindness, which is one of the most important (and rare!) things in a collaboration. Soraya, scientifically, thank you for making me see the "engineering" point of view of the natural products, for teaching me how to write, for being "tiquismiquis" (although it was hard many times!), and for your strong supervision and availability. Personally, thank you for understanding all the difficulties throughout this journey, for your availability 24/7, for trying to help me with my next steps, for the amazing communication and kindness, and for making me part of your family (tío Nikos!). Thank you both for trusting me with the position within a Marie-Curie project 3 years ago, giving me the opportunity to change my life, develop personally and scientifically, and learn a bunch of new things (including a new language!). For all these reasons and a lot more: thank you! They say that collaboration with the supervisors during a Ph.D. thesis is difficult. Maybe they have never worked with Amalia and Soraya!

By extension, I would also like to acknowledge the tutor of this thesis, Dr. María Jesús González García for the administrative management of the IT-DED^3 project, the extension of my contract, the administration of the patent procedure, the communication with any partner whenever needed, as well as the idea of using resveratrol and quercetin for dry eye, which many years later gave birth to the present topic. Similarly, I would like to thank Prof. Dr. Margarita Calonge and Prof. Dr. María José Cocero Alonso for accepting me into their research groups, for trusting me, and for supporting me whenever needed. Additionally, I would like to acknowledge the corrections of Prof. Dr. Calonge in the introduction of this manuscript, teaching me the up-to-date therapy of dry eye better than anyone else. For her important cellular and experimental advice in the monthly meetings, as well as her availability for replying to all questions related to the doctorate program "Ciencias de la Visión", I acknowledge Dr. Yolanda Diebold Luque. Additionally, I would like to thank Dr. Laura García Posadas for the design and performance of the *in vitro* assays on CD4+ T cells, Dr. Itziar Fernández for her guidance and support for the design of experiments and the statistical analyses, as well as Prof. Dr. José Carlos Pastor for his dedication during all these years, which made IOBA one of the most well-known ophthalmological institutes in the country.

I'd also like to extend my gratitude to the scientific groups of iBET (Portugal) and the Ocular Surface group of the University of Cologne (Germany) for their amazing experience during my secondments. From the iBET team, thanks to Prof. Dr. Maria Rosario Bronze and Dr. Naiara Fernández Hernández for their guidance and accessibility despite their busy schedule and obligations, as well as their support and understanding during the COVID-19 outbreak. Prof. Dr. Bronze, thank you for offering us your personal number to help us accelerate the submission of our third article. Dr. Naiara, thank you for your help and kindness throughout my stay in Lisbon, helping me remember this experience as the last "carefree" moment before COVID-19. I also want to acknowledge the great collaboration with Andreia Bento da Silva, who taught me how to use the MassLynx software and performed all HPLC-DAD-MS/MS experiments. From the team of the University of Cologne, I wish to thank Prof. Dr. Philipp Steven and Dr. Uta Gehlsen for the amazing (German!) organization and scientific planning and guidance before, during, and after my secondment. Prof. Dr. Steven, it was an honor to have worked in your team. Thank you for the experience of the weekly meetings with your interesting questions, your trust in me, your excitement with our *in vitro* work (and later with our *in vivo* results), and your interest, inviting the entire team to your house, offering me gifts before my departure, and taking care if I am well installed in Cologne. Dr. Uta, I feel lucky scientifically and personally for meeting you and having worked with you. Before the arrival to Cologne, you took care to introduce me to the right people (your people!) and made sure I was able to find a house. You literally changed my secondment in Cologne. Thank you for teaching me a bunch of new experiments and their principles, for being scientifically present throughout my secondment, for your corrections, and your availability. Also, I will never forget your contribution to my next steps (new positions, CV opinion, etc.). Thanks to both groups and their amazing people, this thesis was able to move forward, completing interdisciplinary experiments.

I am also grateful to Dr. Michael Stern for his important contribution regarding ocular surface pathophysiology and his advice in all the meetings he participated in. Thank you also for your kind words, congratulations, and excitement about the present work, and for believing that these results are compelling. As well, I would like to thank the entire external advisory board of IT-DED^3 (Dr. Jean-Marie Stassen, Dr. Virginia Calder, Dr. Christel Menet, and Prof. Dr. Carina Koppen) for their fruitful advice during the annual meetings, and Prof. Dor. Koen Augustyns for the coordination of the IT-DED^3 project. A big thank you to Paz Yanez for the excellent management of the IT-DED^3 project (project manager role model!), her availability 24/7 regarding any questions or problems appearing, and her kind and helpful personality. Additionally, I would like to acknowledge Maya Berg, the scientific research manager and supervisor of coordination of the IT-DED^3 project, for the revision of all scientific reports and the scientific management of the project during all these 3 years.

Thanks also go to a group of people who helped me every day, without the technical and practical support of whom this thesis could not have been performed. First, special thanks to Carmen García Vázquez for her technical support and training for the cell-based experiments and Luminex bead-based assays, as well as the great support and availability whenever a problem appeared,

and to Isabel Rodríguez Baz for her technical support for the PLE experiments, for her magic hands (which made everything possible) and her kindness and nice words. Further, I would like to thank Araceli Crespo Rodríguez for her professionalism and technical support during ORAC experiments, Beatriz Muñoz for her analyses TPC and TFC, and Mónica Gay Martín for the HPLC assistance. Thanks also to Antonio López García and Maite García Gutiérrez for the organization of the laboratories and the orders of IOBA, and Paulina De la Fuente García, as well as Mari Paz García and Ana Rodríguez, for the administration of the project bureaucracy. From the IOBA team, I cannot forget Dr. Teresa Nieto and Carlos Matesanz, who were both present every day, making the working environment amazing. Dr. Teresa, thank you for your nice personal feedback and advice, as well as your support, during this journey, it was nice working with you every day. From the iBET team, I would like to acknowledge Liliana Rodrigues for her assistance during ORAC assays, Beatriz Anacleto for the HPLC analyses, and the entire group of researchers for receiving me warmly and making me feel comfortable since the beginning. Particularly, I would like to thank Maha Abdallah for the great time we spent in Lisbon and Valladolid, the great support and collaboration during the experiments, as well as the great understanding and friendship we were able to build. From the team of the University of Cologne, I am grateful to Timur Bilgin for our great collaboration during my 3-month stay. Thank you for being so kind, for teaching me many techniques, and for being available 24/7. Moreover, from the team in Germany, I'd like to extend my acknowledgments to Anna-Lena Zachert for her technical support and kindness, Martina Maaß for her guidance during the PCR experiments and her support whenever needed (even after my departure from Germany!), as well as the entire team of the Ocular Surface group; Thomas (thank you for your kindness), Gwen (meet you again in Crete? – Thanks for offering me your CV and cover letter), Karina (I will never forget your cakes!), Alfi (your bike made my life easier in Cologne), Tom (some Tahini maybe?), Bao (it's a pity ROS test didn't work out, but it was worth trying), and Asif (you offered me your working space!), thank you for making me want to come to work every day, it was an amazing environment to work in.

Of course, I would like to thank all people from the Ocular Surface group of IOBA and the High-Pressure Processes group; Alfredo, Amanda, Cristina (Arroyo and Valencia), Andrea, Ana, Andrés, Diego, Cristina Andrés, Elena, Laura (Valencia), Marina, Sara, Ismael, Javier, Kevin, Mario, Marta, and Pablo from IOBA, and Andrea (also for helping me with the free sugar/total polysaccharides analyses), Silvia, Rut (for showing also me how to work with the PLE apparatus), Maira, Tijana, Elaine, Vesna, Marta, Juan, Laura, Reinaldo, Emre, and María from the High-Pressure Processes group. Thank you all for the very nice environment and collaboration. Cristina Arroyo, thank you also for all the information and support you provided me whenever needed during these 3 years (related to the present thesis or not!). Also, I acknowledge the IOBA's nursery team for the blood extraction from the healthy volunteers, to perform the *in vitro* experiments on CD4+ T cells. Further, I would like to personally thank Luna Krstic for the support, collaboration, help, and understanding during our common 3-year journey, as well as the astonishing administration of the orders and any bureaucratic procedure, and the attention to detail. It was very nice working with you these 3 years, we supported each other a lot. These 3 years wouldn't have been the same without your support.

Finally, I couldn't forget thanking a group of people who were present every day in my personal life and supported me tremendously. First, my family (my parents and my sister), who supported all my steps from the beginning, knowing that they were going to see me rarely. I feel grateful to have a family that supports always all my decisions and has helped me continue my studies even during difficult times for them. Additionally, I cannot forget the importance of José in this journey. A part of this Ph.D. belongs to you, without your support this work wouldn't have been the same, I feel lucky to have met you, you are an amazing person. Also, I feel lucky to have a group of friends that support my steps and follow my decisions; Georgia, Natasa, Eirini, Anastasia, and Maria, as well as Pinelopi, Jelly, Dimitra, Dora, and Evi. Georgia, Pinelopi, and Vincent thank you also for your availability and support regarding HPLC-DAD validation and for any additional scientific questions I had these years. Giorgos Papam thank you for the support regarding 3D response surface graphics, Vaggelis thank you for the support in designing the graphical abstract of Chapter 1, Tasou thank you for designing the front and back pages of this thesis and for all the technical support regarding InDesign software, and Giorgos Karampelas thank you for offering me the InDesign software. Rafel thank you for your professional advice and inspiration, you are one of the reasons I achieved the Marie-Curie scholarship. As well, I want to thank Dr. Marina Kritsanida and Dr. Raphaël Grougnet for teaching me all basic natural product chemistry techniques, as the supervisors of my diploma thesis, being also a family for me in Paris. My dedication to science was clearly inspired by them.

Last but not least, I would like to "thank" COVID-19 virus because it taught me that even in the hardest circumstances, you can achieve your goals if you want to follow them.

Author's Biography

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SKILLS

- **Project Management & Problems Solving**
  - 3 industrial and 6 academic projects within Marie-Curie European frameworks
- **Team management**
  - Supervision of students
  - 1 year editor in chief
- **Innovative mindset**
  - 3 first author publications across academia/industry
  - 1 patent submission
- **Reporting**
  - >50 industrial and academic scientific reports or publications
- **First class communication**
  - 7 international conferences
  - 2 Best oral presentation awards
- **Teamwork**
  - 5 academic & 3 industrial groups across 5 different countries

TECHNICAL SKILLS

- Extraction & Purification/Isolation techniques
- Chromatographic & analytical techniques
- *In vitro* assays (cell-based & cell-free)
- *In vivo* assays
- EU-GMP guidelines handling
- Formulation development
- Microorganism cultivation
- Organic chemistry

VOLUNTEERING

Kaleidoskopio Free-press magazine (GR)

Editor in Chief – Cinema column | 2010 – 2011

- Directing a team of 10 different editors towards the subject selection, also deciding its form
- Engagement of the 10 articles
- 8 Articles writing

INTERESTS

- Acting & Directing
- Theatre & Cinema
- Violin
- Journalism
- Crossfit
- Tennis
- Travelling

Nikolaos Katsinas

MPharm, MSc, Marie Sklodowska-Curie Ph.D. candidate

PERSONAL STATEMENT

An experienced and self-motivated scientist of H2020 & FP7 MSCA projects, specialized in natural products chemical engineering combined with pre-clinical *in vitro* & *in vivo* drug discovery and involved in scalable-oriented projects and manufacturing. Development of pipelines for innovative projects, turning academic ideas into commercial concepts. Flexibility to meet deadlines and proven ability to present results coherently with first class communication and writing skills. Demonstrated ability to work effectively as part of a team, under pressure, and engage others towards a common goal.

WORK EXPERIENCE

MARIE SKLODOWSKA-CURIE FELLOW | E.U. project: IT-DED³ — **Universidad de Valladolid - UVa (SP) | 10.2018 – Present**

- Results-oriented investigator: proposed a high value application as ocular surface therapy for an environmentally hazardous agro-industrial by-product. This work led to 3 first author publications and 20 scientific reports, commercializing our concept through 1 patent submission.
- Team player: collaborated with 2 different UVa groups and IT-DED³ partners to achieve research objectives & publications
- Problems solving: established connections among parts of the problems, adding previous industrial experience to the topic's research needs
- Effective communicator: participated in 5 congresses; in one, awarded with the "Best Oral presentation" award by the "Foods-MDPI" journal and in another, voted as "hot topic" by the organization committee
- Prominent researcher: awarded with 2 travel grants for congresses & achieved a Marie Sklodowska-Curie scholarship for the Ph.D. studies

RESEARCH FELLOW | E.U. project: IT-DED³ (internship) — **Uniklinik Köln (GE) | 04.2021 – 07.2021**

- Retentive & fast learner: performed studies in a new area of expertise (*in vivo* studies), leading to a 1 under review first author publication
- Time management: performed all studies in a limited 3-months time schedule, managing to complete the experimental plan
- Flexible co-worker: achieved the goals in various ways, adapting personal and professional style to the situation

RESEARCH FELLOW | E.U. project: IT-DED³ (internship) — **iBET (PT) | 01.2020 – 04.2020**

- Independent researcher: used knowledge acquired during MSc (spectrometric methods) to identify the structure of phenolic compounds
- Time management: applied purification techniques to selected extracts, being able to complete the project within a limited time schedule
- Teamwork: two of the publications achieved was also result of collaboration with 2 different groups of iBET

R&D FORMULATION SCIENTIST — **DEMO Pharmaceuticals SA (GR) | 10.2017 – 10.2018**

- Scalable research management: trained to design sterile pharmaceutical formulations, applying strict industrial experimental planning
- Reporting: participated in the product folder composition and in all reports and procedures prescribed by the guidelines

RESEARCH FELLOW | E.U. project: Microsmetics (internship) — **Givaudan France SAS (FR) | 06.2017 – 07.2017**

- Retentive learner & Applied Research mindset: performed studies in a new area of expertise on a short time schedule, cultivating microorganisms in petri dishes & erlenmeyers and scaling-up the MSc research academic study into bioreactors

RESEARCH FELLOW | E.U. project: Microsmetics — **National and Kapodistrian University of Athens (GR) | 04.2016 – 05.2017**

- Effective researcher: applied analytical, spectrometric and extraction techniques to achieve a bio-guided chemical study of 2 microorganisms, leading to the oral communication of the results in 2 international conferences

TEACHER — **AKMH Private Institute of Professional Training (GR) | 10.2016 – 02.2017**

- Strong interpersonal and teaching skills, independently developing two new courses (Cosmetology II and Control & Evaluation of Cosmetics), benefiting more than 25 students

RESEARCH FELLOW | Erasmus Internship — **Université Paris-Descartes (FR) | 02.2014 – 07.2014**

- Researcher in training: learned analytical & spectrometric methods, together with synthetic and hydrolysis reactions

EDUCATION

Ph.D. candidate with International Mention
University of Valladolid - UVa (SP)
2018 – 2021

MSc in Natural Products Chemistry – Pharmacognosy
National and Kapodistrian University of Athens (GR)
2015 – 2017

Bachelor and Master´s in Pharmacy (MPharm)
National and Kapodistrian University of Athens (GR)
2009 – 2015

LANGUAGES

- Greek (native speaker)
- English (Michigan C1)
- French (DALF C1)
- Spanish (fluent – C2 level)

ACCOMPLISHMENTS

- **3 publications**
  - https://doi.org/10.1021/acssuschemeng.0c09426
  - https://doi.org/10.3390/antiox10071150
  - https://doi.org/10.3390/molecules26196002
- **1 Patent Submission**
  - 28/12/2020 | Application №: P202031306 | Spanish Patent Office
- **"Best Oral Presentation - Foods and Natural Products" award**
  - EMSF 2021 Congress
- **"Best Oral Presentation" award**
  - 2021 | UVa Predoctoral Researchers Day
- **2 Travel Grants**
  - ARVO 2021 & EVER 2019 Congresses
- **Marie Sklodowska-Curie scholarship**
  - 2018 | for the Ph.D. studies
- **IKY Scholarship**
  - 2009 | for the undergraduate studies

Financial Support

This research project has received funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie Initial Training Network (ITN) "IT-DED^3" (H2020-MSCA-ITN-2017) grant agreement No. 765608.

Also, additional financial support has been received:

- Travel grant for Association for Research in Vision and Ophthalmology (ARVO) 2021 Annual Meeting by the Organization Committee
- UVa congress mobility grants for European Association for Vision and Eye Research (EVER) Congress 2019 (October 17-19, Nice, France)

Scientific Dissemination

This doctoral thesis resulted in 5 JCR indexed scientific publications, 3 of them published, one under revision, and another one under preparation:

1. **Katsinas, N.**; Bento da Silva, A.; Enríquez-de-Salamanca, A.; Fernández, N.; Bronze, M.R.; Rodríguez-Rojo, S. Pressurized Liquid Extraction Optimization from Supercritical Defatted Olive Pomace: A Green and Selective Phenolic Extraction Process. *ACS Sustain. Chem. Eng.* **2021**, *9*, 5590–5602, doi:10.1021/acssuschemeng.0c09426. (JCR 2020 Impact Factor: 8.198 – Q1 in Engineering, Chemical)

2. **Katsinas, N.**; Rodríguez-Rojo, S.; Enríquez-de-Salamanca, A. Olive Pomace Phenolic Compounds and Extracts Can Inhibit Inflammatory- and Oxidative-Related Diseases of Human Ocular Surface Epithelium. *Antioxidants* **2021**, *10*, 1150, doi:10.3390/antiox10071150. (JCR 2020 Impact Factor: 6.313 – Q1 in Biochemistry & Molecular Biology)

3. **Katsinas, N.**; Enríquez-de-Salamanca, A.; Bento da Silva, A; Bronze, M.R.; Rodríguez-Rojo, S. Olive pomace phenolic compounds stability and safety evaluation: from raw material to future ophthalmic applications. *Molecules* **2021**, *26*, 6002, doi:10.3390/molecules26196002. (JCR 2020 Impact Factor: 4.412 – Q2 in Chemistry, Multidisciplinary)

4. **Katsinas, N.**; Gehlsen, U.; García-Posadas, L.; Rodríguez-Rojo, S.; Steven, P.; González-Garcia, M.J.; Enríquez-de-Salamanca, A. Olive Pomace Phenolic Compounds: From an Agro-industrial By-product to a Promising Ocular Surface Protection for Dry Eye Disease. *Int. J. Mol. Sci.* Submitted. (JCR 2020 Impact Factor: 5.924 – Q1 in Biochemistry & Molecular Biology)

5. García-Posadas, L.; Romero-Castillo, I.; **Katsinas, N.**; Krstic, L.; Diebold, Y. Characterization and Functional Performance of a New Human Conjunctival Epithelial Cell Line. Manuscript under preparation.

Additionally, results derived from this work have also been presented in the following congresses:

Oral Presentations

- **Katsinas, N.**; Rodríguez Rojo, S.; García-Vázquez, C.; González-Garcia, M.J.; Enríquez-de-Salamanca, A. *Olive pomace phenolic compounds can inhibit inflammatory and oxidative–related diseases of human ocular surface epithelium*. **Pan-American Research Day: Anterior Segment Virtual Meeting.** (July 10, 2021)

- **Katsinas, N.**; Enríquez-de-Salamanca, A.; Cocero, M.J.; Bento da Silva, A.; Fernández, N.; Bronze, M.R.; Rodríguez-Rojo, S. *Selective phenolic-rich extracts from olive pomace by sequential supercritical CO_2 extraction and Pressurized Liquid Extraction with ethanol-water mixtures.* **18th European Virtual Meeting on Supercritical Fluids (EMSF).** (May 4-6, 2021) Awarded with the **"Best Oral Presentation – Foods and Natural Products" award by the Foods-MDPI journal.**

- **Katsinas, N.**; Bento da Silva, A.; Cocero, M.J.; Fernández, N.; Bronze, M.R.; Enríquez-de-Salamanca, A.; Rodríguez-Rojo, S. *Selective and intensified extraction of olive pomace phenolic compounds using green solvents.* **24th Virtual International Congress of Chemical and Process Engineering (CHISA).** (March 15-18, 2021)

Poster Presentations

- **Katsinas, N.**; Rodríguez Rojo, S.; García-Vázquez, C.; González-Garcia, M.J.; Enríquez-de-Salamanca, A. *In vitro evaluation of olive pomace phenolic compounds as therapeutic agents for the dry eye disease*. **1st European Dry Eye Society Virtual Congress (EuDEC).** (June 18-19, 2021)

- **Katsinas, N.**; Rodríguez Rojo, S.; García-Vázquez, C.; González-Garcia, M.J.; Enríquez-de-Salamanca, A. *Olive pomace phenolic compounds can inhibit inflammatory and oxidative–related diseases of human ocular surface epithelium.* **18th Virtual Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO).** (May 1-7, 2021) (Citation: *Invest. Ophthalmol. Vis. Sci.* **2021**, *62(8)*, 1290).

- **Katsinas, N.**; Cocero, M.J.; Bento da Silva, A.; Enríquez-de-Salamanca, A.; Fernández, N.; Bronze, M.R.; Rodríguez Rojo, S. *Green extraction optimization of phenolic compounds from olive pomace by pressurized ethanol-water.* **1st Greenering International Virtual Conference.** (February 15-16, 2021)

Furthermore, the work performed led to a patent submission (Date: 28/12/2020) in the Spanish Patent Office (Oficina Española de Patentes y Marcas):

COMPUESTOS PARA SU USO EN LA PREVENCION Y/O TRATAMIENTO DE LA INFLAMACION Y EL ESTRÉS OXIDATIVO DEL SEGMENTO ANTERIOR DEL OJO (Patent Nº: P202031306). **Katsinas, N.**; Enríquez-de-Salamanca, A.; Rodríguez-Rojo, S.; González García, M.J.; Cocero, M.J.; Calonge Cano, M. Entity: Universidad de Valladolid (UVa) (100%)

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”

Marie Curie

Abstract

This thesis aimed to valorize an environmentally hazardous agro-industrial by-product, the olive pomace, as source of phenolic compounds as potential therapeutic agents for dry eye disease. The effect of crude olive pomace extracts and their major pure phenolic compounds (oleuropein and hydroxytyrosol), alone or in combination, was studied in the inflammatory and oxidative processes of the ocular surface and evaluating them as future topical ophthalmic products.

For this purpose, a sequential extraction process was applied to olive pomace to obtain extracts rich in the phenolic compounds of interest (expressed as mg per g dry extract), as well as high chemical antioxidant activity. Supercritical carbon CO_2 extraction (to remove the residual olive oil) was followed by pressurized liquid extraction (to recover the phenolic compounds). This last step was optimized through design of experiments (circumscribed central composite design). The factors were temperature, ethanol percentage in water, and solid/liquid ratio. Subsequently, selected olive pomace extracts based on the optimization study, together with pure oleuropein, hydroxytyrosol, and low doses of their mixture were tested *in vitro* on two human ocular surface (corneal and conjunctival) epithelial cell lines for their anti-inflammatory and antioxidant effect, using as stimuli TNF-α to induce inflammation and UV-B to induce oxidative stress, respectively. In addition, the immunosuppressive effect of the most potent extract (namely, OPT3) and pure compound (hydroxytyrosol) was tested on lymphocytes CD4+ T cells activated with phytohemagglutinin-M. Their anti-inflammatory effect was also studied through topical application in a desiccating stress-induced dry eye disease mouse model. Finally, the stability and accessibility of the olive phenolic compounds were assessed under different pretreatment and storage conditions of the raw material. Two extracts were prepared by conventional solid-liquid extraction and used as references: CONV using freeze-dried material (reference for the *in vitro* study) and CONV-2 using freeze-dried material defatted with supercritical carbon CO_2 (reference for the extraction optimization study). Also, for the aqueous phenolic solutions, long-term storage stability at 4 different conditions and ocular surface safety through genotoxicity assay (comet) were performed based on industrial guidelines followed by EMA.

In 3 times shorter extraction time and 1.6 times less solvent consumption compared to CONV-2, an increase of 2 to 5-fold was observed in the phenolic extract richness, depending on the

response. In addition, different optimal extraction conditions were observed for each key compound and three extracts were selected: OPT1, which showed the highest chemical antioxidant activity, OPT2, which had the highest oleuropein content in dry extract, and OPT3, with the highest content in hydroxytyrosol. Among the different extracts screened *in vitro* , CONV and OPT3 demonstrated improved antioxidant and anti-inflammatory activity. On corneal epithelial cells, both extracts and hydroxytyrosol reduced the levels of most measured interleukins/chemokines (IL-1β, IL-6, IL-8, IP-10, and IL-17A) dose-dependently, whereas on conjunctival epithelial cells all treatments decreased IP-10 production. In addition, on both cell lines, all treatments reduced reactive oxygen species production. Compared to CONV, the OPT3 extract demonstrated antioxidant and anti-inflammatory activity at 10 to 40 times and 2 to 40 times lower concentrations, respectively. Thus, it was selected to be further tested *in vivo* and *in vitro* on CD4+ T cells, together with hydroxytyrosol. Both treatments inhibited the proliferation of stimulated CD4+ T cells and decreased corneal fluorescein staining, *IP-10* and *TNF-α* gene expression in lacrimal functional unit tissues, and lymphocyte count in cervical lymph nodes of mice exposed to desiccating stress. Among the pretreatment/storage methods of the raw material, lyophilization and supercritical carbon CO_2 increased the extract richness in the phenolic compounds measured. Hydroxytyrosol and oleuropein demonstrated different stability profiles as pure aqueous solutions or as part of an extract. Additionally, oleuropein also had a distinct degradation profile. No genotoxic effect was detected for any of the treatments.

In conclusion, an efficient, selective, scalable, and sustainable sequential extraction process was proposed, able to maximize the phenolic recovery from an agro-industrial by-product, reducing its environmental impact and using exclusively green solvents. In addition, olive pomace extracts and pure compounds (principally hydroxytyrosol) can modulate the inflammatory, oxidative, and immune responses on a cellular level at the ocular surface. Also, their topical application in a mouse dry eye model enhanced clinical signs and reduced inflammation in the ocular surface. Thus, a high-value application was demonstrated for the olive pomace compounds as potential ocular surface therapy. All treatments were proved to be safe for ophthalmic application at the concentrations selected and their industrial stability evaluation was established, necessary for their future approval as pharmaceutical products.

Table of Contents

Table of Abbreviations

%TDNA	Percentage (%) of DNA present in the comet tail
3,4-DHPEA-DEDA	Decarboxymethyl oleuropein aglycone dialdehyde (or Oleacein)
AA	Antioxidant activity
AAPH	2,2'-azobis(2-methylpropionamidine)dihydrochloride
ADDE	Aqueous deficient dry eye
ANOVA	Analysis of variances
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-cyanine7
BCA	Bicinchoninic acid
BSA	N,O-bis(trimethylsilyl)acetamide
CATE	Catechin equivalents
CCC	Circumscribed central composite (design)
CFS	Corneal fluorescein staining
CONV	Conventional olive pomace extract
DDOC	Dry defatted olive cake
DE	Dry extract
DED	Dry eye disease
DEWS	Dry eye workshop
DMEM/F-12	Dulbecco's modified eagle's medium/Nutrient mixture F-12
DMSO	Dimethyl sulfoxide
DoE	Design of Experiments
DPBS	Dulbecco's phosphate buffered saline
DS	Desiccating stress
EDE	Evaporative dry eye
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin Gallate
EGF	Epidermal growth factor
EMA	European Medicines Agency
ESI-	Electrospray Ionization Source in negative ion mode
EtOH	Ethanol
EtOH%	Percentage (%) of ethanol in water
EtOH%^{2}	Quadratic term of percentage (%) of ethanol in water
EtOH%-S/L	Interaction between percentage (%) of ethanol in water and solid/liquid ratio
EY	Extraction yield
FACS	Flow cytometry analysis
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FD-OP	Freeze-dried olive pomace
FD-OP-DO	Freeze-dried olive pomace after supercritical deoiling/defatting process
FITC	Fluorescein isothiocyanate
FS	Fluorescein sodium
GAE	Gallic acid equivalents
H_2DCF-DA	20,70-dichlorodihydrofluorescein diacetate

HCE	Immortalized human corneal epithelial cells
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine-guanine phosphoribosyl-transferase
HT	Hydroxytyrosol
hTCD4+	Human CD4+ T cells
ICAM-1	Intercellular adhesion molecule 1
ICH	International Council for Harmonization
IFN-γ	Interferon-γ
IL	Interleukin
IM-ConjEpi	Immortalized human conjunctival epithelial cells
IOBA-NHC	Immortalized human conjunctival epithelial cells
IP	Interferon γ-induced protein
IP-10	Interferon γ-induced protein-10
k_{obs}	Degradation constant
LFA-1	Lymphocyte function-associated antigen 1
LFU	Lacrimal functional unit
MeOH	Methanol
MMP-9	Matrix metallopeptidase-9
OL	Oleuropein
OL+HT	Combination of oleuropein and hydroxytyrosol
OLC	Oleacein
OLE	Oleuropein Equivalents
OP	Olive pomace
OPT	Optimized olive pomace extracts
OPT3	Selected optimized olive pomace extract – 3 (enriched in hydroxytyrosol)
ORAC	Oxygen radical absorbance capacity
ORAC-AA	Oxygen Radical Absorbance Capacity Antioxidant Activity
PBMC	Peripheral blood mononuclear cells
PHA-M	Phytohemagglutinin – M form
PLE	Pressurized liquid extraction
PMS	5-Methylphenazinium Methyl Sulfate
qRT-PCR	Quantitative real-time polymerase chain reaction
RH	Relative humidity
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
S/L	Solid/liquid ratio
S/L^2	Quadratic term of solid/liquid ratio
$scCO_2$	Supercritical carbon dioxide
SD	Standard deviation
SEM	Standard error of the mean
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
T	Temperature

T-EtOH%	Interaction between temperature and percentage (%) of ethanol in water
$t_{1/2}$	Half-life period
T^2	Quadratic term of Temperature
TE	Trolox equivalents
TFC	Total flavonoid content
TFOS	Tear Film & Ocular Surface Society
t_{lag}	Lag time
TNF-α	Tumor necrosis factor-α
TP	Tear production
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
T-S/L	Interaction between temperature and solid/liquid ratio
TY	Tyrosol
UV	Ultraviolet
WHO	World Health Organization
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

Motivation

Dry eye is a multifactorial disease, affecting millions of people worldwide. It has a high prevalence, varying from 5 to 48% depending on the population, while its economic and humanistic burden on the society is remarkable due to direct (medical and treatment) and indirect (loss of work productivity) costs, as well as a dramatic decrease in the quality of life of the patients. Its pathophysiology is based on chronic inflammation in the lacrimal functional unit, which is produced by evaporation-induced tear hyperosmolarity. This leads to tear film break up, which along with activated T cells and the inflammatory mediators (cytokines/chemokines) produced by them, cause ocular surface damage. Also, oxidative stress plays a very important role by activating inflammatory processes, whereas reactive oxygen species have been found to be effectors of inflammation. Several management and treatment approaches have been proposed, including topical steroids. The only molecules approved so far as dry eye disease therapy are cyclosporine A and lifitegrast. Their mechanism of action is based on the breakage of the inflammation circle involved in the disease. However, these treatment approaches have side effects (steroids), are not available in many countries (lifitegrast), or need a lot of time to reach a therapeutic effect, while they can also cause eye irritation and vision problems due to their lipid-based formulations (cyclosporine A). Thus, there is still a scarcity of effective therapy and, for this reason, the discovery of novel effective molecules for the management of dry eye constitutes an emerging issue in ophthalmology.

In this context, the use of natural phenolic compounds has been proposed as a therapeutic alternative, due to their "versatile" biological activities. Among them, the olive phenolic compounds were selected based on their demonstrated antioxidant, anti-inflammatory, and immunoregulatory effects, among others. Although these compounds are known to be present in olive oil, it has been proved that 98% of them end up in olive pomace. Olive pomace is the semi-solid by-product, produced during industrial olive oil production after the crushing of olive fruits and the subsequent two-phase centrifugation of olive oil. It is a mixture of vegetable waters with olive pulp and smashed pits and constitutes the major olive by-product, produced in huge quantities (between 7 and 30 million m^3 annually in the Mediterranean basin, the principal olive oil production area). The presence of phenolic compounds in olive pomace increases its chemical and biological demand for oxygen and adds phytotoxic and antimicrobial properties in the raw material, a fact that also prevents the natural degradation of these molecules. These facts, together with the presence of residual olive oil, make the management of olive pomace difficult. Thus, it constitutes an important pollution factor and there is increasing global awareness for its valorization, including physicochemical treatments, biotechnological transformations, and extraction of compounds for the cosmetic industry, among others.

Its valorization as a source of high-value phenolic bioactive compounds would offer a by-product with less oxygen demand and therefore, less environmental impact. Among the bioactive phenolic compounds present in olive pomace, oleuropein and hydroxytyrosol have been reported as the major and most cited with numerous proven biological activities, including anti-inflammatory and antioxidant. Furthermore, these molecules have activities on target cells involved in inflammation.

Hence, this thesis aimed to valorize an environmentally hazardous agro-industrial by-product, as well as its two major pure phenolic compounds (oleuropein and hydroxytyrosol, alone or in combination) as potential topical treatments in ocular surface immune-based inflammatory diseases, such as dry eye.

Organisation of the Doctoral Thesis

This Doctoral Thesis report is presented in the form of a **"compendium of publications"** and applies for the **International-awarded Doctorate Degree**. It has been performed at the University of Valladolid (UVa), under the regulations of the International Doctorate Committee. A three-month academic secondment was performed at the Medical Faculty of the University of Cologne (Cologne, Germany) and a three-month industrial secondment was performed at the Instituto de Biologia Experimental e Tecnologica (iBET) (Oeiras, Portugal). The joint requirements are as follows: (1) the whole manuscript has been written in English, (2) a general summary has been included in Spanish, (3) the thematic unit of the work has been justified, (4) the objectives, methodology, results, discussion, and conclusions have been presented, and (5) three publications in peer-reviewed journals with impact factor have been accomplished. In addition, one more article derived from the work of this thesis is currently under revision and has been included in the manuscript. Also, a submitted patent and some additional experimental results carried out in IOBA or during my stay in the research group of iBET, as well as in collaboration with AVIZOR S.A., are presented in the Appendices. As well, supplementary material of the published work has been included in each chapter.

After the general introduction, the hypothesis, and the objectives, the report is organized into four chapters, each one corresponding to the three published articles and the one submitted, reflecting the different blocks of results obtained. The organization of the chapters is not chronological but presented in a way that facilitates a better understanding of the whole work.

Each chapter of this Ph.D. thesis is based on the following articles:

• **Chapter 1: Pressurized liquid extraction optimization from supercritical defatted olive pomace: a green and selective phenolic extraction process.** The first step of this thesis was the optimal recovery of bioactive phenolic compounds from the raw material (olive pomace) through a sustainable and scalable extraction process. Part of this study was conducted during a three-months secondment in the laboratories of Faculdade de Farmacia da Universidade de Lisboa (FFUL) (Lisbon, Portugal) and iBET (Oeiras, Portugal), under the supervision of Drs. Maria Rosario Bronze and Naiara Fernandez. Chapter 1 describes this part of the study and is based on the manuscript published in *ACS Sustainable Chemistry & Engineering (Katsinas et al., 2021)*.

• **Chapter 2: Olive pomace phenolic compounds and extracts can inhibit inflammatory- and oxidative-related diseases of human ocular surface epithelium.** Then, the *in vitro* antioxidant and anti-inflammatory activity of four differently obtained olive pomace extracts (selected based on the work of Chapter 1) was evaluated, together with their major pure phenolic compounds (hydroxytyrosol and oleuropein), on human corneal and conjunctival epithelial cells. This work is described in Chapter 2 and is based on the manuscript published in *Antioxidants (Katsinas et al., 2021) – Special Issue: Intensification Technologies to Efficiently Extract Antioxidants from Agro-Food Residues.*

• **Chapter 3: Olive pomace phenolic compounds: from an agro-industrial by-product to a promising ocular surface protection for dry eye disease.** The last step of this work was the determination of the anti-inflammatory and immunomodulatory effect of a selected olive pomace extract and its major compound (hydroxytyrosol) on human CD4+ T cells, as well as in a mouse dry eye experimental model. The *in vivo* part of this study was conducted in the animal facilities of the Medical Faculty of the University of Cologne (Cologne, Germany), under the supervision of Dr. Uta Gehlsen and Philipp Steven, MD, during a three-month secondment. Chapter 4 reports the results of this work and is based on a manuscript submitted to *International Journal of Molecular Sciences — Special Issue: Dry Eye Disease—Focus on Drug Discovery and Development.*

• **Chapter 4: Olive pomace phenolic compounds stability and safety evaluation: from raw material to future ophthalmic applications.** Subsequently, two out of the four olive pomace extracts were selected based on their *in vitro* activity and their aqueous solutions were evaluated for their long-term stability and genotoxic effect on human ocular surface cells, together with pure hydroxytyrosol and oleuropein, based on EMA guidelines. The effect of different pretreatment methods on the recovery of the compounds of interest from the raw material was also tested; Degradation by-products were also identified. Part of this study was conducted in the laboratories of Faculdade de Farmacia da Universidade de Lisboa (FFUL) (Lisbon, Portugal), under the supervision of Dr. Maria Rosario Bronze. Chapter 3 summarizes these studies and is based on the manuscript published in *Molecules (Katsinas et al., 2021) — Special Issue: Plant Waste Management: Bioactive Compounds with Therapeutic Role in Disease Prevention.*

Introduction

1. Lacrimal Functional Unit and Ocular Surface

The lacrimal functional unit (LFU) is a complex apparatus, being composed of the ocular surface, the main and accessory lacrimal glands, and the interconnecting neural network (sensory and motor) (Figure 1).[1] The ocular surface comprises the eyelids, the cornea, the conjunctiva, the meibomian and lacrimal glands, and the tear film (limbus and overlying), which are well interconnected through an epithelium with nervous, immune, vascular, and endocrine connections.[2,3] The LFU is an integrated system, in which the sensory tissues and the secretory glands are well connected and control the tear secretion, regulating the homeostasis of the ocular surface.[3]

Free nerve endings cover the entire surface of the cornea. Their stimulation generates nerve impulses mediated by the ophthalmic branch of the Trigeminal Nerve (V). These impulses then reach the mid-brain (pons) through the trigeminal ganglion, they synapse, and the signal produced is merged to the cortical and other neural input.[1] The efferent nerve fiber sends impulses through the pterygopalatine ganglion to the lacrimal glands (main and accessory).[3] Several studies have reported that the free nerve endings present in the conjunctival goblet cells and the meibomian glands also follow the same route.[4,5] Therefore, the three major tear film components: mucin, lipid, and aqueous, seem to be secreted to the ocular surface in a controlled manner.[1]

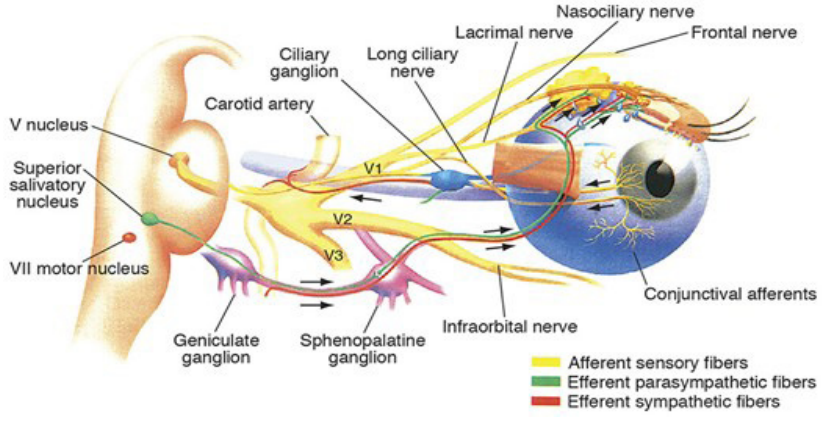


Figure 1. The anatomy of the Lacrimal Functional Unit (LFU) (obtained from Stern et al.[9]).

The principal role of the LFU is to produce a stable tear film, necessary for the health and function of the ocular surface. The ocular surface epithelial tissues are exposed to the outside environment, such as atmospheric oxygen and ultraviolet (UV) rays.[6] Through a normal and stable tear film, the ocular surface is smooth and able to maintain epithelial cell health from external environmental and microbial factors.[7] Also, the good quality of the image projected onto the retina can be ensured.[8] However, the tear film structure or composition can be modified by possible pathological changes (injuries or dysfunctions) in the LFU. This can lead to the loss of nourishment and lubrication (offered by the tear film) of the corneal and conjunctival epithelia, leaving them exposed and resulting in inflammatory processes in the LFU. These processes can cause ocular surface disorders, such as dry eye disease (DED).

The treatment of inflammatory disorders in the LFU is mainly based on topical drugs, due to their easy access to the ocular surface. Their objective is the downregulation of the inflammation in the cornea and conjunctival epithelia, as well as in the lacrimal glands. Thus, to find effective therapeutic agents for LFU disorders, first, it is important to understand the anatomy and physiology of this ophthalmic area.

1.1 Tear Film

As mentioned previously, the tear film provides lubrication to the ocular surface, which protects the corneal and conjunctival epithelial cells from the forces produced by the eyelids during blinking, a function that refreshes the tears continuously. Also, the tear film acts protectively against irritants, allergens, or pollutants, not only by reflex tearing but also through antimicrobial components present in it, such as enzymes (e.g., peroxidase, lysozyme) and proteins (e.g., lactoferrin), among others. Furthermore, since the cornea is a non-vascular structure, the tear film serves as a supplier of peptides/proteins, growth factors, electrolytes, and glucose for the ocular surface, also removing free radicals and wastes.[2]

The traditionally described structure of the tear film is composed of three layers: the lipid (superficial), the aqueous (middle), and the mucous (base).[2,10,11] More recent studies have proposed a 2-layers tear film: a lipid-superficial and a mucin/aqueous, with the latest having a form of glycocalyx gel with decreasing mucin concentration from the base (epithelium) to the surface (lipid layer).[2,9,10,12]

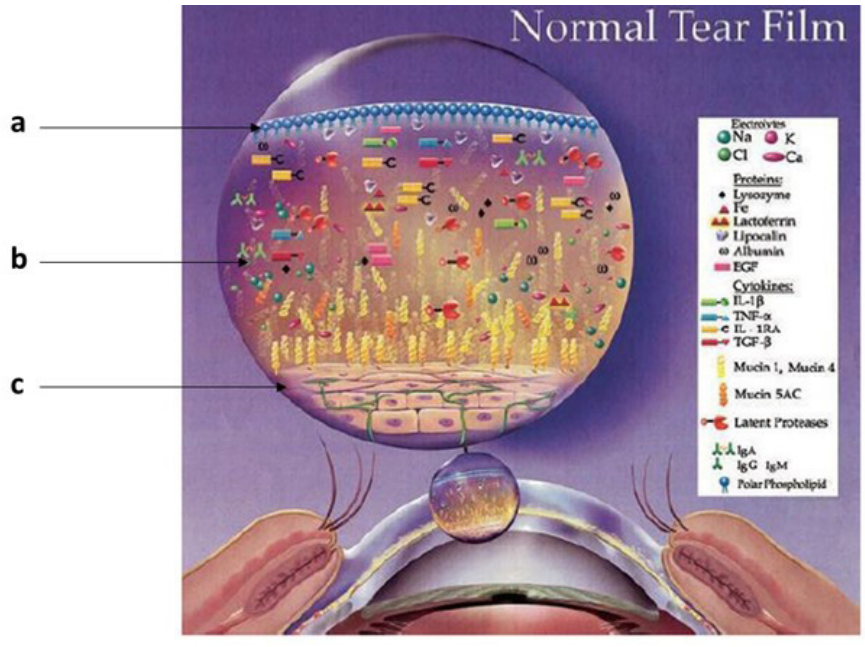


Figure 2. The normal tear film: main components and two-layer structure (**a.** lipid layer, **b.** mucin/aqueous glycocalyx layer, and **c.** corneal epithelium) (modified from Stern et al.[7]).

The lipid layer is secreted by the meibomian glands and is spread onto the tear film by the surface tension forces of blinking. It plays a crucial role in stabilizing the tear film and has also been thought to play an important role in minimizing tear evaporation.[12] In addition, it is the principal barrier from environmental particles and microorganisms, as well as skin lipids, while it also reduces the surface tension of the aqueous layer.[13] The thickness of this layer varies from 15-157 nm.[12]

The aqueous layer is secreted by the Krauze and Wolfring glands (accessory lacrimal glands), while the aqueous tear production is performed by the main lacrimal glands.[2,12,14] The aqueous layer constitutes approximately 90% of the tear film.[13] Apart from water, it consists of electrolytes, vitamins, amino acids, calcium, urea, bicarbonate, and magnesium, as well as proteins, cytokines, immunoglobulins, and growth

factors.[15,16] Among the proteins, albumin, tear-specific prealbumin, defensins, lactoferrin, lysozyme, lipocalin, immunoglobulins A, G, and M, and glycoproteins participate in the antimicrobial activity and immune defense of the ocular surface.[10,16,17] The ocular surface health is maintained through the presence of the growth factors, peptides, vitamins, electrolytes, and protease inhibitors,[2,10] while the presence of retinol (secreted by the lacrimal gland) is necessary for the maintenance of the cornea and the goblet cells.[16,18-20] The aqueous part participates in ocular surface lubrication, removal of contaminants, and nourishing the cornea.[10,21]

The mucous layer is secreted by the corneal and conjunctival epithelia, the lacrimal gland, and the conjunctival goblet cells.[2,10,12,16,22,23] Apart from secreted and transmembrane mucins, it also consists of immunoglobulins, glucose, leukocytes, urea, salts, cellular debris, and enzymes.[2,10,16,22,24] The gel-forming mucins form a glycocalyx cover that prevents pathogen adhesion, lubricates the ocular surface, and acts as clearing molecules. This layer also contributes to tear stability, acts as a barrier for corneal and conjunctival epithelium and is believed to be involved in cellular signaling.[13]

1.2 Conjunctiva

The conjunctiva is a thin and semi-transparent mucous membrane of 33 microns thickness located in the inside of the eyelids, covering also the sclera.[25,26] It is extensively vascularized and has a high number of lymphatic vessels. It protects and lubricates the eye through the secretion of mucus and tears (by the accessory lacrimal glands located in the conjunctiva). It is also a barrier for microbial entrance into the eye and plays a key role in immune surveillance.[26,27] Its structure and main components are presented in Figure 3.

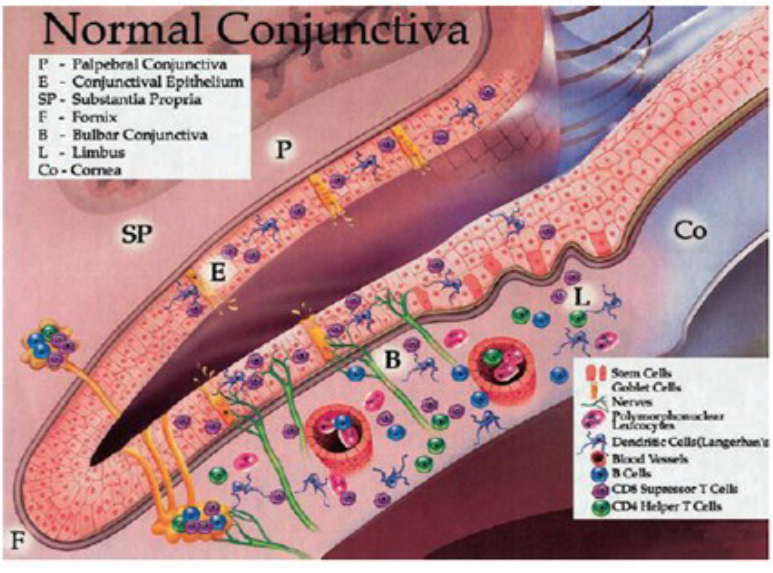


Figure 3. The normal conjunctiva: main components (obtained from Calonge et al.[25]).

The conjunctiva is divided into three parts: the palpebral, the bulbar, and the forniceal. The palpebral (also named tarsal) conjunctiva lines the eyelids, is firmly adhered to the tarsal plate and can be further divided into the marginal, tarsal, and orbital. The bulbar conjunctiva is located on the eyeball, enveloping the anterior sclera and being bound to the underlying sclera through Tenon's capsule. The bulbar conjunctiva is further divided into two parts: the scleral and the limbal. The scleral conjunctiva is extended from the fornix to the limbus, while the limbal conjunctiva is a ring located around the cornea, being a transition zone between the conjunctival and the corneal epithelia. Finally, the forniceal conjunctiva (also named *cul de sac*) is a transition

zone between the palpebral and bulbar conjunctivas. It can be further divided into four regions: the superior, the inferior, the lateral, and the medial. In contrary to the bulbar, the forniceal conjunctiva is loose and flexible, permitting movements of the eyeball and eyelids. The ducts of the accessory and lacrimal glands end up in the forniceal conjunctiva.[25,26]

Histologically, the conjunctiva consists of an epithelial layer and an underlying flexible connective tissue, which is further divided into the deeper layer and the substantia propia (Fig. 4). The epithelial layer is non-keratinized and is composed of stratified columnar and squamous epithelium, containing also interspersed goblet cells, together with T- and B-cell lymphocytes, lymphatic channels, melanocytes, Langerhans cells, epithelial layer blood vessels, accessory lacrimal glands, and fibrous tissue. The deeper layer and the substantia propria (also called conjunctival submucosa) are composed of fibrous and superficial lymphoid tissue, respectively. The substantia propria is a connective tissue, which exists only in the conjunctiva (and not in any other tissue of the eye) and comprises plasma and mast cells, lymphocytes, and neutrophils. The deeper fibrous layer consists of nerves, vessels, and the glands of Krause.[25,26,28,29]

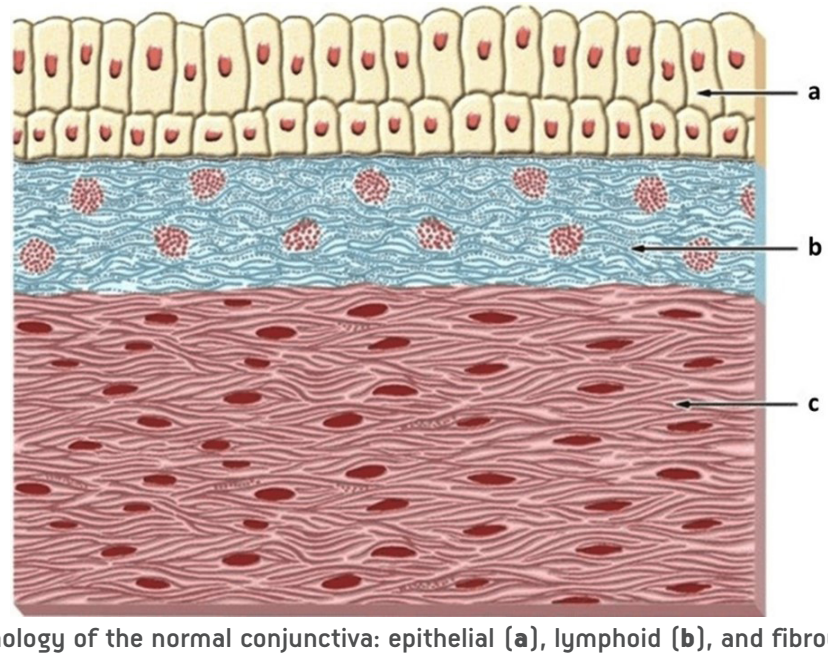


Figure 4. Histopathology of the normal conjunctiva: epithelial (**a**), lymphoid (**b**), and fibrous (**c**) layer (adapted from Sapte et al.[30]).

The thickness of the conjunctival epithelium is of 3 to 5 cell layers thick, containing cuboidal basal cells, which become flattened towards the surface. Close to the fornix area, the conjunctiva has the highest amount of goblet cells, while their number decreases away from that area. Furthermore, the fornix has a big number of immune cells, like T and B lymphocytes, which form the conjunctiva-associated lymphoid tissue.[26,28]

1.3 Cornea

The cornea constitutes the outer layer of the eyeball, together with the sclera. The anterior corneal surface is covered by the tear film and the posterior is in contact with the aqueous humor-filled anterior chamber.[31] The cornea is an avascular transparent tissue, which has as a principal purpose the protection of the structures inside the eye.[32] Its transparency makes the cornea the major refractive component of the eye, contributing

to two-thirds of its refractive power.[33,34] Also, it does not contain blood vessels, as they could interfere with its transparency. Thus, it receives its nourishment from the aqueous humor and the tears.[34]

The cornea is horizontally oval, convex, and aspheric, and is divided into three parts: the central, the peripheral, and the limbus (Fig. 5A).[33] The horizontal diameter is 11-12 mm, while the vertical is 9-11 mm.[35] Regarding thickness, there is a gradual increase from the central to the peripheral cornea. The thickness of the central cornea varies from 551 to 565 μm, while the peripheral from 612 to 640 μm.[36,37] A decrease in corneal thickness has been observed with age. Cellular and acellular components are included in the cornea. Epithelial and endothelial cells, as well as keratocytes, are the major cellular components, while the acellular include glycosaminoglycans and collagen.[33]

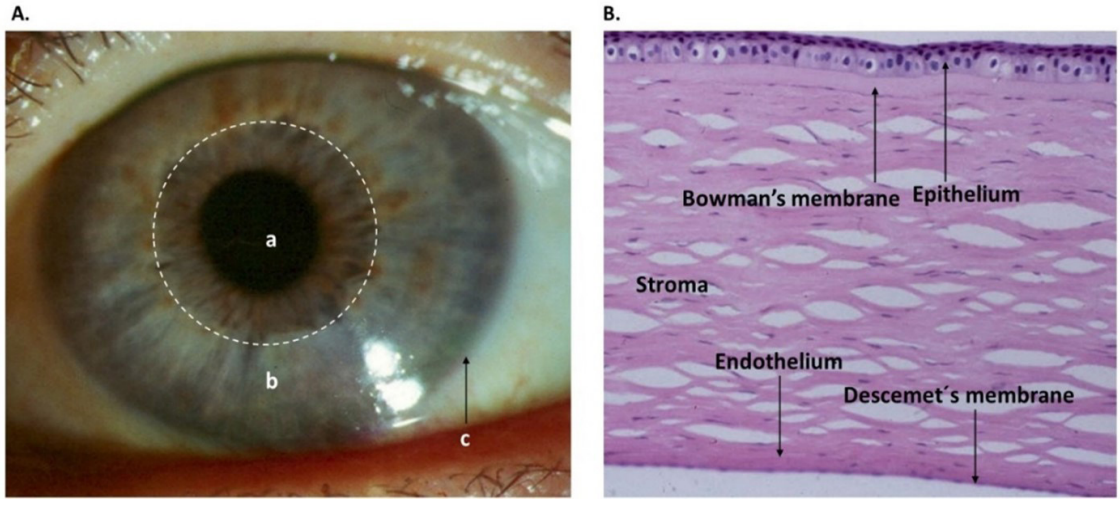


Figure 5. The cornea. **A.** Its 3 parts from the center to the periphery (**a.** Central, **b.** Peripheral, and **c.** Limbus cornea), and **B.** Histopathology showing its layers (Images kindly offered by Prof. Dr. Margarita Calonge, IOBA).

The cornea is arranged in 5 basic layers (Fig. 5B): the epithelium, the Bowman's membrane, the stroma, the Descemet's membrane, and the endothelium. Recently, a well-defined acellular corneal layer is getting attention, named as pre-Descemet's cornea, being the target of lamellar surgeries.[38] The endothelium is the innermost layer of the cornea. It is extremely thin and includes endothelial cells, which help to keep the cornea clear by pumping the excess fluid from the inside of the eye to the stroma. The Descemet's membrane is above the endothelium and is a thin but strong collagen-composed tissue, which protects the eye from infection and injuries. The stroma covers the Descemet's membrane, consists principally of water and collagen, and contributes to 90% of the thickness of the cornea, determining its strength, form, and elasticity. The Bowman's layer is a transparent and collagen-based tissue, lying between the epithelium and the stroma and contributing to the maintenance of the corneal shape. The epithelium is the outermost layer of the cornea, contributing to 10% of the tissue's thickness. It is composed of 5-7 cell layers and has a 50 μm thickness. It provides a smooth surface through its uniformity and is composed of non-keratinized, stratified squamous epithelial tissue. The epithelium is in direct contact with the mucin layer of the tear film, which is produced by the goblet cells present in the conjunctiva. It has 3 types of cells: the superficial (2-3 layers of polygonal cells), the wing (2-3 layers of thawing cells), and the basal (single layer of cuboidal or columnar cells). The basal cells are active mitotically, having abundant organelles. Apart from being a barrier to microbes and chemicals, the epithelium also comprises Langerhans cells which offer important immunological functions. In addition, it contributes to the refractive power of the eye, through its uniformity.[33,34]

DED was defined as a disease only approximately 30 years ago.[39] Nowadays, DED is one of the most frequent reasons for patients to visit ophthalmologists, affecting hundreds of millions of people worldwide.[40] It is a symptomatic disease, with more severe cases being associated with remarkable pain, limitations in daily activities, decreased vitality, poor general health, or even depression.[41] Thus, it significantly affects the quality of life of the patients.

2.1 Definition and Classification

By the mid-1990s, the understanding of DED reached a critical point, publishing the first definition in 1995.[42] This first definition identified the importance of tear film quality and quantity as a cause of DED. In 2007, the name "dysfunctional tear syndrome" characterized the DED, introducing also the word "inflammation" for the first time in the definition of the disease. Thus, according to Tear Film & Ocular Surface Society (TFOS) Dry Eye Workshop (DEWS)-I,[43] "*DED is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface*". Later, it became clear that not only inflammation and hyperosmolarity but also neurosensory abnormalities are crucial for the pathophysiology, understanding, and management of the disease, whereas it was acknowledged that clinical demonstration of the pathophysiology of DED was necessary for its diagnosis.[39] Hence, the current definition was formed according to TFOS DEWS-II,[39] as "*a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles*". Thus, it is a complex disorder that cannot be characterized by a single symptom, sign, or process, while changes in the chemical composition of the tear fluids occur.

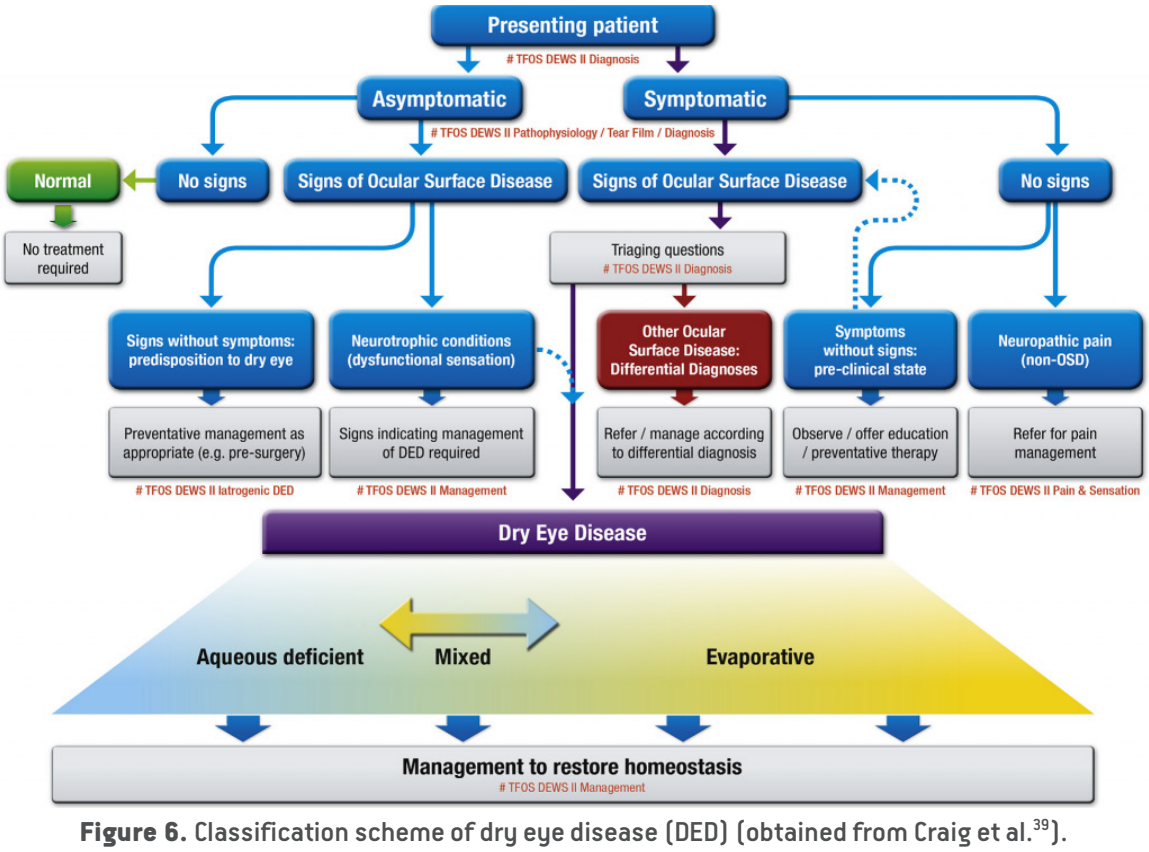


Figure 6. Classification scheme of dry eye disease (DED) (obtained from Craig et al.[39]).

The clinical classification of DED (Fig. 6) is based on both symptoms and signs, whereas triaging questions and ancillary testing can be used for its differentiation from other ocular surface diseases to symptomatic or no-symptomatic patients. Etiologically, DED is divided into aqueous deficient (ADDE) and evaporative (EDE) dry eye, with EDE being more common compared to ADDE. In ADDE, the function of the lacrimal gland is affected, while in EDE the eyelid (e.g., meibomian gland dysfunction or blink-abnormalities) or the ocular surface (e.g., mucin deficiency or use of contact lenses) functions are altered.[39] Thus, in ADDE, the tear secretion is reduced, while in EDE the tear secretion is normal, however, there is excessive tear evaporation. ADDE can be further subcategorised into Sjögren and non-Sjögren DED. Sjögren DED is associated with autoimmune disorders that affect any exocrine glands in combination with other systemic diseases (i.e., rheumatoid arthritis). Non-Sjögren DED is not related to systemic autoimmunity, but lacrimal glands dysfunction. EDE can be further classified in surface-related EDE and meibomian gland dysfunction-related EDE. In surface-related EDE tear film instability can be stimulated by conditions that affect the ocular surface. In meibomian gland dysfunction-related EDE, tear hyperosmolarity is caused by a deficiency of the lipid layer in the tear film.[39,43]

2.2 Prevalence and Risk Factors

Over the last 10 years, a lot of new information has been published reporting the prevalence of the DED in Asia, America, and Europe. Based on the available data, the global prevalence of the DED has been estimated at 12%, varying from 5% (North America) to 48% (Africa) depending on the population, with or without symptoms.[44] If based on signs alone, the global prevalence is adjusted at 30%, reaching up to 75% in some countries.[40,44] Although very few studies have been performed for young populations (below 40 years old), the findings until now indicate that DED is also prevalent in this group.[40]

Among the risk factors, sex and age play a very important role. In particular, the prevalence of DED symptoms increases with age, as there is evidence suggesting that a decrease in tear fluid production occurs with age.[45,46] Also, women are more likely to be diagnosed with DED compared to men, suggesting the influence of sex hormones for DED.[45,47,48] Differences in the lacrimal glands have been attributed to the effect of androgens,[49] while the function of every human cell, organ, and tissue has been proved to be based on sex-related differences.[48] The race has also been proposed as a probable risk factor for DED, as more severe signs and symptoms occur in Asian participants compared to Caucasians.[50] In another study, it was found that 95% of women with DED had white ethnicity.[51] Further, several diseases and dysfunctions constitute risk factors for the development of DED; Sjögren syndrome,[52-54] meibomian gland dysfunction,[55,56] connective tissue disease,[57] and androgen deficiency consistently affect the DED.[58,59] Diabetes,[60] infections,[61] acne rosacea,[62] thyroid deficiency,[63] psychiatric diseases,[47,64] and allergic conjunctivitis[65] probably affect the development of DED, whereas menopause[66] and ocular sarcoidosis[67] inconclusively affect it. In addition, several therapies are included in the consistent, probable, or inconclusive risk factors of DED, such as hormone replacement therapy,[68] hematopoietic stem cell transplantation,[69] refractive surgery,[70] as well as the intake of several medications (contraceptives, antihistamines, anticholinergics, antidepressants, diuretics, anxiolytics, β -blockers, and isotretinoin).[71-73] Risk factors involved in the impairment of DED include environmental conditions (pollution and low humidity)[74] and everyday activities (contact lens wear, computer use, smoking, and alcohol),[47,75-77] while pregnancy[78] and low fatty acid intake[79] also probably affect the development of the disease.

2.3 Economic Burden

The psychological consequences and the physical impact on vision and ocular pain produced by DED generate a considerable impairment of the quality of life of the patients.[80-82] This leads to the implication of the health resources available in each country, such as doctors, physicians, medications, etc.[83] In Europe, the annual medical costs attributed to DED vary from US $0.27 million (France) to US $1.10 million (United Kingdom) (for the years 2003-2004),[84] whereas in the US they were estimated at US $3.84 billion (data 1999).[85,86] Regarding treatment costs, in Europe, they vary from US $22,000 (France) to US $535,000 (United Kingdom) annually (data for the years 2003-2004).[84] The total direct (medical and treatment) costs were approx. US $134.84 million per year in Germany (for the period 2008-2015)[87] and US $34.92 million annually in Spain (for the period 1997-2005).[88] When costs are calculated per patient, it can be noticed that the economic impact for society is high. In Asia, the medical costs for DED were estimated at US $530 per patient and the treatment costs at US $357 per patient for the period 2005-2008,[89] while in the US the treatment costs varied from US $299 to US $357 per patient (having a remarkable increase of 443% within the years 2001-2006).[90,91] The total direct costs for Canada were approx. US $4038 per patient (data 2018)[92] and US $465.54 per patient for China for the years 2018-2019.[93] More recent studies revealed that the average duration of hospitalization due to severe DED in Spain was 9.6 days, exerting an annual cost of US $8376 per patient, while the total direct costs were remarkably increased (ca. 518% - from US $4.9 to US $34.4 million) during the study period (1997-2015), together with the annual cost per patient.[88] The total direct costs for Canada were approx. US $4038 per patient (data 2018)[92] and US $465.54 per patient for China for the years 2018-2019.[93] More recent studies revealed that the average duration of hospitalization due to severe DED in Spain was 9.6 days, exerting an annual cost of US $8376 per patient, while the total direct costs were remarkably increased (ca. 518% - from US $4.9 to US $34.4 million) during the study period (1997-2015), together with the annual cost per patient.[88]

However, the most important economic burden generated by DED is the one produced by the decrease of work productivity (indirect costs).[83,94,95] For 2008, the total indirect costs due to DED in the US were calculated at US $11,302 per patient, giving a total of US $55.4 billion.[96] In Asia, the costs related to productivity loss at work were estimated at US $741 per patient for the year 2011,[97] while in Canada at US $38,534 per patient (data 2018).[92] Severe DED symptoms have been found to cause a significant reduction of work productivity and activity impairment, not only intra-individually but also inter-individually.[95]

2.4 Pathophysiology

As already mentioned, two types of DED are recognized: ADDE and EDE. Both are caused by evaporation-induced tear hyperosmolarity, which damages the ocular surface directly and leads to the release of inflammatory mediators and proteases from ocular surface epithelial cells. This causes goblet and epithelial cell loss and damage in the epithelial glycocalyx of the tear film. Thus, tear film instability is generated and subsequent tear film breakup. This breakup along with activated T-cells, the inflammatory mediators produced by them, and the tear hyperosmolarity itself lead to ocular surface damage (Fig. 7).[98] Therefore, the central pathophysiological feature of DED is inflammation in the LFU, creating a chronic inflammation circle (Fig. 8).

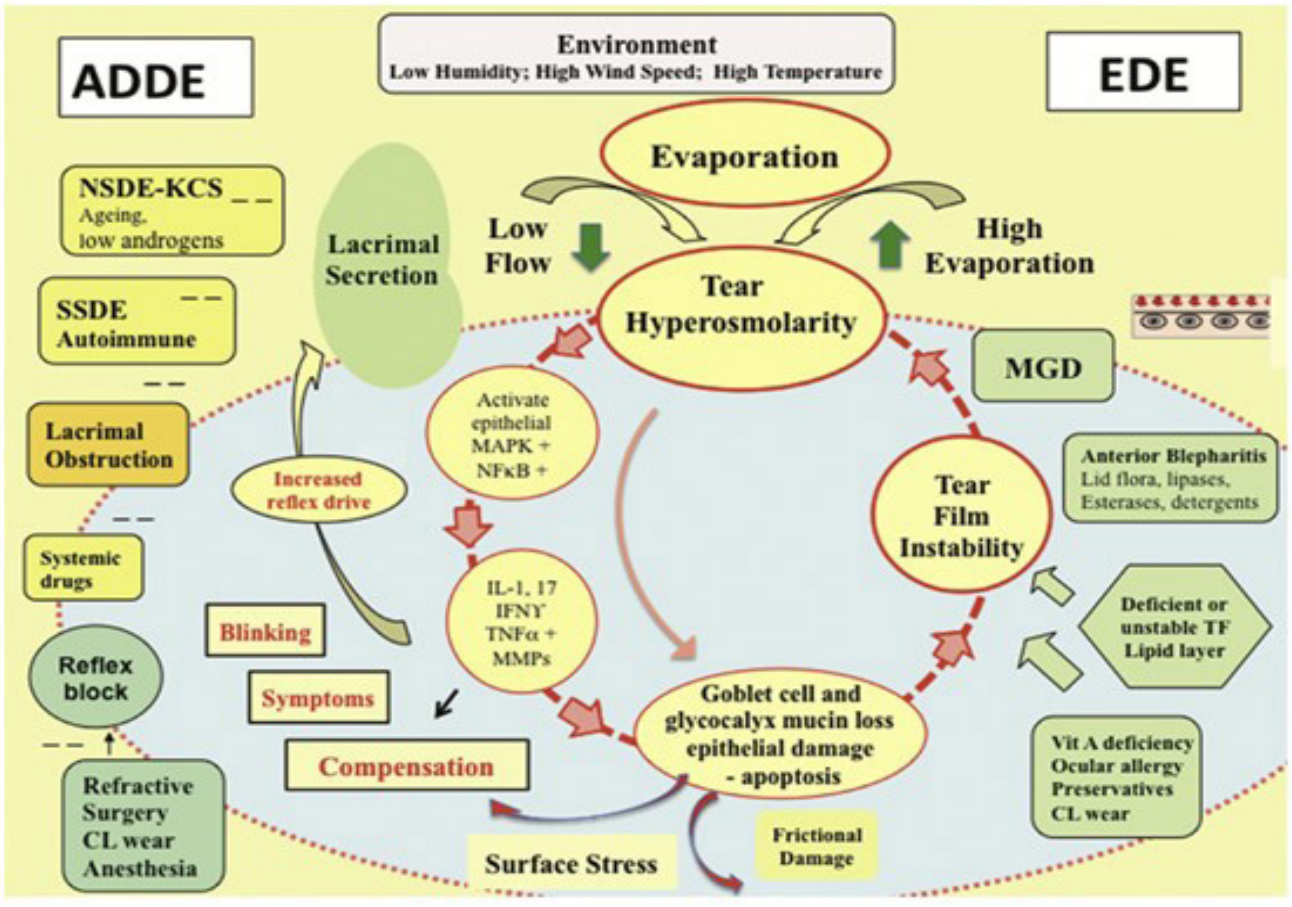


Figure 7. Pathophysiology of dry eye disease (DED): aqueous deficient (ADDE) and evaporative (EDE) dry eye (obtained from Bron et al.[98]).

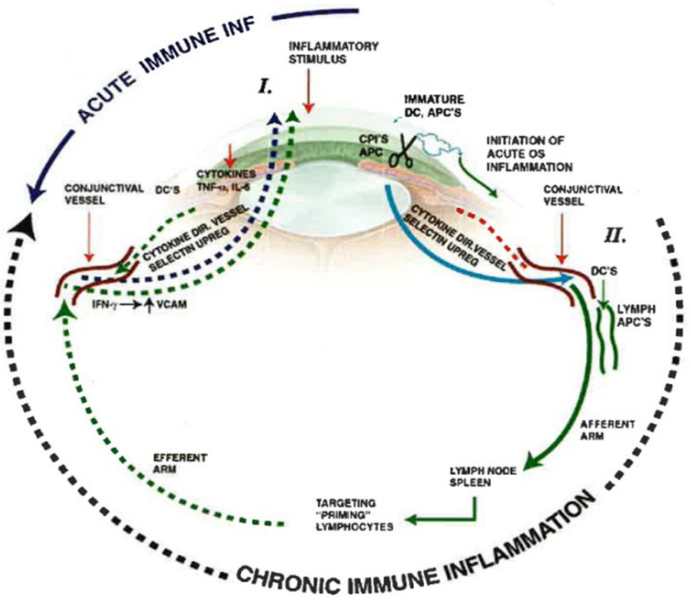


Figure 8. Inflammation circle of dry eye disease (DED) (obtained from Stern et al.[9]).

Increased levels of pro-inflammatory cytokines/chemokines have been related to the pathophysiology of DED.[99-102] Elevated secretion of interleukin (IL)-6 and IL-8 has been detected in the tears and conjunctival epithelium of patients suffering from more severe forms of DED, while IL-6 was high in tears of patients with Sjögren and non-Sjögren DED.[103-111] Upregulation of IL-6 correlated negatively with Schirmer score[106] and positively with tear film breakup time and clearance, alterations of the corneal surface, the density of the conjunctival goblet cell,[108] symptoms' severity, corneal fluorescein staining (CFS), and conjunctival lissamine score.[106] Upregulated levels of IL-8 were detected in the tears and conjunctival epithelium of patients with Sjögren syndrome.[104,107] In addition, tumor necrosis factor (TNF)-α levels were elevated in the conjunctival epithelium of patients with Sjögren syndrome,[104] as well as in the tear fluid of patients with dysfunctional tear syndrome[106] and DED.[108,111,112] Elevated levels of TNF-α were also found to correlate with Sjögren and non-Sjögren DED,[113] as well as DED diagnostic tests.[114] Moreover, IL-1β tear levels have been found to correlate positively with CFS and conjunctival lissamine staining scores in DED patients,[106] as well as with parameters associated with meibomian gland dysfunction[115] and DED diagnostic tests.[114] A negative correlation was reported between IL-1β levels and Schirmer test score,[106] while high levels of IL-1β and lower levels of precursors IL-1β were detected in the tear fluid[105,106,111,116,117] and conjunctiva of DED patients.[105] IL-1β and IL-6 were also upregulated in the tear fluid of DED patients with associated blepharitis,[117] whereas increased levels of interferon γ-induced protein (IP)-10 were observed in conjunctiva and tears of Sjögren and non-Sjögren DED patients.[118,119] Regarding experimental DED, high levels of TNF-α, IL-6, and IP-10 were detected in the corneal and conjunctival epithelium of mice exposed to desiccating stress.[120,121] Increased levels of IL-17A have also been reported in the tears of desiccating stress-induced mice[109] and the conjunctiva of patients with DED, proving the key role of IL-17A in the conjunctival epithelial disruption.[122] Furthermore, elevated levels of epidermal growth factor (EGF) were found to be significantly decreased in the tear fluid of patients with meibomian gland dysfunction, corneal subepithelial fibrosis, and meibomian gland orifice metaplasia,[123] as well as in tears of patients with DED.[103,106,119,124-126] Additionally, matrix metalloproteinase (MMP)-9 has been proved to be an important diagnostic tool for DED, as it correlated with DED diagnostic tests and ocular inflammation in DED patients.[127] Several studies have also reported increased levels of MMP-9 in the tears of DED patients.[114,117,125,126,128-130] Further, other cytokines, such as IL-2, IL-4, IL-5, IL-10, and interferon (IFN)-γ, have been identified in the ocular surface of DED patients.[102-111]

Lymphocytes (principally activated Th1 and also Th17 T cells) have been proved to play a key role in the pathophysiology of DED.[131,132] Activated T cells have been detected in the conjunctiva of DED patients,[104,105,107,133] while CD4+ T cell infiltration levels in the conjunctival epithelium are also related to the response to the experimental eye dryness.[134] Furthermore, clinical symptoms of conjunctival inflammation in patients with Sjögren syndrome or non-Sjögren keratoconjunctivitis sicca is depended on T cell activation.[135] The importance of CD4+ T cells for the pathophysiology of DED is also proved by the fact that DED in naïve mice can be developed through adoptive transfer of CD4+ T cells from mice with established DED.[136,137]

On the other hand, the involvement of oxidative stress in the pathophysiology of DED has also been described. Oxidative stress is the loss of balance between the pro-oxidant and the antioxidant procedure of the cells, that can cause oxidative damage.[138] The ocular surface is continuously exposed to atmospheric oxygen and UV rays that can lead to oxidative damage and subsequent oxidative stress-stimulated ocular surface diseases.[6] Oxidative damage has been proved to be involved in the pathophysiology of Sjögren and non-Sjögren

DED.[139-144] Also, high levels of reactive oxygen species (ROS) in tears and meibomian glands have been related to ocular surface tissue damage and inflammation.[145] ROS production is related to lipid peroxidation-related membrane damage and DED inflammatory processes.[141]

Thus, inflammation and oxidative stress in the LFU are highly connected and are the principal biological processes involved in the pathophysiology of DED.

2.5 *In vitro* Models

In drug discovery, translatability plays a very important role, as it determines whether a study is promising or not.[146] *In vitro* models are extensively being used to study the pathophysiological mechanisms of human diseases and to interpret the translatability of a treatment.[146,147] The controlled experimental conditions of an *in vitro* study constitute one of its most important advantages, allowing to study in-depth the molecular pathways of the disease in a short time and at a lower cost (compared to the expensive and time-consuming animal models).[147] In addition, the *in vitro* models highly contribute to the "3Rs" guideline (replacement, reduction, and refinement) for ethical animal research by avoiding the use of animals and by using fewer animals through the obtention of more information regarding the safety and efficacy of the treatments before moving to the animal experimentation.[148] Nevertheless, single or maximum two cell type cell cultures can be used, being the principal limitation of these studies.[147] Cells cannot represent the complexity of a multicellular developed organism. Thus, they cannot predict the metabolic profile of an administered treatment.[146] However, currently, several works are proposing 3 dimensional *in vitro* models that aim to enhance the correspondence of the model with the tissue of origin.[149]

Several *in vitro* models have been used to study the inflammatory and oxidative stress-related pathophysiological mechanisms of DED. Epithelial cells can act as mediators of immune responses, although they are not the principal target.[150] Thus, numerous studies have reported that in inflammatory-related ocular surface diseases, the epithelium of the ocular surface is a potential therapeutic target.[151-155] On an immortalized human conjunctival epithelial cell line (IOBA-NHC),[156] Enriquez-de-Salamanca et al.[157] examined the changes in cytokine/chemokine secretion in response to pro-inflammatory cytokines. Based on the different cytokines tested, TNF-α demonstrated the strongest induction on cytokine secretion time-dependently. Therefore, the importance of conjunctival epithelial cells in the regulation of ocular surface inflammation was demonstrated, contributing to its understanding. Similar results were demonstrated on an SV40 immortalized human corneal epithelial cell line (HCE), which was characterized by Araki-Sasaki et al.[158] and was proved to demonstrate high correspondence to the tissue of origin (normal corneal epithelial cells). Twenty-four hours of TNF-α treatment significantly increased the secretion of cytokines/chemokines related to DED (IL-6, IL-8, and IP-10) by both HCE and IOBA-NHC cell lines.[159] In addition, the same study by Abengozar-Vela et al.[159] also examined a model of oxidative stress, including exposure of HCE and IOBA-NHC cells to UV-B radiation and subsequent measurement of the free ROS produced. It was demonstrated that 15 seconds of exposure to UV-B light with an intensity of 107.25 mJ/cm^2 significantly increased the levels of intracellular ROS on both cell lines. Similarly, Pauloin et al.[160] used a UV-B-induced apoptosis and inflammation model on an immortalized HCE cell line, studying its intervention on the levels of cytokine secretion and oxidative stress biomarkers. It was shown that ROS production, as well as IL-6 and IL-8 secretion, were stimulated significantly.

Therefore, *in vitro* models of inflammation and oxidative stress on human ocular surface epithelial cells can contribute to the understanding of the pathophysiological pathways involved in the DED.

2.6 *In vivo* Dry Eye Model

The most reliable data are obtained through experiments in humans. However, ethical issues arise, as previous knowledge of toxicological effects and possible adverse reactions of the tested treatments are needed to minimize the risks before the administration to humans. Consequently, to approach the complexity of a living organism, which cannot be achieved through an *in vitro* model, animal models were developed, constituting one of the most important tools for researchers to discover the pathogenesis of diseases and develop new therapies. Nevertheless, *in vivo* data do not always represent the activity and fate of an administered treatment in humans, as there is not always correspondence. Also, animal models cannot represent all characteristics of an induced human disease but only some of its aspects.[146]

Several animal models of DED exist based on the different forms and pathophysiological mechanisms of the disease, contributing highly to its understanding. One of the most important experimental DED models for EDE is the so-called environmental or desiccating stress (DS) mouse model. This model was firstly developed by Dursun et al.[161] and included mice exposed to low humidity and constant airflow (DS conditions), together with scopolamine administration 3 times per day. These mice developed histopathological and clinical signs similar to DED patients. In particular, tear production (TP) was reduced, together with tear clearance and goblet cell density, while CFS, corneal epithelial permeability, cytokine levels (IL-1β, TNF-α, IFN-γ, and IL-17) in tear fluid, substance P, calcitonin-gene-related peptide, and T cell ocular surface infiltration were increased. Also, changes in conjunctival epithelial morphology and apoptosis on the ocular surface were observed. Similarly, Barabino et al.[162] proved that mice exposed to DS had decreased TP, combined with increased CFS and goblet cell loss. Interestingly, CD4+ T cells isolated from lymph nodes and spleen of DS-exposed mice, when transferred to recipient (naïve) mice, increased their cytokine levels and reduced their TP, tear turnover, and goblet cell density.[9,136] Therefore, the importance of this model on the DED establishment and understanding was confirmed. Scopolamine administration in this model was performed using patches, which was later adapted to pumps by Gehlsen et al.[163] Several DED studies have been performed using this DS/scopolamine model, having a usual duration from 10 to 14 days. The mice selected were C57BL/6, the conditions included humidity below 30% and constant airflow, while the scopolamine was administered subcutaneously with a continuous dose.[159,164,165]

Additional models have also been developed, including different animal species and addressing the multifunctional pathophysiology of DED. For EDE, several additional mouse, rat, and rabbit models exist. Meibomian glands' absence or atrophy in mice led to corneal epithelial defects and ulceration, keratitis, keratinization, neovascularization, conjunctivitis, blepharitis, periocular crusts and wounds, and pruritus.[166] In rats, induction of reduced blinking and environmental stress by constant airflow and jogging board led to increased CFS.[167] In rabbits, mechanical prevention from blinking using speculum inducted dry spots and CFS,[168] exposure of the ocular surface to sodium hypochloric acid increased tear break-up time and punctuate CFS,[169] while the closure of meibomian orifices by cauterization reduced goblet cell density and corneal epithelial glycogen levels, and increased conjunctival inflammatory cells and tear film osmolarity.[170] For ADDE, mouse, rabbit,

canine, and monkey models exist. Regarding mouse models, non-obese diabetic mice with predominantly CD4+ T cell infiltration in the lacrimal glands did not decrease TP and showed no signs of ocular surface diseases,[171] Id3-deficient mice with T cell-dominant lacrimal gland infiltration had periocular skin lesions (due to eye scratching),[172] while Neurturin-deficient mice with defects in the enteric, sensory nervous and autonomic systems demonstrated reduced corneal sensation, and mucin production, and increased ocular surface inflammation.[173] Rabbits with autoimmune dacryoadenitis (Sjögren syndrome) induced by injection of activated lymphocytes[174] and neuronal pathway dysfunction through blockage of lacrimal gland innervation by topical atropine,[175] had increased CFS. In terms of canine models,[176-179] signs of DED and keratoconjunctivitis sicca with reduced TP were produced through spontaneous DED model and surgical-induced DED (mechanical removal of the main lacrimal gland), respectively. Removal of the lacrimal gland in a monkey model did not affect TP and no further ocular surface disease signs appeared.[180]

Thus, all these studies confirm that DED animal models allow us to understand in-depth the pathophysiology of DED. Each model reflects specific aspects of DED and thus, no ideal model exists.[181] The DS-exposed mouse model with scopolamine administration was considered a reliable model to test new therapeutic agents for DED at a pre-clinical level.

2.7 Management and Therapy

The multifactorial etiology of DED makes its management highly complicated. The principal objective of DED management is to restore the ocular surface's and tear film's homeostasis. This could, at least in theory, be achieved by breaking the vicious cycle of the disease. Although several therapies may be appropriate for one specific aspect of the patient's condition, the management of DED involves chronic prevention and management of the disease instead of short-term treatment. Algorithms are often set up to indicate adequate treatment based on the stage of the disease. However, in DED this approach is complicated due to the variation of the severity and the character of the disease among the patients.[182] In general, DED management starts with easily accessible and low-risk over-the-counter treatments (for early-stage/mild cases), and advances to more complicated therapies for more severe cases.[182] In this chapter, available therapies for DED are going to be discussed, focusing specifically on drug-based therapies.

For tear insufficiency, tear replacement is performed using lubricants, which can be artificial[183-186] or biological tear substitutes.[187,188] Tear conservation is also an alternative used, achieved either by punctal occlusion (blocking the drainage of tears)[189,190] or by moisture chamber spectacles and humidifiers.[191] Tear stimulation has also been found to be effective, achieved by topical secretagogues (such as diquafosol tetrasodium),[192,193] lipid stimulators (such as Insulin-like growth factor 1)[194] or nasal neurostimulators,[195,196] or oral secretagogues (such as cholinergic agonists, i.e., pilocarpine[197] and cevimeline[198]).

When meibomian gland dysfunction is an important component of DED, which is frequent, in addition to the anti-inflammatory therapies that will be mentioned below, all measures helping maintain meibomian glands open and producing a, as normal as possible, meibum must be adopted. These include manual lid hygiene[199-201]

that patients would perform at home daily and/or "in-office" ways to deliver it, such as thermal pulsation systems[202] and/or intense pulsed light systems.[203] Additionally, if DED is associated with anterior blepharitis, antibiotics[204] or products for a potential Demodex infestation[205,206] must be used. If there is a component of abnormal tear distribution (abnormal eyelid position of dynamics), specific measures must be taken.[207,208]

Although all these approaches temporally improve DED symptoms, they do not break the inflammation circle involved in DED. Thus, the most important advance in DED therapy is the administration of anti-inflammatory treatments. Among them, topical glucocorticoids, non-glucocorticoid immunomodulators, lymphocyte function-associated antigen 1 (LFA-1) antagonists, and macrolide antibiotics are currently used for the management of the inflammatory process in DED.[182]

Topical steroids, such as methylprednisolone, fluorometholone, or dexamethasone, among others, are widely used for the therapy of many inflammatory diseases, including DED. Numerous clinical studies have been published, reporting the importance of corticosteroids use in the management of DED by breaking the inflammatory circle of DED.[209-218] However, their long-term administration can have several complications, including ocular hypertension and glaucoma, infections, and cataract.[219-223]

Regarding non-glucocorticoid immunomodulators, tacrolimus[224] is used as DED treatment, whereas several biologic molecules (lubricin, recombinant human nerve growth factor, TNF-α-stimulated gene/protein 6, anti-TNF-α, IL-1 receptor antagonist, neuropeptides, and anti-IL-17 therapeutic targets) have been reported as potential future DED treatments.[182] However, cyclosporine A is the principal immunomodulatory drug administered, having at the same time anti-inflammatory properties and actions helping the management of DED.[225-227] It is a naturally derived cyclic polypeptide, produced by the fungus *Beauveria nivea*[228] (also known as *Tolypocladium inflatum*[229]). It is a calcineurin inhibitor, acting by inhibiting lymphocyte activation and infiltration[230-232] and the subsequent release of cytokines (principally IL-2) by them.[227] This is achieved through binding with cyclophilin inside T cells, a reaction that causes inhibition of calcineurin-induced de-phosphorylation of nuclear factor and transcription of cytokine genes.[227] Additionally, p38 and c-Jun N-terminal kinase activation is blocked by cyclosporine A, causing further reduction of IL-2 secretion[233] and subsequent decrease of effector T cell function.[234] It has widely been used in autoimmune diseases[235,236] and to control transplant rejection,[237,238] while topically is useful in autoimmune ocular inflammation and stem cell dysfunction.[239-242] It was approved in 2003 by The American Food and Drug Administration (FDA) for severe DED, as it improved TP and conjunctival goblet cell density. Later, it was also demonstrated that it reduced inflammation biomarkers and tear osmolarity in patients.[243-247] Nevertheless, cyclosporine A topical treatment needs several months to achieve its effect. In particular, at least a 3-month therapy has been considered necessary (reaching even a 16-months treatment),[248] as cyclosporine A acts by preventing T cell activation and not by deactivating the activated T cells, which live 110 days in the human body.[249] Also, cyclosporine A has very low water solubility, complicating its formulation as ocular drops. Emulsions of cyclosporine A have been formulated and released for DED treatment in Europe.[250] However, the oil or surfactants present in them can be irritating for the eye, causing burning, itching, irritation, or even blurry vision in some cases.[226,251]

More recently, LFA-1 antagonists, such as lifitegrast, have been considered an efficient strategy to inhibit DED T cell-mediated inflammation, as they block T cell interaction with other cellular elements. LFA-1 is an integrin (heterodimeric cell surface receptor), having as principal ligand the intercellular adhesion molecule 1 (ICAM-1), which is expressed in endothelial, epithelial, and antigen-presenting cells.[252] When ICAM-1 binds to LFA-1, an immunologic synapse with subsequent T cell activation occurs.[253] Lifitegrast acts by imitating ICAM-1's binding to LFA-1 (competitive antagonist), inhibiting T cell migration, and decreasing cytokine production.[254-256] In 2016, lifitegrast (as 5% ophthalmic drops) was approved by FDA for the treatment of DED. Lifitegrast was found to be effective in improving the eye dryness score (visual analog scale), as well as ocular discomfort. Nevertheless, no effect was observed in inferior corneal staining after 84 days of treatment. Also, side effects were reported, such as instillation site burning, reduced visual acuity, and dysgeusia.[257] Currently, it has received approval in the US, Canada, Australia, and 6 more countries, although not by the European Medicines Agency (EMA).[258]

Several macrolide antibiotics have also been incorporated in the therapeutic strategy of DED. Systemic azithromycin has a stimulatory effect on meibomian gland epithelial cells,[259] being efficient in meibomian gland dysfunction associated with rosacea[260-262] and regulating lid bacterial flora and inflammation.[263-266] However, its ideal dosage is still controversial,[260,261,267] while there is still doubt that oral antibiotics are effective in meibomian gland-related ocular surface diseases.[268] Azithromycin has been also administered topically for DED associated with severe blepharitis with much better toleration.[269] Tetracyclines are broad-spectrum antibiotics, which have also been proposed as DED treatment. They act by decreasing lipolytic exoenzymes and lipase production by bacteria through blockage of aminoacyl-tRNA binding to mRNA ribosome.[270-272] Thus, they reduce meibomian lipid breakdown products, contributing to the improvement of blepharitis and meibomian gland dysfunction, while they also have anti-inflammatory properties, reducing several cytokines in the corneal epithelium.[273-277] Nevertheless, the optimal dosage for tetracyclines has not been established either,[278] while their known side effects (photosensitivity and gastrointestinal disorders) and the safety issues emerging from their long-term use are still an obstacle for their future established use and approval for DED.[182]

3. Natural Therapeutic Agents: Phenolic Compounds

Natural compounds have extensively been used as therapeutic agents[279]; from ancient times, when Greeks or Chinese used plants to treat most of the diseases,[280,281] to nowadays, providing effective drugs.[282] Natural products were widely used in the pharmaceutical industry in the 1800s and 1900s. However, between the 1960s and 1980s, a decline in their use was noticed. This was attributed to many factors, such as the complexity of the isolation, fractionation, and structural identification processes, as well as the heterogeneity of the obtained extract which could lead to a lack of detection of the bioactive compound present in it.[283-285] Scaling-up problems,[284] low reproducibility of samples re-collection,[286,287] large variability in the phytochemical profile of the plants among the different cultivars or varieties,[288,289] together with the appearance of computational chemistry as an alternative to drug discovery,[290] contributed to this decline. In 1970, the discovery of Taxol (paclitaxel), a natural alkaloid isolated from the Pacific yew, as an effective naturally derived anticancer drug, refocused the scientific pharmaceutical interest in natural products.[288,291] This was also supported by the advances in new technologies (i.e., chromatographic and spectroscopic techniques), which enhanced drug discovery from medicinal plants.[292,293]

Nowadays, nature is a recognized source of bioactive molecules. According to World Health Organization (WHO), traditional or alternative medicine is used by up to 80% of people in developed countries.[294] Also, 60% of the available pharmaceutical compounds are derived directly or indirectly from natural molecules, 25% of the drugs prescribed are plant-based, and 121 naturally derived active compounds are being used worldwide.[289,294-296] Interestingly, 11% of the essential drugs are of natural origin or synthetic molecules based on natural compounds.[296] Therefore, the importance of natural products for drug discovery is of utmost scientific and medical interest.

The bioactive compounds present in plants are secondary metabolites. Secondary metabolites are produced either during plant growth or during exposure of the plant to biotic (parasites, bacteria, or fungi attack, animal damage, etc.) or abiotic (UV radiation, extreme temperatures, low water availability, etc.) factors.[297,298] They are produced through several biosynthetic pathways, such as those of malonic, mevalonic, and shikimic acid. They can be divided into two big categories, based on the presence or absence of nitrogen in their structure. Alkaloids, glucosilates, and amines belong to the first category (nitrogen-containing), while phenolic and terpenoid compounds in the second (non-nitrogen-containing).[299]

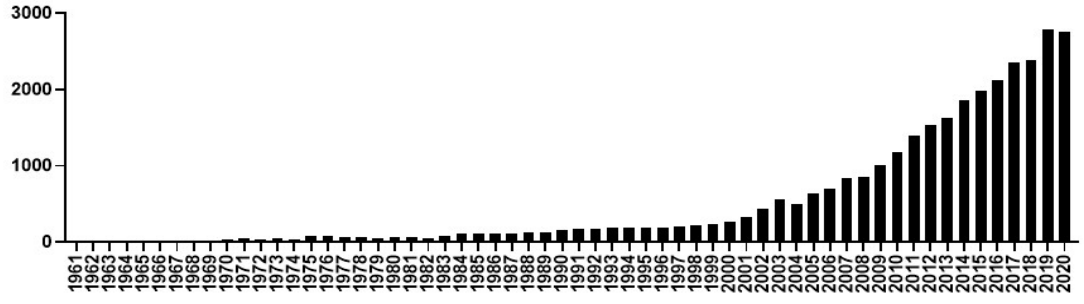


Figure 9. Number of publications related to "biological activities of phenolic compounds": evolution from 1961 to 2020 (Source: PubMed).

Phenolic compounds are widely found in the plant kingdom, being one of the most important molecular groups derived from plants.[300] In plants, they contribute to their color, odor, astringency, taste, and oxidative stability.[301] Structurally, they have at least one aromatic ring coupled with one or more

hydroxyl groups (phenol). They can be classified as simple phenols and polyphenols, based on the number of phenol groups in their structure.[300] Polyphenols include tannins, lignans, secoiridoids, coumarins, flavonoids, and phenolic acids.[298] More than 800 different phenolic compounds have been isolated and identified.[301] Their activities change based on the position and number of hydroxyl groups, as well as the substitution of the aromatic rings.[300] For the human, they exert numerous biological properties, including anti-inflammatory, antimicrobial, antioxidant, antiallergenic, and antiatherogenic.[302,303]

Thus, there is a current recognition of plant phenolic compounds not only by the scientific community as shown by the increasing number of publications related, especially in the last 20 years (Fig. 9), but also by the public, as polyphenols are abundant in fruits, vegetables, seeds, and produced healthy foods and beverages,[304] being one of the most important ingredients of a healthy diet.

3.1 Olive Phenolic Compounds: Structures and Biological Activities

The olive tree, genus *Olea* (Oleaceae), comprises more than 40 varieties of shrubs and trees, located in Africa, Asia, Oceania, and south Europe.[305] Among them, the *Olea europea* is found in the Mediterranean area and is extensively cultivated for its fruit, from which the olive oil and table olives are produced.[306] The cultivation of *Olea europea* starts in the Copper Age.[307] Later, in ancient Greece, its role was of chief importance, being part of the Greeks' social and religious life. According to Greek mythology, the origin of the name of the city of Athens is related to the olive tree, as Athena, the Goddess of wisdom, won the possession of the Attica region (where Athens belongs) to Poseidon, the God of the sea and earthquakes, because the gift she offered (an olive tree) was considered more precious than that of Poseidon (salt spring) by the jury formed by the other ten Gods of Olympus.[308] Today, the olive tree is one of the most important crops throughout the world, as its hardiness and consumption are extensive.[307] In traditional and folk medicine, olive oil and fruits have been widely used, including antiseptic and analgesic for abdominal pain and rheumatism, among others.[309,310] Nowadays, numerous studies have highlighted the importance of the consumption of olive oil for the human health, being the principal dietary fat ingredient of the well-known healthy Mediterranean diet.[311,312] A negative correlation has been reported between olive oil consumption and heart (coronary and atherosclerosis) and cancer (breast and colorectal) diseases.[313,314]

The beneficial properties of the consumption of olive oil for the human health were attributed to its unsaturated fatty acids, which have demonstrated beneficial activities for the cardiovascular system.[315-317] Currently, these properties have also been related to olive oil's phenolic content.[313,318-326] Over the latest 20 years, the olive phenolic compounds have been elucidated and found to have a wide range of biological activities, including prevention of low-density lipoprotein oxidation, anti-inflammatory effect, and protection of external agents.[327] They can be divided into 13 classes, which include 222 different molecules.[328] The classes are simple phenols, hydroxybenzoic acids, methoxyphenols, hydroxyphenylacetic acids, hydroxycinnamic acids, glucosides, secoiridoids, irridoids, coumarins, flavonoids, lignans, hydroxy-isochromans, and phenolic fatty acid esters, being the most important and characteristic of the *Olea europea* the secoiridoids (Fig. 10).

Simple phenols have at least one hydroxymethyl group connected to an aromatic ring and/or one to two hydroxyl groups,[328,329] while methoxyphenols are phenols including a methoxy group in their aromatic structure.[328] Hydroxybenzoic acids are derivatives of benzoic acid, with a general structure of the C6-C1 type[330] and one to three hydroxyl groups.[328] Similarly, hydroxycinnamic and hydroxyphenylacetic acids are C6-C3 type cinnamic acid derivatives[331] with one to three hydroxyl groups[328] or phenylacetic acid derivatives with one or two hydroxyl groups,[328] respectively. Glycosides are molecules comprising a sugar connected to another functional group.[332] Glucosides are glycosides including glucopyranose and can be attached either to the heterocyclic or the two phenyl rings.[328] Iridoids are monoterpenoids with a general form of cyclopentan-[c]-pyran.[333] The cleavage of the bond in the cyclopentane ring generates the secoiridoids,[334] which are characterized by the presence of elenolic acid in their structure.[328] Coumarins are benzopyrone derivatives,[335] similar to hydroxyiso-chromans, and are hydroxylated in the benzene ring.[328] Flavonoids comprise a 15-carbon skeleton, consisting of two phenyl rings and a heterocyclic one, which contains the bound oxygens.[336] Lignans are monolignol dimers of higher molecular weight, comprising a 2,3-dibenzylbutane in their structure.[328,337] They can be free or glucosylated.[328] Fatty acid esters result from the combination of alcohol with fatty acid. When the alcohol is a phenolic ring, they form the phenolic fatty acid esters.[338]

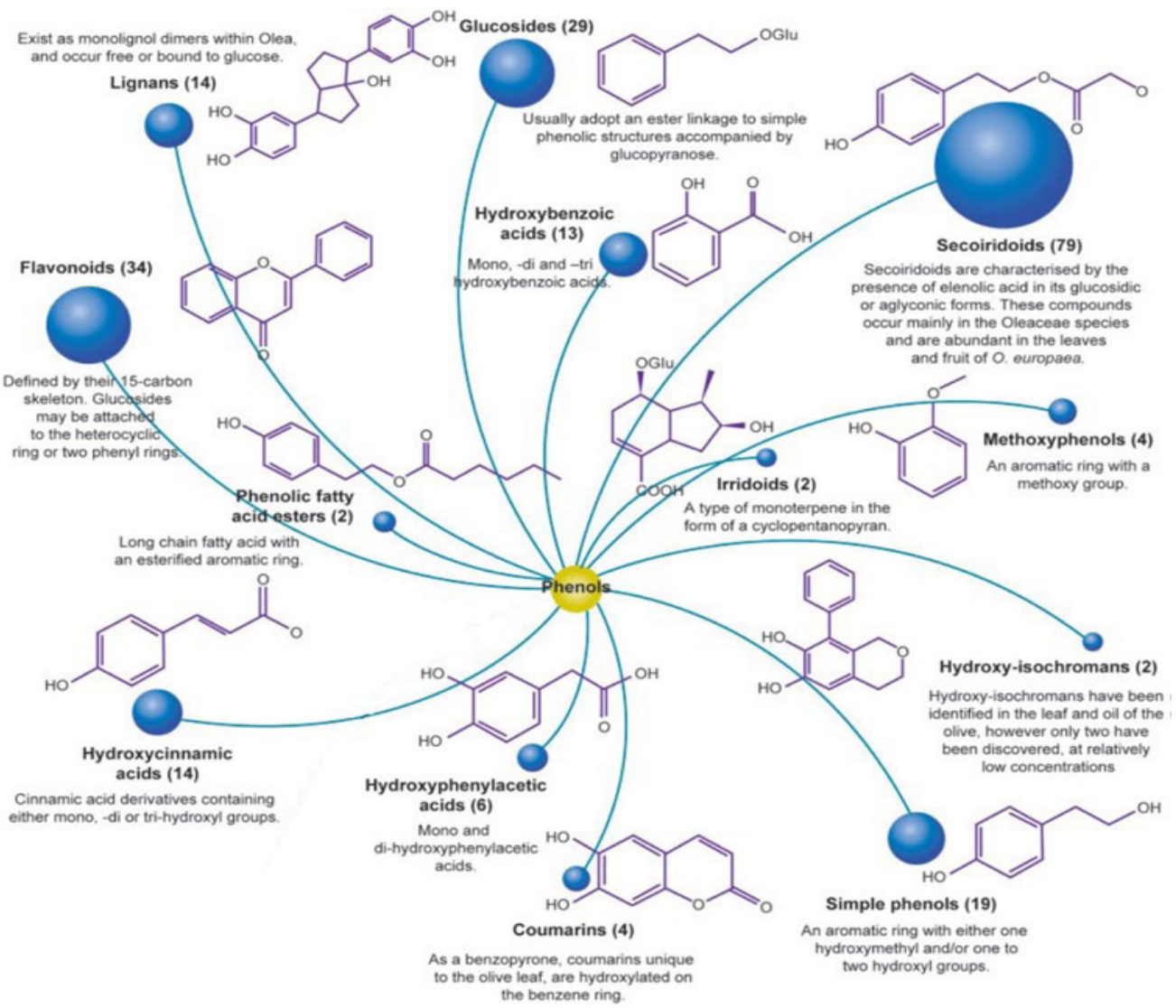


Figure 10. 13 classes of olive phenolic compounds: number of compounds and key-information for each class, together with representative molecular structures (adapted from Bonvino et al.[328]).

However, only 1-2% of the phenolic compounds of the olive fruit end up in the olive oil, as the rest 98% of them goes to the by-products produced during the olive oil production,[339] which will be analyzed in detail in chapters 3.2 and 3.3. Apart from the olive fruit, the olive leaves also constitute a part of the olive tree rich in phenolic compounds.[340,341] Interestingly, monographs of olive oil and leaves exist in most pharmacopeias (European, British, and the US) nowadays,[342-344] confirming the important biological activities of these molecules for the human health. Among the olive phenolic categories, simple phenols and secoiridoids are the most abundant[328] and thus, include most of the cited bio-active compounds.[345] Nevertheless, other important compounds, such as acids (e.g., caffeic, vanillic, elenolic, and *p*-coumaric), glucosides (e.g., verbascoside), and flavonoids (e.g., rutin), contribute to the biological properties of the olive products, adding antioxidant,[346-350] anti-inflammatory,[351-353] anti-microbial and antiviral,[354-356] chemoprotective,[347,357-359] cardioprotective,[360-364] and anti-depressive[365] activities to the material.

Regarding the biological properties of secoiridoids, ***oleuropein (OL)*** is the major representative, exerting *in vitro* and *in vivo* antioxidant,[366,367] *in vivo* anti-inflammatory,[351] endocrinal,[368] and hypoglycemic,[319] *in vitro* cytostatic[369] and molluscicidal[370], *in vitro*[371,372] and *in vivo*[373,374] cardioprotective/antiatherogenic and *ex vivo* antihypertensive[375] properties, as well as enzyme modulation[376] and antimicrobial/antiviral effects.[354,355,377-379] ***Oleacein (OLC)*** (also known as ***decarboxymethyl OL aglycone dialdehyde – 3,4-DHPEA-DEDA***) is also a highly investigated secoiridoid. For OLC, the *in vitro* antioxidant,[380] anti-inflammatory,[381] anti-tumor,[380] and anti-cancer[382] activities have been reported, while enhancement of insulin sensitivity *in vitro* and *in vivo*[383] has also been demonstrated.

Regarding simple phenols, ***hydroxytyrosol (HT)*** and ***tyrosol (TY)*** have been extensively reported. They both exert antioxidant, anti-inflammatory, and antiatherogenic/cardioprotective activities *in vitro*,[320,357,384-389] *in vivo*[321,351,384,390-392], or in humans[393]. Additionally, HT has demonstrated chemo-preventive,[394-396] antimicrobial,[378,397] and skin-bleaching[398] activities *in vitro*. The strongest biological activity of HT compared to TY has been attributed to the presence of the 3-hydroxyl group, which plays a very important role in the affinity of the molecule with the substrates.[399] Figure 11 presents the chemical structures of the phenolic compounds mentioned.

The beneficial effects of HT and OL in ocular disorders have also been demonstrated. On human retinal pigment epithelial cells, HT reduced the bevacizumab-stimulated increase of angiogenin secretion[400] and the acrolein-induced oxidative stress and mitochondrial dysfunction,[401] acting preventively in smoking- and age-related macular degeneration. On the same cell line, its antioxidant activity was proved to be related to the Nrf2-Keap1 pathway[402] and the activation of mitochondrial biogenesis and phase II detoxifying enzymes.[403] In rats with diabetic retinopathy, induced using intravenous injection of streptozotocin, HT inhibited the reduction of the number of retinal ganglion cells, exerting a neuroprotective effect, enhancing also some cardiovascular biomarkers.[404] Patents for eye drops comprising HT have also been published,[405] targeting trigeminal ganglion,[406] treating or preventing age-related macular degeneration, as well as protecting the eyesight.[407] In terms of OL, its prophylactic consumption can decrease intraocular pressure during anesthesia in rabbits, acting protectively for the eye.[408] Thus, there is increasing recognition for HT and OL by the scientific community, as shown by the increasing number of publications related in the last 40 years (Fig. 12).

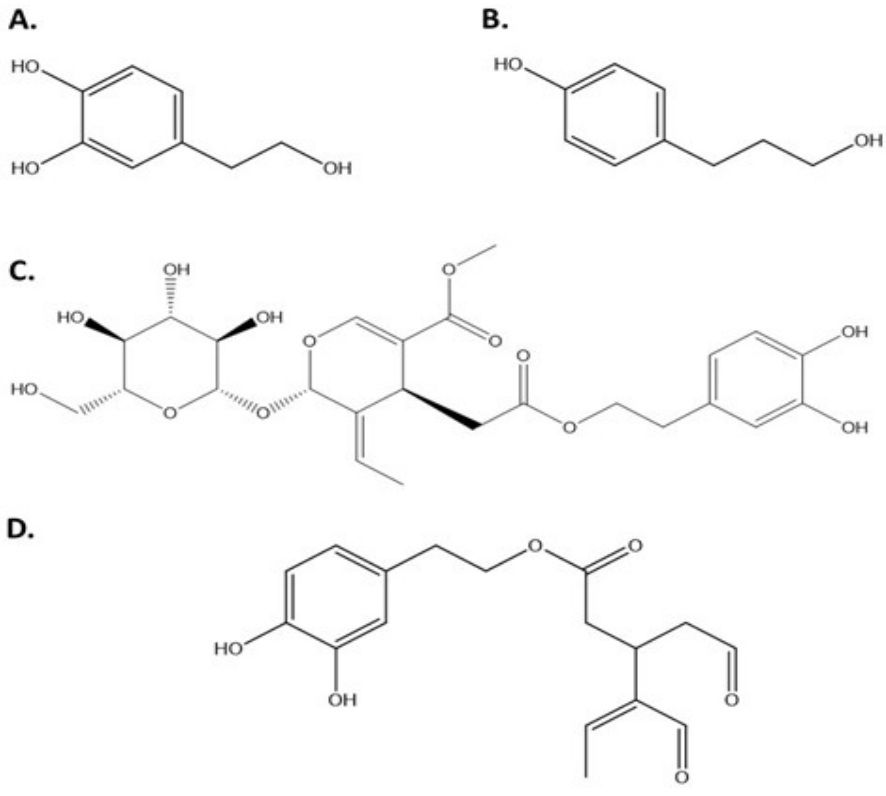


Figure 11. Chemical structures of the most investigated olive phenolic compounds: **A.** Hydroxytyrosol (HT), **B.** Tyrosol (TY), **C.** Oleuropein (OL), and **D.** Oleacein (OLC).

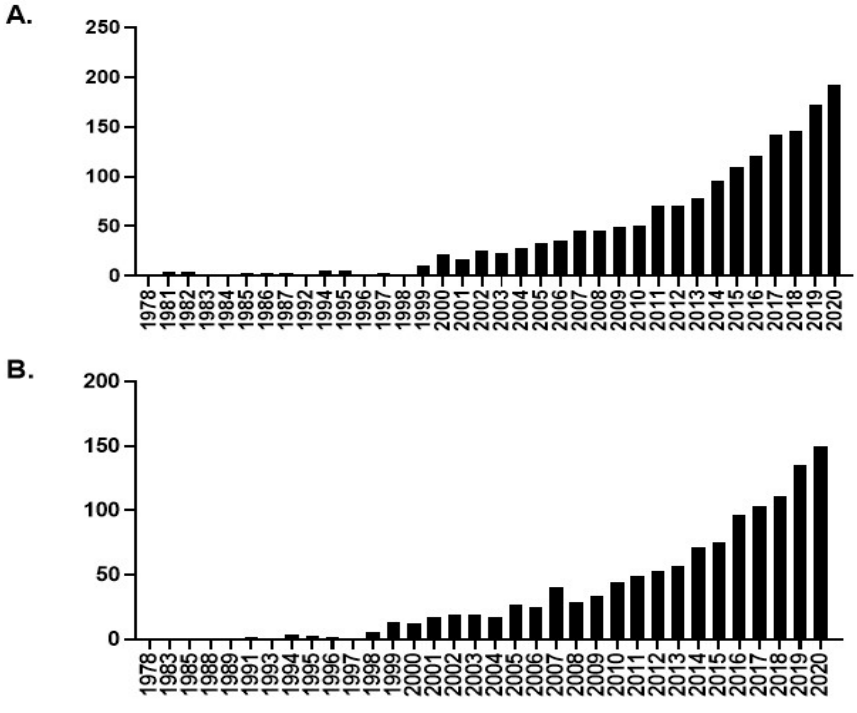


Figure 12. Evolution of the number of publications related to: **A.** "hydroxytyrosol" (HT) and **B.** "oleuropein" (OL), from 1978 to 2020 (Source: PubMed).

3.2 Olive Oil Industrial Production and By-products

The cultivation of olive groves covers a surface of 10.6 million hectares (data 2019), producing approximately 20,069,835 tons of olive oil per year for the decade 2010-2019.[409] Each olive tree offers between 15 and 40 kg of olive fruits per year. Most of the total olive oil production (ca. 72%) is performed by the Mediterranean countries, with Spain, Italy, Greece, Tunisia, and Turkey being the main olive oil-producing countries. Morocco, Algeria, and Portugal have also a well-established olive oil industry. In total, approximately 25,000 olive mills exist worldwide. USA, Argentina, Australia, and the Middle East are the principal olive oil producers outside the Mediterranean area.[409,410] Over the last decades, olive oil production has increased remarkably by 20%.[409]

The olive oil is principally located in the vacuoles of mesocarpal cells of the olive fruit, in small drops. It is also found in the colloidal system of the cell's cytoplasm, the epicarp, and the endosperm, but to a lesser extent.[411] Its extraction systems can be divided into two categories: the traditional pressing method and the centrifugation method (modern process). Figure 13 presents the typical modern extraction process of olive oil. The main steps of oil production are feeding, rejection of the leaves, washing, crushing, and malaxation (or slow mixing). Then, the olive oil is separated either by pressure or centrifugation.[308] All operations must be performed the fastest possible, as a long processing time could lead to the oxidation and acidity of the material, affecting its final quality.[412,413]

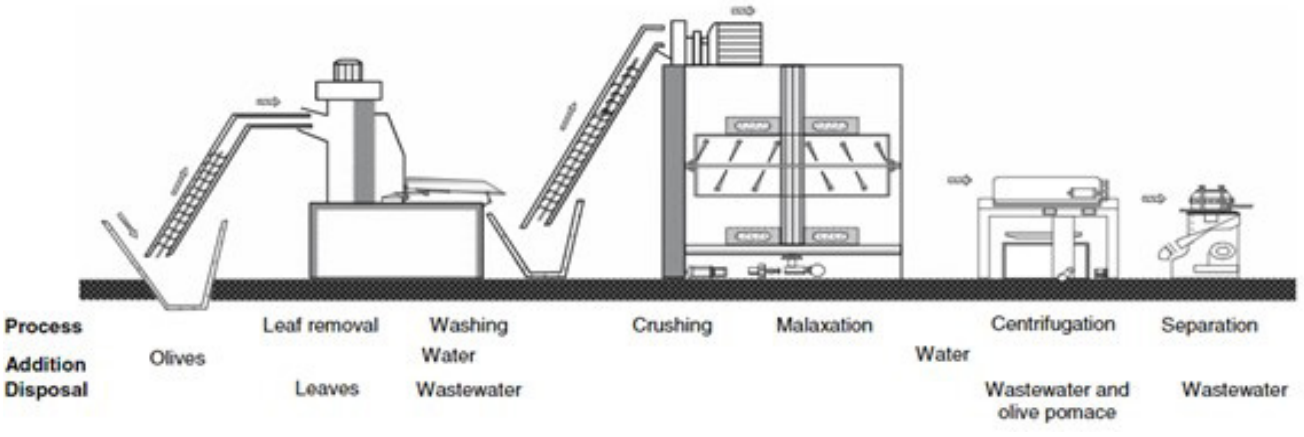


Figure 13. Typical layout of the modern olive oil extraction process (obtained from Kapellakis et al.[308]).

After their collection, olives are placed into the feeding hopper of the olive mill. Washing with water and leaves removal are performed in this step with the aid of a moving belt, to reject any external material that could contaminate the final product or harm the machine. Then, crushing the fruits is the main step of the process, as the cell walls of the mesocarpal cells break and the oil is released from the vacuoles. This step is performed in a big bowl containing two or three wheels rotating at high speed. Subsequently, the resulting paste from the crushing step is mixed slowly and constantly for 30 minutes (malaxation step), to increase the recovery of the obtained oil by the coalescence of small oil drops into larger ones; thus, helping to separate the oil from the water phase, breaking up the emulsion drops formed by the mixture of oil with water. Malaxators are made of stainless steel, to avoid oxidation, and have double walls being run by heating water in between, to maintain a temperature slightly above standard room value (up to 30 °C) to lower the oil viscosity.[308,414]

The large mixture produced after the malaxation step comprises olive oil, water, scattered cellular pieces of crushed olive fruits, and pieces of olive stones (pits). To extract the olive oil by this mixture, a pressure process and two- or three-phase centrifugation process can be applied. The pressure process is the oldest method and is performed placing the olive paste obtained by the previous stage in diaphragms, which are consequently placed in moving trolleys with a central shaft. The trolleys and their load are placed under hydraulic pressure, something that makes the liquids (water and oil) run out from the solid residue. Centrifugation is a more recent method of separation of the olive oil from the paste. It is performed using a horizontal decanter (centrifuge), which has a rotating bowl and an inner screw, resulting in the separation of the solid from the liquid (oil and olive mill wastewaters) part. There are two types of decanters: two- and three-phase.[308,415] The three-phase centrifugation system needs the addition of water, which aids the obtention of oil locked inside microgels or emulsions. It was introduced in the 1970s to increase oil yield. During this process, the oil, the olive mill wastewaters, and the solid waste (olive pulp and stones) are produced, with the latest being separated from the two liquid phases. The liquid phases are further centrifuged vertically, to isolate the oil without the presence of the mill wastewaters. However, the large amounts of wastewaters produced (1.25-1.75 times more water used, compared to the traditional pressure separation method) led to the development of the two-phase centrifugation process. This system has lower water demands, as it uses washing water, providing only the oil and producing principally a solid residue with high humidity, the olive pomace (OP), while fewer wastewaters.[416,417] Currently, the two-phase centrifugation system is the one used for the separation of the olive oil from the bulk from the biggest olive oil producer countries.[418] Thus, the olive by-products consist of the OP, the mill wastewaters, the leaves, and the stones (Figure 14).

The olive stones are recovered in the oil separation step, while the by-products generated by the table olive industry are even more, as they are usually rejected before further processing. Regarding the stones obtained by the olive oil production, they are usually crushed in small pieces, and they represent only 10% of by weight of the fruit. They consist of cellulose (ca. 28-40%), lignin (ca. 25-37%), and hemicelluloses (ca. 19-32%), along with fats (polyunsaturated fatty acids, e.g., linoleic acid) and proteins (i.e., essential amino acids).[340,341]

The leaves are also a lignocellulosic by-product, obtained in two points of the olive oil production: during the pruning (harvesting), i.e., before the washing step, and during the washing step of leaves removal. In the first case, they constitute approximately 25% of the pruning and are usually discarded into the soil (together with branches) or burned. They have a high humidity (ca. 51%), and they consist of extractives (37%, with cellulose, hemicelluloses, and lignin being 6%, 4%, and 40% of them, respectively), carbohydrates (ca. 27%), proteins (ca. 7%), fibers (ca. 7%), oil (ca. 3%), phenolic compounds (ca. 2-3%), and ash (ca. 2%).[340,341]

The olive mill wastewaters are generated during the washing of the fruits, as well as the horizontal and the vertical centrifugation.[341,415] They are an organic waste (ca. 4-16% of organic compounds) rich in toxic phenols, principally composed of water (ca. 83-92%), as well as sugars (ca. 2-4%), mineral salts, and metals (ca. 0.4-3%, with nitrogen, potassium, magnesium, and phosphorus being the principal ingredients), but also high-value ingredients, i.e., ca. 2-15% of phenolic compounds of low (HT, TY, *p*-coumaric acid, syringic acid, ferulic acid, etc.) or high (tannins, anthocyanins, etc.) molecular weight.[340,415] They have a low pH (ca. 5), high electrical conductivity, and dark color due to lignin polymerization with polyphenols.

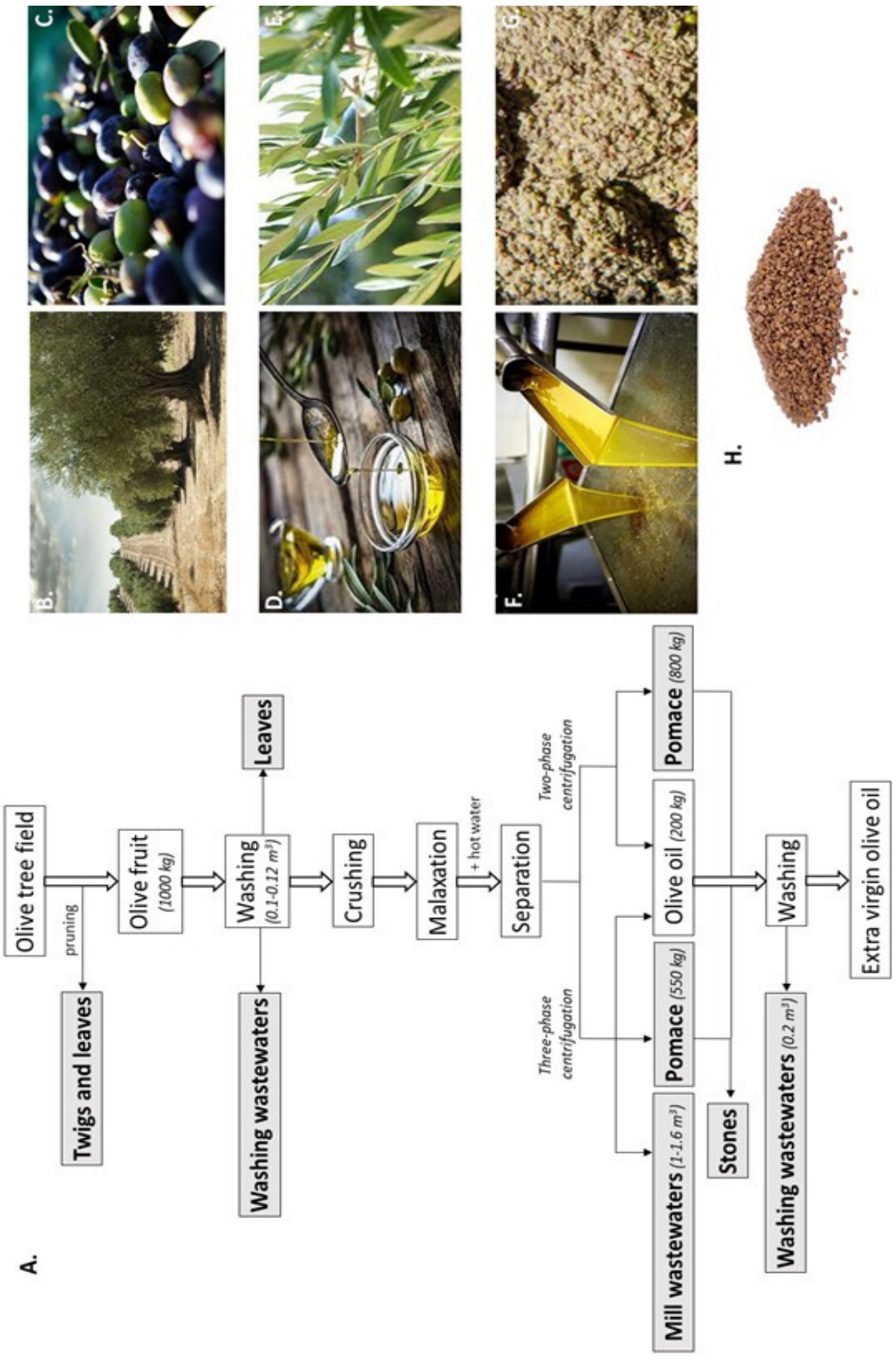


Figure 14. Olive oil production and by-products. **A.** Steps of the two- and three-phase separation process and the different by-products generated (adapted from Romero-García et al.[341] and Roig et al.[421]). **B.** Olive tree field. **C.** Olive fruit. **D.** Olive oil. **E.** Olive twigs and leaves. **F.** Washing oil wastewaters. **G.** Olive pomace (OP). **H.** Olive stones.

The OP is the semi-solid by-product and constitutes a mixture of vegetation and process waters, olive stones, and pulp. By weight, it is the principal by-product produced by the principally used two-phase separation method. 1,000 kg of processed olive fruit provide only 200 kg of olive oil, while the rest 800 kg is converted into OP.[341] Between 7 and 30 million m^3 of OP per year are produced only in the Mediterranean basin, which are stored in open-air bags.[341,419] It is an organic waste with phytotoxic and antimicrobial properties, high salinity, and humidity (ca. 55-70%), while it is also rich in phenolic compounds. It also comprises polysaccharides (ca. 39%), cellulose (ca. 30%), xyloglucans (ca. 15%), glucuronoxylans (ca. 14%), and mannans (ca. 2%), as well as carbohydrates, metals (potassium, calcium, and sodium) and salts, which lower its pH (ca. 5), as well as a high quantity of phytochemicals, which are antioxidant compounds (tocopherols and ca. 1-4% of phenolic compounds). Furthermore, the remaining 80% of the weight of the olive fruits end up in the OP, together with the washing waters, making the content of the OP in the remaining olive oil important as well (ca. 2-3%).[340,341,419,420]

3.3 Environmental Impact and Valorization Strategies of Olive By-products

As already mentioned, the two-phase separation system is the most used in Europe, generating OP as the main by-product.[421] During the processing, as also already mentioned, only 1-2% of the olive phenolic compounds end up in olive oil, as the rest 98% goes to the solid by-product (OP).[339] The presence of phenolic compounds in them makes their treatment difficult, resulting in a by-product with high biological and chemical demand in oxygen.[341] Thus, they constitute a major pollution factor for the atmosphere, the water, and the soil.[415] In the beginning, the two-phase system had been characterized as more ecological, due to the reduction of the production of wastewaters. However, the resulting OP has much higher humidity and cannot be managed easily. Thus, this characterization cannot be considered complete, as the load in this by-product is more concentrated and polluting.[308] Also, the produced OP comprises a high amount of remaining oil, which means that it must be further processed to recover it.[308] The recovery of the remaining oil produces the so-called "OP oil", which is available in the market. Its recovery is usually performed by drying the OP in ovens at high temperatures and using toxic organic solvents (hexane),[422] creating the risk of generation of carcinogenic substances.[308,422] For this reason, alternative methods have been proposed for the extraction of the OP oil, avoiding high temperatures and organic solvents, such as extraction with supercritical carbon CO_2.[423]

Therefore, there is increasing global awareness for the valorization of the olive by-products, and principally the OP. Regarding washing wastewaters, they can be used as fertilizers on soils and crops, giving a partial solution to the problem. However, several valorization strategies have been applied, including its evaporation, physicochemical treatments, extraction of valuable compounds (principally phenolic compounds), and biotechnological transformation into biofuel, biogas, or textile dye.[340,421] Regarding OP, its soil disposal is the principal alternative. However, large areas are required, and several environmental problems are produced, such as bad odor, insect proliferation, and leaching.[421] This method has been prohibited by many national regulations.[419] Composting the OP (mixing it with natural organic residues, i.e., leaves and twigs) has also been proposed to increase soil fertility in farms. However, its semi-solid texture hinders the elimination of its phytotoxic properties, thus the process lasts for at least 18 weeks.[424] The valorization strategies of OP also include physicochemical treatments, biotechnological transformations, and extraction of compounds useful for the

cosmetic industry, wastewater purification, and solid residues composting industry.[421,425] All these strategies can be performed in the defatted OP (after the removal of the OP oil).[341,426]

One of the most important valorization strategies for OP is the recovery of its high-value phenolic compounds, which can have numerous applications for the human health, as previously reported. Among the OP phenolic compounds, HT and OL are the principal molecules found, together with vanillin, apigenin, rutin, and luteolin.[427,428] Also, several simple phenols (including TY, 4-vinylphenol, and 4-methylcatechol[429,430]), hydroxybenzoic acid derivatives (quinic acid, shikimic acid, gallic acid, vanillic acid, etc.[429,431]), hydroxyphenylacetic acids (*p*-hydroxyphenylacetic acid and 2,5-dihydroxyphenylacetic acid[430,432]), hydroxycinnamic acids (rosmarinic, chlorogenic, ferulic, caffeic, *p*- and *o*-coumaric, etc.[430,432]), phenolic fatty acid esters (e.g., deoxyloganic acid lauryl ester[433]), glucosides (hydroxytyrosol glucosides, verbascoside and its derivatives, oleoside, etc.[431,432]), flavonoids (luteolin glycosides, isorhoifolin, apigenin glucosides, taxifolin, quercetin, delphinidin, hesperitin, etc.[430-432,434]), lignans (pinoresinol and its derivatives, 3-acetyloxy berchemol, etc.[429,430,435]), and secoiridoids (including OLC, OL aglycone, and derivatives, secologanin, secologanic acid, nüzhenide, etc.[430-432]) have been reported. Thus, the recovery of the OP phenolic compounds is not only beneficial for the human health with numerous biological applications but also contributes to the valorization of an agro-industrial by-product, reducing its environmental impact and contributing highly to the sustainable development of the olive oil chain.

3.4 Obtention of Phenolic Compounds from the Raw Material

To recover the phenolic compounds from vegetable materials, several steps are involved, i.e., feed preparation, solvent extraction, purification, and drying. In the feed preparation step, the material is prepared by removing any unwanted external component, maximizing the subsequent extraction efficiency (effectively using the available volume in the extractor only with the material of interest), and increasing the solid/liquid ratio for the same volume of solvent use. Also, if the material is frozen, thawing it before extraction is usually required. However, the extraction constitutes the most important step of the polyphenols' recovery process. The key factors for the extraction are the technique and the solvents, leading to the maximum recovery of the compounds of interest, and reducing the presence of unwanted substances.[436]

The solvent highly affects the extraction efficiency. The type of solvent, together with the solid/solvent ratio and the time of contact with the plant matrix are among the most important factors influencing the extraction of plant bio-actives.[437-439] For phenolic recovery, organic solvents have been mostly proposed, with acetone, methanol, and ethanol, among them. Acetone is non-polar and thus, cannot recover the medium polarity phenolic compounds,[440] whereas methanol is highly toxic.[441] On the other hand, ethanol has been considered a green organic solvent alternative for phenolic recovery.[442] However, pure organic solvents cannot enter entirely into vegetable cells, due to dehydration that occurs, and thus, they cannot recover polyphenols effectively.[443] For this reason, hydroalcoholic mixtures are proved to be the most suitable and are mainly used as solvents. Ethanol reduces the polarity and dielectric constant values in the aqueous mixture, leading to an increased phenolic extraction.[444] In addition, these mixtures are non-toxic, sustainable, and low cost (a big part of the solvent mixture is water).[436,445,446] Hence, solubility and environmental aspects should both be taken into account during solvent selection. In this context, extraction processes can also be based on

the use of "green" solvents, such as deep eutectic, which are a new class of ionic liquid analogs with low toxicity, high biodegradability, low-cost preparation, and simple synthesis with no further necessary purification.[447-449] The use of deep eutectic solvents for phenolic extraction from OP has already been proposed.[428]

In terms of extraction techniques, phenolic compounds are traditionally recovered using simple equipment. These techniques are heat reflux, maceration, Soxhlet, percolation, infusion, and decoction. In infusion (often called steeping), the material remains suspended in the selected solvent over time. In maceration, the raw material remains in contact with the solvent during a certain time at room temperature. In decoction, boiling the plant material is performed, while in percolation, the solvent is passed through the raw material. In heat reflux, the system (solvent and raw material) is heated for a specific amount of time, while the vapor produced is returned to the liquid by cooling it using a condenser. Soxhlet was invented by Frantz von Soxhlet in 1879 and is an apparatus based on the recycling of a small quantity of solvent, permitting the recovery of a large amount of material. However, all these techniques need a high amount of solvent and thus, they are not environmentally friendly and industrially appropriate. Also, long extraction times are used, which together with the high temperatures applied, increase the possibilities of degradation for the thermally labile compounds.[436,450-452]

As new technologies emerge, "green", cheaper, and more efficient extraction techniques have been proposed, reducing organic solvents and shortening the extraction time. The total cost of the extraction process can be remarkably decreased by using intensified extraction technologies, such as pressurized liquid extraction, supercritical fluid extraction, pulsed electric field extraction, and microwave-, ultrasound-, or enzyme-assisted extraction.[436]

Pulsed electric field extraction is a "green" extraction method of phytochemicals from the raw material and is performed by applying moderate to high-intensity electric pulses on the sample, which is placed between two electrodes. By applying the electric pulses, the cell membranes brake, while the temperature is maintained low. Its non-thermal nature makes this technique ideal for thermally sensitive materials, while its selectivity and ability to keep stable the extraction parameters (electric field strength, pulse duration, number of pulses, and size of membrane pores) are also included in its advantages.[436,453] Application of this technique to the OP have enhanced the recovery of the phenolic content by 13.3% with no negative impact on the OP oil quality,[454] while Andreou et al.[455] demonstrated that the phenolic compounds recovery from the OP increases by 91.6% compared to conventional extraction methods using hydroalcoholic mixtures.

Enzyme-assisted extraction is also a "green" method, able to enhance the phenolic recovery from the plant material by adding enzymes in the extraction solution. Enzymes (cellulose, pectinase, protease, etc.) can soften or break the cell walls, allowing better interaction between solvent and active ingredients. This technique is effective for the recovery of phenolic compounds that are bound to carbohydrates or proteins within the cells or on the cell walls. The process is performed by mixing the plant material with the solvent (mostly water) and the enzyme and incubating at 35-50 °C. Hence, the advantage of this technique is the avoidance of organic solvents. Also, its performance at low temperatures makes it ideal for thermally labile compounds. However, the cost of the enzyme is high and the extraction time is prolonged, varying from 1 to 48 hours, being its principal drawback for its future scalability.[436]

Ultrasound-assisted extraction is performed by applying sound waves with frequency higher than the human hearing ability (varying from 20 kHz to 2 MHz). Ultrasounds act like a piston on the liquid solvent and plant tissues, as their shape changes when sound waves travel through them, while they return to their initial shape in the absence of waves. This generates cavitation bubbles, which release energy, creating spots of high pressure and temperature. Thus, the matrix is ruptured, and the active ingredients come in direct contact with the solvent, enhancing the mass transfer in a green way. In addition, less solvent consumption is achieved, and many solvents can be used, reducing the extraction cost.[436,456] Applied to the OP, the ultrasounds can increase the recovery of phenolic compounds and antioxidant activity of the obtained extract either at 30 °C and 75 min of extraction time, or at 60 °C and 60min of extraction time (optimal conditions).[339,457] Similarly, Sahin and Samli[458] enhanced the antioxidant activity and the phenolic content of the obtained olive leaf extract by applying 1 h of extraction time and using water-ethanol mixtures. The combination of ultrasounds with enzyme extraction has also been found to increase the phenolic recovery from olive wastes.[459]

Microwave-assisted extraction is one of the most advanced "green" methods, widely used for the recovery of phenolic compounds. The frequencies of the electromagnetic radiation of the microwaves vary from 300 MHz to 300 GHz. The principle of this technique is based on heating the extraction solution dielectrically (caused by dipole rotation) or by ionic conduction (caused by ions alignment with the electromagnetic field), although both mechanisms could occur simultaneously. The advantages of this method are the low cost of the apparatus, as well as the quick and homogeneous heating, performed from the inside of the extraction solution and not by an external fluid or bath.[460] However, the selection of the solvents is very important in this technique, as several factors can affect it, i.e., affinity with active components, as well as ability to absorb microwave radiation (some solvents cannot be heated by this method, due to their lack of microwaves absorbing capacity).[436,460] The application of a microwave pre-treatment to the OP has already been described[461] and was found to increase the recovery of TY, HT, and OL during the subsequent conventional extraction. Also, microwaves were found to extract similar phenolic content from OP compared to traditional solvent extract in shorter extraction times and fewer solvent volumes. When combined with enzymes, a higher phenolic recovery was achieved, compared to conventional extraction.[462] Compared to ultrasounds, microwaves were found to be more effective in the recovery of HT, maslinic acid, and oleanolic acid from the OP.[463]

Extraction processes can also be based on the use of "green" solvents, like eutectic[447-449] (as already mentioned) or supercritical. A fluid is characterized as supercritical when it retains at the same time the properties of gas and liquid. This is achieved above a certain temperature (called "critical"), at which the pressure cannot influence the formation of liquid from the gas phase, and thus, no liquid-phase boundaries or surface tension exist. These properties give to the fluid good diffusivity into the solid matrix (gas-like properties) and at the same time high ability of dissolution of active materials (liquid-like properties). Therefore, with this technique, the extraction of active compounds from plant materials is very effective. The most used fluid for supercritical fluid extraction (SFE) is carbon CO_2. CO_2 has many advantages; it is cheap at an industrial level, chemically inert, non-toxic, non-flammable, easily available in high quantity and quality, suitable for lab or pilot operations, and can be easily separated from the extract and recycled. Also, the critical temperature of CO_2 is very close to the ambient (31 °C), which means that no extreme heating is required for this fluid, while it is easily separated from the plant matrix after the extraction procedure, only by reducing the pressure in

the separator, which collects the final extracts.[436] The ***supercritical carbon CO_2 ($scCO_2$) extraction*** is principally used for the recovery of non-polar compounds, such as lipids and waxes, also from the olive fruits.[464,465] Coupled with more polar co-solvents, such as EtOH, it is efficient in recovering also phenolic compounds in the oil obtained from the OP.[466] Nevertheless, the recovery of non-polar compounds from biomass by $scCO_2$ is of utmost importance, as it is part of a holistic biorefinery concept.[467,468] Compared to conventional defatting processes, $scCO_2$ offers an oil product with improved properties, as it has numerous biological applications (e.g., pharmaceuticals, foods, cosmetics, and nutraceuticals), and no subsequent expensive and harmful refining treatments are needed (due to its good dissipation from the extracts at atmospheric pressure), something necessary in the case of toxic organic solvents (i.e., hexane).[464,469] The use of $scCO_2$ for the recovery of the remaining non-polar compounds from the OP has already been proposed by Difonzo et al.[470] and Tirado et al.,[423] confirming that $scCO_2$ can be a green alternative solution for the obtention of the remaining OP oil.

As $scCO_2$ is useful only for non-polar compounds, pressurized or subcritical liquids can be used for more polar components. ***Pressurized liquid extraction (PLE)*** is based on the principle that the temperature of the boiling point is proportional to the pressure applied. Thus, by applying high pressure before the elevation of the temperature in the extraction procedure, the solvent remains in liquid state even at high temperatures.[436] Generally, the elevation of extraction temperature enhances the recovery of extractives, while several studies have proved that the solubility of phenolic compounds in the solvent increases through the temperature/pressure increase achieved by PLE.[472-474] In addition, the enhanced solvent diffusivity and the reduced solvent viscosity lead to high extraction yields in a short time and low solvent volumes, due to a higher mass transfer.[475] Hence, PLE is an ideal technique for these molecules. It is also an energy-saving method, as less heat is required to generate high temperatures. Thus, a higher quantity of phenolic compounds is recovered in the generated extract, spending less energy. Also, the extraction temperature can vary from 50 to 200 °C, while the extraction equipment is simple, scalable, and low cost.[436,445,446] PLE has already been applied to olive leaves[476,477] and OP[478-480] to recover their high-value phenolic compounds.

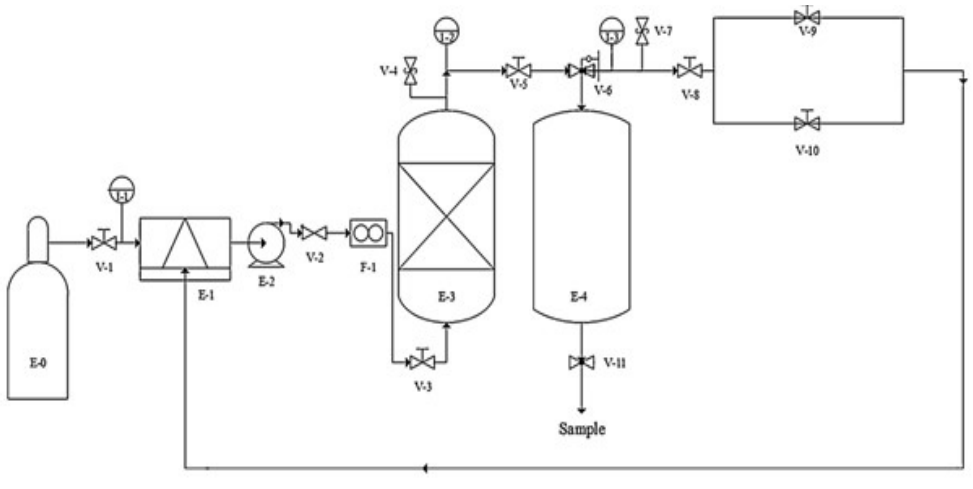


Figure 15. Representative supercritical carbon dioxide extraction ($scCO_2$) apparatus. E-0: tank for CO_2, E-1: refrigerator, E-2: pump, E-3: extractor, E-4: separator, F-1: flowmeter. (obtained from: Mustapa et al.[471]).

4. Industrial guidelines for botanical pharmaceutical product approval

For the approval of a drug substance as a pharmaceutical product, it should first comply with several guidelines and regulations regarding stability and toxicity. For a botanical drug, there is also uncertainty about its active components, due to its high heterogeneity. In many cases, its constituents have very low concentrations, especially in the final product. To ensure its therapeutic consistency, apart from chemical test control and biological assay results, botanical raw material control should be performed. This includes identification of the plant species via microscopic, macroscopic, or organoleptic methods and appropriate and standardized storage conditions.[481,482] It has been proved that the richness of the plant material in phenolic compounds can vary depending on geographical, climatic, and varietal factors. For example, olive oils from south Spain produced from the picual variety, have higher richness in phenolic compounds compared to north varieties such as arbequina, due to the higher hydric stress stimulated by the more severe climate.[483,484] However, pretreatment and storage of the material also affect its final retention in polyphenols. Storage temperature and duration, presence of light, oxidation, and drying or freezing have already been studied as possible factors that affect the stability of phenolic compounds in the raw material.[485-489]

Botanical drugs are classified in their whole like the active pharmaceutical ingredient in the drug product. The classifications include standardized, quantified, or "other" extracts. The standardized extracts have a declared content of components of a certain chemical group with known therapeutic activity, e.g., silymarines for *Silybum marianum*. The quantified extracts have a defined range of components with a known contribution to the therapeutic activity, e.g., hypericines for *Hypericum perforatum*. "Other" extracts that do not have known active components are defined by their production process and specifications, e.g., ratio of botanical substance to genuine botanical preparation. The choice of markers to be measured should be performed considering the literature, the monographs of available pharmacopeias, the analytical feasibility of the marker, the suitability of the marker for stability studies, and the availability, quality, and cost of the reference standards. Additionally, the shelf-life specifications are of chief importance for the product to be released in the market. The common limits for the botanical drug products are similar to those including chemically synthesized active pharmaceutical ingredients, having an assay limit of ± 5 % of the declared value.[481]

The long-term storage to study the shelf-life of the pharmaceutical product is described by the guidance CPMP/ICH/2736/99, which is followed by EMA and determines the different thermal and moisture storage conditions in which the product should remain stable. The same regulation applies to an herbal pharmaceutical product. The selection of the conditions in this guideline has as objective to cover the storage, shipment, and subsequent use of the final product.[490] Four different conditions are described: a temperature of 5 ± 3 °C (with no humidity), a temperature of 25 ± 2 °C (with 60 ± 5% relative humidity), a temperature of 30 ± 2 °C (with 65 ± 5% relative humidity), and a temperature of 40 ± 2 °C (with 75 ± 5% relative humidity). Long-term, accelerated, and intermediate studies exist. The general case describes as a long-term study the storage at 25 °C and 30 °C for 12 months, while storage at 40 °C for 6 months is considered an accelerated study. Also, 30 °C storage for 6 months can be considered as an intermediate study, depending on the drug substance. However, for drug products intended for storage in a refrigerator, 12-months storage should be performed at 5 °C, together with accelerated storage of 6-months at 25 °C. For a freezer-stored drug product, 12-months stability at – 20 °C should be ensured. Thus, the long-term, accelerated, and intermediate storage conditions depend on the intended storage conditions of the future product and can vary between 6 and 12 months.

Additionally, before the development of clinical studies and the subsequent approval of the medicinal product in the market, a non-clinical safety assessment should be performed. It includes toxicological, pharmacokinetic, genotoxicity, and carcinogenicity studies.[482] The genotoxicity assays are described by the International Council forHarmonisation (ICH) guideline S2 (R1) followed by EMA and can be *in vitro* or *in vivo*, detecting genetic damage by various mechanisms induced by the compounds.[491] These assays enable hazard identification concerning damage to DNA and its fixation. In general, the assessment includes mutagenicity in bacterial strains and genotoxicity in mammalian cells *in vitro* and/or *in vivo*. Among them, the comet assay (also called DNA strand brake assay) is proposed as a reliable and widely used technique to detect *in vitro* genotoxicity.[491,492]

References

1. Stern, M.E.; Gao, J.; Siemasko, K.F.; Beuerman, R.W.; Pflugfelder, S.C. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp. Eye Res.* **2004**, *78*, 409–416, doi:10.1016/j.exer.2003.09.003.
2. Holland, E.J.; Mannis, M.J.; Lee, W.B. Ocular surface disease: cornea, conjunctiva and tear film: expert consult-online and print; Saunders E.; Philadelphia, PA, USA, 2013;
3. Stern, M.E.; Beuerman, R.W.; Fox, R.I.; Gao, J.; Mircheff, A.K.; Pflugfelder, S.C. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. *Cornea* **1998**, *17*, 584–589, doi:10.1097/00003226-199811000-00002.
4. Diebold, Y.; Rios, J.D.; Hodges, R.R.; Rawe, I. Presence of nerves and their receptors in mouse and human conjunctival goblet cells. *Investig. Ophthalmol. Vis. Sci.* **2001**, *42*, 2270–2282.
5. LeDoux, M.S.; Zhou, Q.; Murphy, R.B.; Greene, M.L.; Ryan, P. Parasympathetic innervation of the meibomian glands in rats. *Investig. Ophthalmol. Vis. Sci.* **2001**, *42*, 2434–2441.
6. Wenk, J.; Brenneisen, P.; Meewes, C.; Wlaschek, M.; Peters, T.; Blaudschun, R.; Ma, W.; Kuhr, L.; Schneider, L.; Scharffetter-Kochanek, K. UV-induced oxidative stress and photoaging. *Curr. Probl. Dermatol.* **2001**, *29*, 83–94, doi:10.1159/000060656.
7. Stern, M.E.; Beuerman, R.W.; Pflugfelder, S.C. The normal tear film and ocular surface. In *Dry Eye and Ocular Surface Disorders*; CRC Press: Boca Raton, FL, USA, 2004; pp. 41–62.
8. Beuerman, R.W.; Mircheff, A.; Pflugfelder, S.C.; Stern, M.E. The lacrimal functional unit. In *Dry Eye and Ocular Surface Disorders*; CRC Press: Boca Raton, FL, USA, 2004; pp. 11–39.
9. Stern, M.E.; Schaumburg, C.S.; Pflugfelder, S.C. Dry eye as a mucosal autoimmune disease. *Int. Rev. Immunol.* **2013**, *32*, 19–41, doi :10.3109/08830185.2012.748052.
10. Stahl, U.; Willcox, M.; Stapleton, F. Osmolality and tear film dynamics. *Clin. Exp. Optom.* **2012**, *95*, 3–11, doi:10.1111/J.1444-0938.2011.00634.X.
11. Dartt, D.; Willcox, M. Complexity of the tear film: Importance in homeostasis and dysfunction during disease. *Exp. Eye Res.* **2013**, *117*, 1, doi:10.1016/J.EXER.2013.10.008.
12. Willcox, M.D.P.; Argüeso, P.; Georgiev, G.A.; Holopainen, J.M.; Laurie, G.W.; Millar, T.J.; Papas, E.B.; Rolland, J.P.; Schmidt, T.A.; Stahl, U.; et al. TFOS DEWS II Tear Film Report. *Ocul. Surf.* **2017**, *15*, 366–403, doi:10.1016/J.JTOS.2017.03.006.
13. Kopacz, D.; Niezgoda, L.; Fudalej, E.; Nowak, A.; Maciejewicz, P. Tear Film – Physiology and Disturbances in Various Diseases and Disorders. In *Ocular Surface Diseases - Some Current Date on Tear Film Problem and Keratoconic Diagnosis*; IntechOpen: London, UK, 2020; pp. 1–18 ISBN 978-1-83880-960-7.
14. Rolando, M.; Zierhut, M. The Ocular Surface and Tear Film and Their Dysfunction in Dry Eye Disease. *Surv. Ophthalmol.* **2001**, *45*, S203–S210, doi:10.1016/S0039-6257(00)00203-4.
15. Iwata, S. Chemical composition of the aqueous phase. *Int. Ophthalmol. Clin.* **1973**, *13*, 29–46, doi:10.1097/00004397-197301310-00005.
16. Davidson, H.J.; Kuonen, V.J. The tear film and ocular mucins. *Vet. Ophthalmol.* **2004**, *7*, 71–77, doi:10.1111/J.1463-5224.2004.00325.X.
17. Huang, L.C.; Jean, D.; Proske, R.J.; Reins, R.Y.; McDermott, A.M. Ocular Surface Expression and In Vitro Activity of Antimicrobial Peptides. *Curr. Eye Res.* **2009**, *32*, 595–609, doi:10.1080/02713680701446653.
18. Ubels, J.L.; Foley, K.M.; Rismondo, V. Retinol secretion by the lacrimal gland. *Investig. Ophthalmol. Vis. Sci.* **1986**, *27*, 1261–1268.
19. Ubels, J.L.; Rismondo, V.; Osgood, T.B. The relationship between secretion of retinol and protein by the lacrimal gland. *Investig. Ophthalmol. Vis. Sci.* **1989**, *30*, 952–960.
20. Pfister, R.R.; Renner, M.E. The corneal and conjunctival surface in vitamin A deficiency: a scanning electron microscopy study. *Invest. Ophthalmol. Vis. Sci.* **1978**, *17*, 874–883.
21. Dartt, D.A. Regulation of Tear Secretion. *Adv. Exp. Med. Biol.* **1994**, *350*, 1–9, doi:10.1007/978-1-4615-2417-5_1.
22. Gipson, I.K.; Argüeso, P. Role of mucins in the function of the corneal and conjunctival epithelia. *Int. Rev. Cytol.* **2003**, *231*, 1–49, doi:10.1016/S0074-7696(03)31001-0.
23. Dilly, P.N. Structure and Function of the Tear Film. *Adv. Exp. Med. Biol.* **1994**, *350*, 239–247, doi:10.1007/978-1-4615-2417-5_41.
24. Gipson, I.K. Distribution of mucins at the ocular surface. *Exp. Eye Res.* **2004**, *78*, 379–388, doi:10.1016/S0014-4835(03)00204-5.
25. Calonge, M.; Stern, M.E. The conjunctiva and tear film maintenance. In *Dry Eye and Ocular Surface Disorders*; CRC Press: Boca Raton, FL, USA, 2004; pp. 89–109.
26. Shumway, C.L.; Motlagh, M.; Wade, M. Anatomy, Head and Neck, Eye Conjunctiva. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island, FL, USA, 2021.
27. Puro, D.G. Role of ion channels in the functional response of conjunctival goblet cells to dry eye. *Am. J. Physiol. Cell Physiol.* **2018**, *315*, C236–C246, doi:10.1152/AJPCELL.00077.2018.
28. Wotherspoon, A.C.; Hardman-Lea, S.; Isaacson, P.G. Mucosa-associated lymphoid tissue (MALT) in the human conjunctiva. *J. Pathol.* **1994**, *174*, 33–37, doi:10.1002/PATH.1711740106.
29. Takahashi, Y.; Watanabe, A.; Matsuda, H.; Nakamura, Y.; Nakano, T.; Asamoto, K.; Ikeda, H.; Kakizaki, H. Anatomy of secretory glands in the eyelid and conjunctiva: A photographic review. *Ophthal. Plast. Reconstr. Surg.* **2013**, *29*, 215–219, doi:10.1097/IOP.0B013E-3182833DEE.

30. Sapte, E.; Costea, C.F.; Carauleanu, A.; Danca, C.; Dumitrescu, G.F.; Dimitriu, G.; Chihaia, M.A.; Buzduga, C.M.; Cucu, A.; Turliuc, M.D. Histological, immunohistochemical and clinical considerations on amniotic membrane transplant for ocular surface reconstruction. *Rom. J. Morphol. Embryol.* **2017**, *58*, 363–369.
31. Tai, L.Y.; Khaw, K.W.; Ng, C.M.; Subrayan, V. Central corneal thickness measurements with different imaging devices and ultrasound pachymetry. *Cornea* **2013**, *32*, 766–771, doi:10.1097/ICO.0B013E318269938D.
32. DelMonte, D.W.; Kim, T. Anatomy and physiology of the cornea. *J. Cataract Refract. Surg.* **2011**, *37*, 588–598, doi:10.1016/J.JCRS.2010.12.037.
33. Sridhar, M.S. Anatomy of cornea and ocular surface. *Indian J. Ophthalmol.* **2018**, *66*, 190, doi:10.4103/IJO.IJO_646_17.
34. Corneal Conditions | National Eye Institute Available online: https://www.nei.nih.gov/learn-about-eye-health/eye-conditions-and-diseases/corneal-conditions#0 (accessed on Oct 13, 2021).
35. Rüfer, F.; Schröder, A.; Erb, C. White-to-white corneal diameter: normal values in healthy humans obtained with the Orbscan II topography system. *Cornea* **2005**, *24*, 259–261, doi:10.1097/01.ICO.0000148312.01805.53.
36. Fares, U.; Otri, A.M.; Al-Aqaba, A.; Dua, H.S. Correlation of central and peripheral corneal thickness in healthy corneas. *Contact Lens Anterior Eye* **2011**, *35*, 39–45, doi:10.1016/j.clae.2011.07.004.
37. Feizi, S.; Jafarinasab, M.R.; Karimian, F.; Hasanpour, H.; Masudi, A. Central and peripheral corneal thickness measurement in normal and keratoconic eyes using three corneal pachymeters. *J. Ophthalmic Vis. Res.* **2014**, *9*, 296–304, doi:10.4103/2008-322X.143356.
38. Dua, H..; Faraj, L.A.; Said, D.G.; Gray, T.; Lowe, J. Human corneal anatomy redefined: a novel pre-Descemet's layer (Dua's layer). *Ophthalmology* **2013**, *120*, 1778–1785, doi:10.1016/J.OPHTHA.2013.01.018.
39. Craig, J.P.; Nichols, K.K.; Akpek, E.K.; Caffery, B.; Dua, H.S.; Joo, C.K.; Liu, Z.; Nelson, J.D.; Nichols, J.J.; Tsubota, K.; et al. TFOS DEWS II Definition and Classification Report. *Ocul. Surf.* **2017**, *15*, 276–283, doi:10.1016/J.JTOS.2017.05.008.
40. Stapleton, F.; Alves, M.; Bunya, V.Y.; Jalbert, I.; Lekhanont, K.; Malet, F.; Na, K.-S.; Schaumberg, D.; Od, S.D.; Uchino, M.; et al. TFOS DEWS II Epidemiology Report. *Ocul. Surf.* **2017**, *15*, 334–365, doi:10.1016/j.jtos.2017.05.003.
41. Craig, J.P.; Nelson, J.D.; Azar, D.T.; Belmonte, C.; Bron, A.J.; Chauhan, S.K.; de Paiva, C.S.; Gomes, J.A.P.; Hammitt, K.M.; Jones, L.; et al. TFOS DEWS II Report Executive Summary. *Ocul. Surf.* **2017**, *15*, 802–812, doi:10.1016/j.jtos.2017.08.003.
42. Lemp, M. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. *CLAO J.* **1995**, *21*, 221–232.
43. Lemp, M.A.; Baudouin, C.; Baum, J.; Dogru, M.; Foulks, G.N.; Kinoshita, S.; Laibson, P.; McCulley, J.; Murube, J.; Pflugfelder, S.C.; et al. The definition and classification of dry eye disease: Report of the definition and classification subcommittee of the international Dry Eye WorkShop (2007). *Ocul. Surf.* **2007**, *5*, 75–92, doi:10.1016/S1542-0124(12)70081-2.
44. Papas, E.B. The global prevalence of dry eye disease: A Bayesian view. *Ophthalmic Physiol. Opt.* **2021**, *41*, 1254–1266, doi:10.1111/OPO.12888.
45. Schaumberg, D.A.; Sullivan, D.A.; Dana, M.R. Epidemiology of Dry Eye Syndrome. Lacrimal Gland, Tear Film, and Dry Eye Syndromes. In *Lacrimal Gland. Tear Film, and Dry Eye Syndromes 3. Advances in Experimental Medicine and Biology;* Springer: Boston, MA, 2002; pp. 989–998.
46. Moss, S.E.; Klein, R.; K Klein, B.E. Prevalence of and Risk Factors for Dry Eye Syndrome. *Arch. Ophthalmol.* **2000**, *118*, 1264–1268.
47. Inomata, T.; Iwagami, M.; Nakamura, M.; Shiang, T.; Yoshimura, Y.; Fujimoto, K.; Okumura, Y.; Eguchi, A.; Iwata, N.; Miura, M.; et al. Characteristics and Risk Factors Associated with Diagnosed and Undiagnosed Symptomatic Dry Eye Using a Smartphone Application. *JAMA Ophthalmol.* **2020**, *138*, 58–68, doi:10.1001/JAMAOPHTHALMOL.2019.4815.
48. Sullivan, D.A.; Rocha, E.M.; Aragona, P.; Clayton, J.A.; Ding, J.; Golebiowski, B.; Hampel, U.; McDermott, A.M.; Schaumberg, D.A.; Srinivasan, S.; et al. TFOS DEWS II Sex, Gender, and Hormones Report. *Ocul. Surf.* **2017**, *15*, 284–333, doi:10.1016/J.JTOS.2017.04.001.
49. Sullivan, D.A.; Wickham, L.A.; Rocha, E.M.; Kelleher, R.S.; da Silveira, L.A.; Toda, I. Influence of gender, sex steroid hormones, and the hypothalamic-pituitary axis on the structure and function of the lacrimal gland. *Adv. Exp. Med. Biol.* **1998**, *438*, 11–42, doi:10.1007/978-1-4615-5359-5_2.
50. Craig, J.; Lim, J.; Han, A.; Tien, L.; Xue, A.L.; Wang, M.T.M. Ethnic differences between the Asian and Caucasian ocular surface: A co-located adult migrant population cohort study. *Ocul. Surf.* **2019**, *17*, 83–88, doi:10.1016/J.JTOS.2018.09.005.
51. Rexrode, K.M.; Lee, I.M.; Cook, N.R.; Hennekens, C.H.; Buring, J.E. Baseline characteristics of participants in the Women's Health Study. *J. Womens. Health Gend. Based. Med.* **2000**, *9*, 19–27, doi:10.1089/152460900318911.
52. Argueso, P.; Balaram, M.; Spurr-Michaud, S.; Keutmann, H.T.; Reza Dana, M.; Gipson, I.K. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 1004–1011.
53. Danjo, Y.; Hamano, T. Observation of precorneal tear film in patients with Sjögren's syndrome. *Acta Ophthalmol. Scand.* **1995**, *73*, 501–505, doi:10.1111/J.1600-0420.1995.TB00324.X.
54. Virtanen, T.; Konttinen, Y.T.; Honkanen, N.; Härkönen, M.; Tervo, T. Tear fluid plasmin activity of dry eye patients with Sjögren's syndrome. *Acta Ophthalmol. Scand.* **1997**, *75*, 137–141, doi:10.1111/J.1600-0420.1997.TB00109.X.
55. Mathers, W.D.; Shields, W.J.; Sachdev, M.S.; Petroll, W.M.; Jester, J.V. Meibomian gland dysfunction in chronic blepharitis. *Cornea* **1991**, *10*, 277–285, doi:10.1097/00003226-199107000-00001.
56. Goto, E.; Endo, K.; Suzuki, A.; Fujikura, Y.; Matsumoto, Y.; Tsubota, K. Tear Evaporation Dynamics in Normal Subjects and Subjects with Obstructive Meibomian Gland Dysfunction. *Invest. Ophthalmol. Vis. Sci.* **2003**, *44*, 533–539, doi:10.1167/IOVS.02-0170.
57. Bjerrum, K.B. Snake-like chromatin in conjunctival cells of normal elderly persons and of patients with primary Sjögren's syndrome and other connective tissue diseases. *Acta Ophthalmol. Scand.* **1995**, *73*, 33–36, doi:10.1111/J.1600-0420.1995.TB00009.X.
58. Krenzer, K.L.; Dana, M.R.; Ullman, M.D.; Cermak, J.M.; Tolls, D.B.; Evans, J.E.; Sullivan, D.A. Effect of androgen deficiency on the human meibomian gland and ocular surface. *J. Clin. Endocrinol. Metab.* **2000**, *85*, 4874–4882, doi:10.1210/JC.85.12.4874.

59. Sullivan, B.D.; Evans, J.E.; Dana, M.R.; Sullivan, D.A. Impact of Androgen Deficiency on the Lipid Profiles in Human Meibomian Gland Secretions. *Adv. Exp. Med. Biol.* **2002**, *506 A*, 449–458, doi:10.1007/978-1-4615-0717-8_63.
60. Zhang, X.; Zhao, L.; Deng, S.; Sun, X.; Wang, N. Dry Eye Syndrome in Patients with Diabetes Mellitus: Prevalence, Etiology, and Clinical Characteristics. *J. Ophthalmol.* **2016**, *2016*, 1–7, doi:10.1155/2016/8201053.
61. Alves, M.; Angerami, R.N.; Rocha, E.M. Dry eye disease caused by viral infection: review. *Arq. Bras. Oftalmol.* **2013**, *76*, 129–132, doi:10.1590/S0004-27492013000200016.
62. McCulley, J.P.; Dougherty, J.M. Blepharitis associated with acne rosacea and seborrheic dermatitis. *Int. Ophthalmol. Clin.* **1985**, *25*, 159–172, doi:10.1097/00004397-198502510-00010.
63. Gilbard, J.P.; Farris, R.L. Ocular surface drying and tear film osmolarity in thyroid eye disease. *Acta Ophthalmol.* **1983**, *61*, 108–116, doi:10.1111/J.1755-3768.1983.TB01401.X.
64. Liang, C.-Y.; Cheang, W.-M.; Wang, C.-Y.; Lin, K.-H.; Wei, L.-C.; Chen, Y.-Y.; Shen, Y.-C. The association of dry eye syndrome and psychiatric disorders: a nationwide population-based cohort study. *BMC Ophthalmol.* **2020**, *20*, 123, doi:10.1186/S12886-020-01395-Z.
65. Hom, M.M.; Nguyen, A.L.; Bielory, L. Allergic conjunctivitis and dry eye syndrome. *Ann. allergy, asthma Immunol.* **2012**, *108*, 163–166, doi:10.1016/J.ANAI.2012.01.006.
66. Peck, T.; Olsakovsky, L.; Aggarwal, S. Dry Eye Syndrome in Menopause and Perimenopausal Age Group. *J. Midlife. Health* **2017**, *8*, 51–54, doi:10.4103/JMH.JMH_41_17.
67. Pasadhika, S.; Rosenbaum, J.T. Ocular Sarcoidosis. *Clin. Chest Med.* **2015**, *36*, 683, doi:10.1016/J.CCM.2015.08.009.
68. Schaumberg, D.A.; Buring, J.E.; Sullivan, D.A.; Dana, M.R. Hormone Replacement Therapy and Dry Eye Syndrome. *JAMA* **2001**, *286*, 2114–2119, doi:10.1001/JAMA.286.17.2114.
69. Ogawa, Y.; Okamoto, S.; Wakui, M.; Watanabe, R.; Yamada, M.; Yoshino, M.; Ono, M.; Yang, H.Y.; Mashima, Y.; Oguchi, Y.; et al. Dry eye after haematopoietic stem cell transplantation. *Br. J. Ophthalmol.* **1999**, *83*, 1125–1130, doi:10.1136/BJO.83.10.1125.
70. Denoyer, A.; Landman, E.; Trinh, L.; Faure, J.-F.; Auclin, F.; Baudouin, C. Dry Eye Disease after Refractive Surgery: Comparative Outcomes of Small Incision Lenticule Extraction versus LASIK. *Ophthalmology* **2015**, *122*, 669–676, doi:10.1016/J.OPHTHA.2014.10.004.
71. Fraunfelder, F.T.; Sciubba, J.J.; Mathers, W.D. The role of medications in causing dry eye. *J. Ophthalmol.* **2012**, *2012*, 1–8, doi:10.1155/2012/285851.
72. Aragona, P.; Cannavo, S.P.; Borgia, F.; Guarneri, F. Utility of studying the ocular surface in patients with acne vulgaris treated with oral isotretinoin: A randomized controlled trial. *Br. J. Dermatol.* **2005**, *152*, 576–578, doi:10.1111/J.1365-2133.2005.06389.X.
73. 2007 TFOS report of the international dry eye workshop (DEWS). *Ocul. Surf.* **2007**, *5*, 163–178.
74. Berg, E.J.; Ying, G.; Maguire, M.G.; Sheffield, P.E.; Szczotka-Flynn, L.B.; Asbell, P.A.; Shen, J.F.; Group, the D.S.R. Climatic and Environmental Correlates of Dry Eye Disease Severity: A Report From the Dry Eye Assessment and Management (DREAM) Study. *Transl. Vis. Sci. Technol.* **2020**, *9*, 25, doi:10.1167/TVST.9.5.25.
75. Kojima, T. Contact Lens-Associated Dry Eye Disease: Recent Advances Worldwide and in Japan. *Invest. Ophthalmol. Vis. Sci.* **2018**, *59*, DES102–DES108, doi:10.1167/IOVS.17-23685.
76. Xu, L.; Zhang, W.; Zhu, X.-Y.; Suo, T.; Fan, X.-Q.; Fu, Y. Smoking and the risk of dry eye: a Meta-analysis. *Int. J. Ophthalmol.* **2016**, *9*, 1480–1486, doi:10.18240/IJO.2016.10.19.
77. You, Y.-S.; Qu, N.-B.; Yu, X.-N. Alcohol consumption and dry eye syndrome: a Meta-analysis. *Int. J. Ophthalmol.* **2016**, *9*, 1487–1492, doi:10.18240/IJO.2016.10.20.
78. Yenerel, N.M.; Küçümen, R.B. Pregnancy and the Eye. *Turkish J. Ophthalmol.* **2015**, *45*, 213–219, doi:10.4274/TJO.43815.
79. Miljanovic, B.; Trivedi, K.A.; Dana, M.R.; Gilbard, J.P.; Buring, J.E.; Schaumberg, D.A. Relation between dietary n-3 and n-6 fatty acids and clinically diagnosed dry eye syndrome in women. *Am. J. Clin. Nutr.* **2005**, *82*, 887–893, doi:10.1093/AJCN/82.4.887.
80. Nepp, J. Psychosomatic aspects of dry eye syndrome. *Ophthalmologe* **2016**, *113*, 111–119, doi:10.1007/S00347-015-0187-3.
81. Galor, A.; Covington, D.; Levitt, A.E.; McManus, K.T.; Seiden, B.; Felix, E.R.; Kalangara, J.; Feuer, W.; Patin, D.J.; Martin, E.R.; et al. Neuropathic ocular pain due to dry eye is associated with multiple comorbid chronic pain syndromes. *J. Pain* **2016**, *17*, 310–318, doi:10.1016/J.JPAIN.2015.10.019.
82. Uchino, M.; Schaumberg, D.A. Dry Eye Disease: Impact on Quality of Life and Vision. *Curr. Ophthalmol. Rep.* **2013**, *1*, 51–57, doi:10.1007/S40135-013-0009-1.
83. Reddy, P.; Grad, O.; Rajagopalan, K. The Economic Burden of Dry Eye A Conceptual Framework and Preliminary Assessment. *Cornea* **2004**, *23*, 751–761, doi:10.1097/01.ico.0000134183.476.
84. Clegg, J.; Guest, J.; Lehman, A.; Smith, A. The annual cost of dry eye syndrome in France, Germany, Italy, Spain, Sweden and the United Kingdom among patients managed by ophthalmologists. *Ophthalmic Epidemiol.* **2006**, *13*, 263–274, doi:10.1080/09286580600801044.
85. Schaumberg, D.A.; Dana, R.; Buring, J.E.; Sullivan, D.A. Prevalence of Dry Eye Disease among US Men: Estimates from the Physicians' Health Studies. *Arch. Ophthalmol.* **2009**, *127*, 763–768, doi:10.1001/ARCHOPHTHALMOL.2009.103.
86. Schaumberg, D.A.; Sullivan, D.A.; Buring, J.E.; Dana, M.R. Prevalence of dry eye syndrome among US women. *Am. J. Ophthalmol.* **2003**, *136*, 318–326, doi:10.1016/S0002-9394(03)00218-6.
87. Siffel, C.; Hennies, N.; Joseph, C.; Lascano, V.; Horvat, P.; Scheider, M.; Ganzera, F. Burden of dry eye disease in Germany: a retrospective observational study using German claims data. *Acta Ophthalmol.* **2020**, *98*, e504–e512, doi:10.1111/AOS.14300.
88. Darbà, J.; Ascanio, M. Economic impact of dry eye disease in Spain: A multicentre retrospective insurance claims database analysis. *Eur. J. Ophthalmol.* **2021**, *31*, 328–333, doi:10.1177/1120672120907315.

89. Mizuno, Y.; Yamada, M.; Shigeyasu, C. Annual direct cost of dry eye in Japan. *Clin. Ophthalmol.* **2012**, *6*, 755–760, doi:10.2147/OPTH.S30625.
90. Galor, A.; Zheng, D.D.; Arheart, K.L.; Lam, B.L.; Perez, V.L.; McCollister, K.E.; Ocasio, M.; McClure, L.A.; Lee, D.J. Dry eye medication use and expenditures: data from the medical expenditure panel survey 2001 to 2006. *Cornea* **2012**, *31*, 1403–1407, doi:10.1097/ICO.0B013E31823CC0B7
91. Dalzell, M.D. Dry eye: prevalence, utilization, and economic implications. *Manag Care* **2003**, *12*, 9–13.
92. Chan, C.; Ziai, S.; Myageri, V.; Burns, J.G.; Prokopich, C.L.; Chan@, C. Economic burden and loss of quality of life from dry eye disease in Canada. *BMJ Open Ophthalmol.* **2021**, *6*, e000709, doi:10.1136/bmjophth-2021-000709.
93. Luo, Y.; Yang, W.; Qi, M.; Wang, Y.; Li, S.; Wang, M.; Zeng, Q. Annual direct economic burden and influencing factors of dry eye disease in Central China. *Ophthalmic Epidemiol.* **2021**, 1–8, doi:10.1080/09286586.2021.1959618.
94. McDonald, M.; Patel, D.A.; Keith, M.S.; Snedecor, S.J. Economic and Humanistic Burden of Dry Eye Disease in Europe, North America, and Asia: A Systematic Literature Review. *Ocul. Surf.* **2016**, *14*, 144–167, doi:10.1016/j.jtos.2015.11.002.
95. Greco, G.; Pistilli, M.; Asbell, P.A.; Maguire, M.G. Association of Severity of Dry Eye Disease with Work Productivity and Activity Impairment in the Dry Eye Assessment and Management Study. *Ophthalmology* **2021**, *128*, 850–856, doi:10.1016/J.OPHTHA.2020.10.015.
96. Yu, J.; Asche, C. V; Fairchild, C.J. The Economic Burden of Dry Eye Disease in the United States: A Decision Tree Analysis. *Cornea* **2011**, *30*, 379–387, doi:10.1097/ico.0b013e3181f7f363.
97. Yamada, M.; Mizuno, Y.; Shigeyasu, C. Impact of dry eye on work productivity. *Clin. Outcomes Res.* **2012**, *4*, 307–312, doi:10.2147/CEOR.S36352.
98. Bron, A.J.; de Paiva, C.S.; Chauhan, S.K.; Bonini, S.; Gabison, E.E.; Jain, S.; Knop, E.; Markoulli, M.; Ogawa, Y.; Perez, V.; et al. TFOS DEWS II pathophysiology report. *Ocul. Surf.* **2017**, *15*, 438–510, doi:10.1016/J.JTOS.2017.05.011.
99. Roda, M.; Corazza, I.; Reggiani, M.L.B.; Pellegrini, M.; Taroni, L.; Giannaccare, G.; Versura, P. Dry Eye Disease and Tear Cytokine Levels—A Meta-Analysis. *Int. J. Mol. Sci.* **2020**, *21*, 3111, doi:10.3390/IJMS21093111.
100. Enriquez-De-Salamanca, A.; Bonini, S.; Calonge, M. Molecular and cellular biomarkers in dry eye disease and ocular allergy. *Curr. Opin. Allergy Clin. Immunol.* **2012**, *12*, 523–533, doi:10.1097/ACI.0B013E328357B488.
101. Enriquez-de-Salamanca, A.; Calonge, M. Cytokines and chemokines in immune-based ocular surface inflammation. *Expert Rev. Clin. Immunol.* **2008**, *4*, 457–467, doi:10.1586/1744666X.4.4.457.
102. Hagan, S.; Martin, E.; Enriquez-de-Salamanca, A. Tear fluid biomarkers in ocular and systemic disease: potential use for predictive, preventive and personalised medicine. *EPMA J.* **2016**, *7*, 15, doi:10.1186/S13167-016-0065-3.
103. Tishler, M.; Yaron, I.; Geyer, O.; Shirazi, I.; Naftaliev, E.; Yaron, M. Elevated tear interleukin-6 levels in patients with Sjogren syndrome. *Ophthalmology* **1998**, *105*, 2327–2329, doi:10.1016/S0161-6420(98)91236-2.
104. Pflugfelder, S.C.; Jones, D.; Ji, Z.; Afonso, A.; Monroy, D. Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjogren's syndrome keratoconjunctivitis sicca. *Curr. Eye Res.* **1999**, *19*, 201–211, doi:10.1076/ceyr.19.3.201.5309.
105. Solomon, A.; Dursun, D.; Liu, Z.; Xie, Y.; Macri, A.; Pflugfelder, S.C. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. *Investig. Ophthalmol. Vis. Sci.* **2001**, *42*, 2283–2292.
106. Lam, H.; Bleiden, L.; de Paiva, C.S.; Farley, W.; Stern, M.E.; Pflugfelder, S.C. Tear Cytokine Profiles in Dysfunctional Tear Syndrome. *Am. J. Ophthalmol.* **2009**, *147*, 198–205, doi:10.1016/j.ajo.2008.08.032.
107. Jones, D.; Monroy, D.; Ji, Z.; Atherton, S.; Pflugfelder, S. Sjögren's syndrome: Cytokine and Epstein-Barr viral gene expression within the conjunctival epithelium. *Invest. Ophthalmol. Vis. Sci.* **1994**, *35*, 3493–3504.
108. Yoon, K.C.; Jeong, I.Y.; Park, Y.G.; Yang, S.Y. Interleukin-6 and tumor necrosis factor-α levels in tears of patients with dry eye syndrome. *Cornea* **2007**, *26*, 431–437, doi:10.1097/ICO.0b013e31803dcda2.
109. Paiva, C.S.; Chotikavanich, S.; Pangelinan, S.B.; Zheng, X.; Butler, M.; Farley, W.; Stern, M.; Li, D.; Pflugfelder, S. Desiccating Stress Promotes Th-17 Pattern Response in the Conjunctiva. *Invest. Ophthalmol. Vis. Sci.* **2008**, *49*, 5420–5420.
110. Benitez-del-Castillo Sanchez, J.; Morillo-Rojas, M.D.; Galbis-Estrada, C.; Pinazo-Duran, M.D. Determinacion de mediadores de la respuesta inmune e inflamacion en lagrimas: cambios en ojo seco y glaucoma frente a poblacian sana. *Arch. Soc. Esp. Oftalmol.* **2017**, *92*, 210–217, doi:10.1016/J.OFTAL.2016.12.009.
111. Massingale, M.L.; Li, X.; Vallabhajosyula, M.; Chen, D.; Wei, Y.; Asbell, P.A. Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea* **2009**, *28*, 1023–1027, doi:10.1097/ICO.0B013E3181A16578.
112. Boehm, N.; Riechardt, A.I.; Wiegand, M.; Pfeiffer, N.; Grus, F.H. Proinflammatory Cytokine Profiling of Tears from Dry Eye Patients by Means of Antibody Microarrays. *Invest. Ophthalmol. Vis. Sci.* **2011**, *52*, 7725–7730, doi:10.1167/IOVS.11-7266.
113. Lee, S.Y.; Han, S.J.; Nam, S.M.; Yoon, S.C.; Ahn, J.M.; Kim, T.I.; Kim, E.K.; Seo, K.Y. Analysis of Tear Cytokines and Clinical Correlations in Sjögren Syndrome Dry Eye Patients and Non–Sjögren Syndrome Dry Eye Patients. *Am. J. Ophthalmol.* **2013**, *156*, 253, doi:10.1016/J.AJO.2013.04.003.
114. Vandermeid, K.R.; Su, S.P.; Ward, K.W.; Zhang, J.Z. Correlation of Tear Inflammatory Cytokines and Matrix Metalloproteinases with Four Dry Eye Diagnostic Tests. *Invest. Ophthalmol. Vis. Sci.* **2012**, *53*, 1512–1518, doi:10.1167/IOVS.11-7627.
115. Landsend, E.C.S.; Utheim, O.A.; Pedersen, H.R.; Aass, H.C.D.; Lagali, N.; Dartt, D.A.; Baraas, R.C.; Utheim, T.P. The Level of Inflammatory Tear Cytokines is Elevated in Congenital Aniridia and Associated with Meibomian Gland Dysfunction. *Invest. Ophthalmol. Vis. Sci.* **2018**, *59*, 2197–2204, doi:10.1167/IOVS.18-24027.
116. Zhao, H.; Li, Q.; Ye, M.; Yu, J. Tear Luminex Analysis in Dry Eye Patients. *Med. Sci. Monit.* **2018**, *24*, 7595–7602, doi:10.12659/MSM.912010.

117. Acera, A.; Rocha, G.; Vecino, E.; Lema, I.; Duran, J.A. Inflammatory Markers in the Tears of Patients with Ocular Surface Disease. *Ophthalmic Res.* **2008**, *40*, 315–321, doi:10.1159/000150445.
118. Yoon, K.C.; Park, C.S.; You, I.C.; Choi, H.J.; Lee, K.H.; Im, S.K.; Park, H.Y.; Pflugfelder, S.C. Expression of CXCL9, -10, -11, and CXCR3 in the tear film and ocular surface of patients with dry eye syndrome. *Investig. Ophthalmol. Vis. Sci.* **2010**, *51*, 643–650, doi:10.1167/iovs.09-3425.
119. Enriquez-de-Salamanca, A.; Castellanos, E.; Stern, M.E.; Fernandez, I.; Carreno, E.; Garcia-Vazquez, C.; Herreras, J.M.; Calonge, M. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol. Vis.* **2010**, *16*, 862–873.
120. Corrales, R.M.; Villarreal, A.; Farley, W.; Stern, M.E.; Li, D.Q.; Pflugfelder, S.C. Strain-related cytokine profiles on the murine ocular surface in response to desiccating stress. *Cornea* **2007**, *26*, 579–584, doi:10.1097/ICO.0b013e318033a729.
121. Yoon, K.C.; De Paiva, C.S.; Qi, H.; Chen, Z.; Farley, W.J.; Li, D.Q.; Pflugfelder, S.C. Expression of Th-1 chemokines and chemokine receptors on the ocular surface of C57BL/6 mice: Effects of desiccating stress. *Investig. Ophthalmol. Vis. Sci.* **2007**, *48*, 2561–2569, doi:10.1167/iovs.07-0002.
122. De Paiva, C.S.; Chotikavanich, S.; Pangelinan, S.B.; Pitcher, J.D.; Fang, B.; Zheng, X.; Ma, P.; Farley, W.J.; Siemasko, K.F.; Niederkorn, J.Y.; et al. IL-17 disrupts corneal barrier following desiccating stress. *Mucosal Immunol.* **2009**, *2*, 243–253, doi:10.1038/mi.2009.5.
123. Rao, K.; Farley, W.J.; Pflugfelder, S.C. Association between High Tear Epidermal Growth Factor Levels and Corneal Subepithelial Fibrosis in Dry Eye Conditions. *Invest. Ophthalmol. Vis. Sci.* **2010**, *51*, 844–849, doi:10.1167/IOVS.09-3875.
124. Ohashi, Y.; Ishida, R.; Kojima, T.; Goto, E.; Matsumoto, Y.; Watanabe, K.; Ishida, N.; Nakata, K.; Takeuchi, T.; Tsubota, K. Abnormal protein profiles in tears with dry eye syndrome. *Am. J. Ophthalmol.* **2003**, *136*, 291–299, doi:10.1016/S0002-9394(03)00203-4.
125. Teson, M.; Gonzalez-Garcia, M.J.; Lopez-Miguel, A.; Enriquez-de-Salamanca, A.; Martin-Montanez, V.; Benito, M.J.; Mateo, M.E.; Stern, M.E.; Calonge, M. Influence of a Controlled Environment Simulating an In-Flight Airplane Cabin on Dry Eye Disease. *Invest. Ophthalmol. Vis. Sci.* **2013**, *54*, 2093–2099, doi:10.1167/IOVS.12-11361.
126. Lopez-Miguel, A.; Teson, M.; Martin-Montanez, V.; Enriquez-De-Salamanca, A.; Stern, M.E.; Gonzalez-Garcia, M.J.; Calonge, M. Clinical and Molecular Inflammatory Response in Sjögren Syndrome–Associated Dry Eye Patients Under Desiccating Stress. *Am. J. Ophthalmol.* **2016**, *161*, 133–141, doi:10.1016/J.AJO.2015.09.039.
127. Messmer, E.M.; von Lindenfels, V.; Garbe, A.; Kampik, A. Matrix Metalloproteinase 9 Testing in Dry Eye Disease Using a Commercially Available Point-of-Care Immunoassay. *Ophthalmology* **2016**, *123*, 2300–2308, doi:10.1016/J.OPHTHA.2016.07.028.
128. Chotikavanich, S.; de Paiva, C.S.; de Quan Li; Chen, J.J.; Bian, F.; Farley, W.J.; Pflugfelder, S.C. Production and Activity of Matrix Metalloproteinase-9 on the Ocular Surface Increase in Dysfunctional Tear Syndrome. *Invest. Ophthalmol. Vis. Sci.* **2009**, *50*, 3203–3209, doi:10.1167/IOVS.08-2476.
129. Aragona, P.; Aguennouz, M.; Rania, L.; Postorino, E.; Sommario, M.S.; Roszkowska, A.M.; De Pasquale, M.G.; Pisani, A.; Puzzolo, D. Matrix Metalloproteinase 9 and Transglutaminase 2 Expression at the Ocular Surface in Patients with Different Forms of Dry Eye Disease. *Ophthalmology* **2015**, *122*, 62–71, doi:10.1016/J.OPHTHA.2014.07.048.
130. Lopez-Miguel, A.; Teson, M.; Martin-Montanez, V.; Enriquez-De-Salamanca, A.; Stern, M.E.; Calonge, M.; Gonzalez-Garcia, M.J. Dry Eye Exacerbation in Patients Exposed to Desiccating Stress under Controlled Environmental Conditions. *Am. J. Ophthalmol.* **2014**, *157*, 788–798, doi:10.1016/J.AJO.2014.01.001.
131. Chen, Y.; Dana, R. Autoimmunity in dry eye disease – An updated review of evidence on effector and memory Th17 cells in disease pathogenicity. *Autoimmun. Rev.* **2021**, *20*, 102933, doi:10.1016/J.AUTREV.2021.102933.
132. Pflugfelder, S.C.; Corrales, R.M.; de Paiva, C.S. T helper cytokines in dry eye disease. *Exp. Eye Res.* **2013**, *117*, 118–125, doi:10.1016/J.EXER.2013.08.013.
133. Calonge, M. The Treatment of Dry Eye. *Surv. Ophthalmol.* **2001**, *45*, S227–S239, doi:10.1016/S0039-6257(00)00205-8.
134. Paiva, C.S. De; Villarreal, A.L.; Corrales, R.M.; Rahman, H.T.; Chang, V.Y.; Farley, W.J.; Stern, M.E.; Niederkorn, J.Y.; Li, D.-Q.; Pflugfelder, S.C. Dry Eye–Induced Conjunctival Epithelial Squamous Metaplasia Is Modulated by Interferon-γ. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 2553–2560, doi:10.1167/IOVS.07-0069.
135. Stern, M.E.; Gao, J.; Schwalb, T.A.; Ngo, M.; Tieu, D.D.; Chan, C.C.; Reis, B.L.; Whitcup, S.M.; Thompson, D.; Smith, J.A. Conjunctival T-cell subpopulations in Sjögren's and non-Sjögren's patients with dry eye. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 2609–2614.
136. Niederkorn, J.Y.; Stern, M.E.; Pflugfelder, S.C.; De Paiva, C.S.; Corrales, R.M.; Gao, J.; Siemasko, K. Desiccating stress induces T cell-mediated Sjögren's Syndrome-like lacrimal keratoconjunctivitis. *J. Immunol.* **2006**, *176*, 3950–3957, doi:10.4049/JIMMUNOL.176.7.3950.
137. Abengozar-Vela, A.; Schaumburg, C.S.; Stern, M.E.; Calonge, M.; Enriquez-de-Salamanca, A.; Gonzalez-Garcia, M.J. Topical Quercetin and Resveratrol Protect the Ocular Surface in Experimental Dry Eye Disease. *Ocul. Immunol. Inflamm.* **2019**, *27*, 1023–1032, doi:10.1080/09273948.2018.1497664.
138. Wakamatsu, T.H.; Dogru, M.; Tsubota, K. Tearful relations: Oxidative stress, inflammation and eye diseases. *Arq. Bras. Oftalmol.* **2009**, *71*, 72–79, doi:10.1590/s0004-27492008000700015.
139. Cejkova, J.; Ardan, T.; Jirsova, K.; Jechova, G.; Malec, J.; Simonova, Z.; Cejka, C.; Filipec, M.; Dotrelova, D.; Brunova, B. The role of conjunctival epithelial cell xanthine oxidoreductase/xanthine oxidase in oxidative reactions on the ocular surface of dry eye patients with Sjögren's syndrome. *Histol. Histopathol.* **2007**, *22*, 997–1003, doi:10.14670/HH-22.997.
140. Cejkova, J.; Ardan, T.; Simonova, Z.; Cejka, C.; Malec, J.; Dotrelova, D.; Brunova, B. Decreased expression of antioxidant enzymes in the conjunctival epithelium of dry eye (Sjögren's syndrome) and its possible contribution to the development of ocular surface oxidative injuries. *Histol. Histopathol.* **2008**, *23*, 1477–1483, doi:10.14670/HH-23.1477.

141. Wakamatsu, T.H.; Dogru, M.; Matsumoto, Y.; Kojima, T.; Kaido, M.; Ibrahim, O.M.A.; Sato, E.A.; Igarashi, A.; Ichihashi, Y.; Satake, Y.; et al. Evaluation of lipid oxidative stress status in Sjögren syndrome patients. *Investig. Ophthalmol. Vis. Sci.* **2013**, *54*, 201–210, doi:10.1167/iovs.12-10325.
142. Wakamatsu, T.H.; Dogru, M.; Ayako, I.; Takano, Y.; Matsumoto, Y.; Ibrahim, O.M.A.; Okada, N.; Satake, Y.; Fukugawa, K.; Shimazaki, J.; et al. Evaluation of lipid oxidative stress status and inflammation in atopic ocular surface disease. *Mol. Vis.* **2010**, *16*, 2465–2475, doi:10.1167/iovs.12-10325.
143. Nakamura, S.; Shibuya, M.; Nakashima, H.; Hisamura, R.; Masuda, N.; Imagawa, T.; Uehara, M.; Tsubota, K. Involvement of oxidative stress on corneal epithelial alterations in a blink-suppressed dry eye. *Investig. Ophthalmol. Vis. Sci.* **2007**, *48*, 1552–1558, doi:10.1167/iovs.06-1027.
144. Seen, S.; Tong, L. Dry eye disease and oxidative stress. *Acta Ophthalmol.* **2018**, *96*, e412–e420, doi:10.1111/AOS.13526.
145. Dogru, M.; Kojima, T.; Simsek, C.; Tsubotav, K. Potential role of oxidative stress in ocular surface inflammation and dry eye disease. *Investig. Ophthalmol. Vis. Sci.* **2018**, *59*, DES163–DES168, doi:10.1167/iovs.17-23402.
146. Rezaee, R.; Abdollahi, M. The importance of translatability in drug discovery. *Expert Opin. Drug Discov.* **2017**, *12*, 237–239, doi:10.1080/17460441.2017.1281245.
147. Arantes-Rodrigues, R.; Colaço, A.; Pinto-Leite, R.; Oliveira, P. In vitro and in vivo experimental models as tools to investigate the efficacy of antineoplastic drugs on urinary bladder cancer. *Anticancer Res.* **2013**, *33*, 1273–1296.
148. Kilkenny, C.; Browne, W.J.; Cuthill, I.C.; Emerson, M.; Altman, D.G. Improving bioscience research reporting: The arrive guidelines for reporting animal research. *PLoS Biol.* **2010**, *8*, 1–5, doi:10.1371/JOURNAL.PBIO.1000412.
149. Garcia-Posadas, L.; Diebold, Y. Three-Dimensional Human Cell Culture Models to Study the Pathophysiology of the Anterior Eye. *Pharmaceutics* **2020**, *12*, 1215, doi:10.3390/PHARMACEUTICS12121215.
150. Mitsias, D.I.; Kapsogeorgou, E.K.; Moutsopoulos, H.M. The role of epithelial cells in the initiation and perpetuation of autoimmune lesions: lessons from Sjögren's syndrome (autoimmune epithelitis). *Lupus* **2006**, *15*, 255–261, doi:10.1191/0961203306LU2290RR.
151. Hingorani, M.; Calder, V.L.; Buckley, R.J.; Lightman, S.L. The role of conjunctival epithelial cells in chronic ocular allergic disease. *Exp. Eye Res.* **1998**, *67*, 491–500, doi:10.1006/exer.1998.0528.
152. Irkeç, M.; Bozkurt, B. Epithelial cells in ocular allergy. *Curr. Allergy Asthma Rep.* **2003**, *3*, 352–357, doi:10.1007/S11882-003-0098-2.
153. Stahl, J.L.; Barney, N.P. Ocular allergic disease. *Curr. Opin. Allergy Clin. Immunol.* **2004**, *4*, 455–459, doi:10.1097/00130832-200410000-00020.
154. Zhan, H.; Towler, H.M.A.T.; Calder, V.L. The Immunomodulatory Role of Human Conjunctival Epithelial Cells. *Invest. Ophthalmol. Vis. Sci.* **2003**, *44*, 3906–3910, doi:10.1167/IOVS.02-0665.
155. Calonge, M.; Enriquez-de-Salamanca, A. The role of the conjunctival epithelium in ocular allergy. *Curr. Opin. Allergy Clin. Immunol.* **2005**, *5*, 441–445, doi:10.1097/01.ALL.0000182545.75842.77.
156. Diebold, Y.; Calonge, M.; De Salamanca, A.E.; Callejo, S.; Corrales, R.M.; Saez, V.; Siemasko, K.F.; Stern, M.E. Characterization of a spontaneously immortalized cell line (IOBA-NHC) from normal human conjunctiva. *Invest. Ophthalmol. Vis. Sci.* **2003**, *44*, 4263–4274, doi:10.1167/IOVS.03-0560.
157. Enriquez-de-Salamanca, A.; Calder, V.; Gao, J.; Galatowicz, G.; Garcia-Vazquez, C.; Fernandez, I.; Stern, M.E.; Diebold, Y.; Calonge, M. Cytokine responses by conjunctival epithelial cells: An in vitro model of ocular inflammation. *Cytokine* **2008**, *44*, 160–167, doi:10.1016/j.cyto.2008.07.007.
158. Araki-Sasaki, K.; Ohashi, Y.; Sasabe, T.; Hayashi, K.; Watanabe, H.; Tano, Y.; Handa, H. An SV40-immortalized human corneal epithelial cell line and its characterization. *Investig. Ophthalmol. Vis. Sci.* **1995**, *36*, 614–621.
159. Abengozar-Vela, A.; Calonge, M.; Stern, M.E.; Gonzalez-Garcia, M.J.; Enriquez-De-Salamanca, A. Quercetin and resveratrol decrease the inflammatory and oxidative responses in human ocular surface epithelial cells. *Investig. Ophthalmol. Vis. Sci.* **2015**, *56*, 2709–2719, doi:10.1167/iovs.15-16595.
160. Pauloin, T.; Dutot, M.; Joly, F.; Warnet, J.; Rat, P. High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. *Mol. Vis.* **2009**, *15*, 577–583.
161. Dursun, D.; Wang, M.; Monroy, D.; Li, D.Q.; Lokeshwar, B.L.; Stern, M.E.; Pflugfelder, S.C. A mouse model of keratoconjunctivitis sicca. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 632–638.
162. Barabino, S.; Shen, L.L.; Chen, L.; Rashid, S.; Rolando, M.; Dana, M.R. The controlled-environment chamber: A new mouse model of dry eye. *Investig. Ophthalmol. Vis. Sci.* **2005**, *46*, 2766–2771, doi:10.1167/IOVS.04-1326.
163. Gehlsen, U.; Braun, T.; Notara, M.; Krösser, S.; Steven, P. A semifluorinated alkane (F4H5) as novel carrier for cyclosporine A: a promising therapeutic and prophylactic option for topical treatment of dry eye. *Graefe's Arch. Clin. Exp. Ophthalmol.* **2017**, *255*, 767–775, doi:10.1007/S00417-016-3572-Y.
164. Steven, P.; Schwab, S.; Kiesewetter, A.; Saban, D.R.; Stern, M.E.; Gehlsen, U. Disease-specific expression of conjunctiva associated lymphoid tissue (CALT) in mouse models of dry eye disease and ocular allergy. *Int. J. Mol. Sci.* **2020**, *21*, 1–14, doi:10.3390/IJMS21207514.
165. Gehlsen, U.; Stary, D.; Maass, M.; Riesner, K.; Musial, G.; Stern, M.E.; Penack, O.; Steven, P. Ocular Graft-versus-Host Disease in a Chemotherapy-Based Minor-Mismatch Mouse Model Features Corneal (Lymph-) Angiogenesis. *Int. J. Mol. Sci.* **2021**, *22*, 6191, doi:10.3390/IJMS22126191.
166. Barabino, S.; Dana, M.R. Animal Models of Dry Eye: A Critical Assessment of Opportunities and Limitations. *Invest. Ophthalmol. Vis. Sci.* **2004**, *45*, 1641–1646, doi:10.1167/IOVS.03-1055.

167. Nakamura, S.; Shibuya, M.; Nakashima, H.; Imagawa, T.; Uehara, M.; Tsubota, K. d-β-Hydroxybutyrate Protects against Corneal Epithelial Disorders in a Rat Dry Eye Model with Jogging Board. *Invest. Ophthalmol. Vis. Sci.* **2005**, *46*, 2379–2387, doi:10.1167/IOVS.04-1344.
168. Fujihara, T.; Nagano, T.; Nakamura, M.; Shirasawa, E. Establishment of a rabbit short-term dry eye model. *J. Ocul. Pharmacol. Ther.* **1995**, *11*, 503–508, doi:10.1089/JOP.1995.11.503.
169. Nakamura, S.; Okada, S.; Umeda, Y.; Saito, F. Development of a Rabbit Model of Tear Film Instability and Evaluation of Viscosity of Artificial Tear Preparations. *Cornea* **2004**, *23*, 390–397, doi:10.1097/00003226-200405000-00015.
170. Gilbard, J.P.; Rossi, S.R.; Heyda, K.G. Tear film and ocular surface changes after closure of the meibomian gland orifices in the rabbit. *Ophthalmology* **1989**, *96*, 1180–1186, doi:10.1016/S0161-6420(89)32753-9.
171. Robinson, C.P.; Yamachika, S.; Bounous, D.I.; Brayer, J.; Jonsson, R.; Holmdahl, R.; Peck, A.B.; Humphreys-Beher, M.G. A novel NOD-derived murine model of primary Sjögren's syndrome. *Arthritis Rheumatol.* **1998**, *41*, 150–156, doi:10.1002/1529-0131(199801)41:1<150::AID-ART18>3.0.CO;2-T.
172. Li, H.; Dai, M.; Zhuang, Y. A T Cell Intrinsic Role of Id3 in a Mouse Model for Primary Sjögren's Syndrome. *Immunity* **2004**, *21*, 551–560, doi:10.1016/J.IMMUNI.2004.08.013.
173. Golden, J.P.; Demaro, J.A.; Osborne, P.A.; Milbrandt, J.; Johnson, E.M. Expression of Neurturin, GDNF, and GDNF Family-Receptor mRNA in the Developing and Mature Mouse. *Exp. Neurol.* **1999**, *158*, 504–528, doi:10.1006/EXNR.1999.7127.
174. Guo, Z.; Song, D.H.; Azzarolo, A.M.; Schechter, J.E.; Warren, D.W.; Wood, R.L.; Mircheff, A.K.; Kaslow, H.R. Autologous Lacrimal–Lymphoid Mixed-cell Reactions Induce Dacryoadenitis in Rabbits. *Exp. Eye Res.* **2000**, *71*, 23–31, doi:10.1006/EXER.2000.0855.
175. Burgalassi, S.; Panichi, L.; Chetoni, P.; Saettone, M.F.; Boldrini, E. Development of a simple dry eye model in the albino rabbit and evaluation of some tear substitutes. *Ophthalmic Res.* **1999**, *31*, 229–235, doi:10.1159/000055537.
176. Kaswan, R.L.; Salisbury, M.A.; Ward, D.A. Spontaneous Canine Keratoconjunctivitis Sicca: A Useful Model for Human Keratoconjunctivitis Sicca: Treatment with Cyclosporine Eye Drops. *Arch. Ophthalmol.* **1989**, *107*, 1210–1216, doi:10.1001/ARCHOPHT.1989.01070020276038.
177. Kaswan, R.L.; Martin, C.L.; Dawe, D.L. Keratoconjunctivitis sicca: immunological evaluation of 62 canine cases. *Am. J. Vet. Res.* **1985**, *46*, 376.
178. Kaswan, R.; Pappas, C.; Wall, K.; Hirsh, S.G. Survey of Canine Tear Deficiency in Veterinary Practice. *Adv. Exp. Med. Biol.* **1998**, *438*, 931–939, doi:10.1007/978-1-4615-5359-5_132.
179. Kaswan, R. Characteristics of a Canine Model of KCS: Effective Treatment with Topical Cyclosporine. *Adv. Exp. Med. Biol.* **1994**, *350*, 583–594, doi:10.1007/978-1-4615-2417-5_99.
180. Maitchouk, D.Y.; Beuerman, R.W.; Ohta, T.; Stern, M.; Varnell, R.J. Tear production after unilateral removal of the main lacrimal gland in squirrel monkeys. *Arch. Ophthalmol.* **2000**, *118*, 246–252, doi:10.1001/ARCHOPHT.118.2.246.
181. Huang, W.; Tourmouzis, K.; Perry, H.; Honkanen, R.; Rigas, B. Animal models of dry eye disease: Useful, varied and evolving (Review). *Exp. Ther. Med.* **2021**, *22*, 1394, doi:10.3892/ETM.2021.10830.
182. Jones, L.; Downie, L.E.; Korb, D.; Benitez-del-Castillo, J.M.; Dana, R.; Deng, S.X.; Dong, P.N.; Geerling, G.; Hida, R.Y.; Liu, Y.; et al. TFOS DEWS II Management and Therapy Report. *Ocul. Surf.* **2017**, *15*, 575–628, doi:10.1016/J.JTOS.2017.05.006.
183. Dogru, M.; Nakamura, M.; Shimazaki, J.; Tsubota, K. Changing trends in the treatment of dry-eye disease. *Expert Opin. Investig. Drugs* **2013**, *22*, 1581–1601, doi:10.1517/13543784.2013.838557.
184. Dogru, M.; Tsubota, K. Pharmacotherapy of dry eye. *Expert Opin. Pharmacother.* **2011**, *12*, 325–334, doi:10.1517/14656566.2010.518612.
185. Tong, L.; Petznick, A.; Lee, S.; Tan, J. Choice of artificial tear formulation for patients with dry eye: Where do we start? *Cornea* **2012**, *31*, S32–S36, doi:10.1097/ICO.0B013E318269CB99.
186. Pucker, A.D.; Ng, S.M.; Nichols, J.J. Over the counter (OTC) artificial tear drops for dry eye syndrome. *Cochrane Database Syst. Rev.* **2016**, *2016*, CD009729, doi:10.1002/14651858.CD009729.PUB2.
187. Hondur, A.M.; Akcam, H.T.; Karaca, E.E.; Yazici Eroglu, H.; Aydin, B. Autologous Serum Eye Drops Accelerate Epithelial Healing After LASEK. *Curr. Eye Res.* **2015**, *41*, 15–19, doi:10.3109/02713683.2014.1002051.
188. Ziakas, N.G.; Boboridis, K.G.; Terzidou, C.; Naoumidi, T.L.; Mikropoulos, D.; Georgiadou, E.N.; Georgiadis, N.S. Long-term follow up of autologous serum treatment for recurrent corneal erosions. *Clin. Experiment. Ophthalmol.* **2010**, *38*, 683–687, doi:10.1111/J.1442-9071.2010.02304.X.
189. Kaido, M.; Ishida, R.; Dogru, M.; Tsubota, K. Visual function changes after punctal occlusion with the treatment of short but type of dry eye. *Cornea* **2012**, *31*, 1009–1013, doi:10.1097/ICO.0B013E31823F8CFC.
190. Chen, F.; Wang, J.; Chen, W.; Shen, M.; Xu, S.; Lu, F. Upper Punctal Occlusion versus Lower Punctal Occlusion in Dry Eye. *Invest. Ophthalmol. Vis. Sci.* **2010**, *51*, 5571–5577, doi:10.1167/IOVS.09-5097.
191. Shen, G.; Qi, Q.; Ma, X. Effect of Moisture Chamber Spectacles on Tear Functions in Dry Eye Disease. *Optom. Vis. Sci.* **2016**, *93*, 158–164, doi:10.1097/OPX.0000000000000778.
192. Fujihara, T.; Murakami, T.; Nagano, T.; Nakamura, M.; Nakata, K. INS365 Suppresses Loss of Corneal Epithelial Integrity by Secretion of Mucin-like Glycoprotein in a Rabbit Short-term Dry Eye Model. *J. Ocul. Pharmacol. Ther.* **2004**, *18*, 363–370, doi:10.1089/10807680260218524.
193. Shigeyasu, C.; Hirano, S.; Akune, Y.; Yamada, M. Diquafosol Tetrasodium Increases the Concentration of Mucin-like Substances in Tears of Healthy Human Subjects. *Curr. Eye Res.* **2015**, *40*, 878–883, doi:10.3109/02713683.2014.967871.

194. Ding, J.; Sullivan, D.A. The Effects of Insulin-like Growth Factor 1 and Growth Hormone on Human Meibomian Gland Epithelial Cells. *JAMA Ophthalmol.* **2014**, *132*, 593–599, doi:10.1001/JAMAOPHTHALMOL.2013.8295.
195. Cohn, G.S.; Corbett, D.; Tenen, A.; Coroneo, M.; McAlister, J.; Craig, J.P.; Gray, T.; Kent, D.; Murray, N.; Petsoglou, C.; et al. Randomized, Controlled, Double-Masked, Multicenter, Pilot Study Evaluating Safety and Efficacy of Intranasal Neurostimulation for Dry Eye Disease. *Invest. Ophthalmol. Vis. Sci.* **2019**, *60*, 147–153, doi:10.1167/IOVS.18-23984.
196. Craig, J.P.; Friedman, N.; Torkildsen, G.; Ackermann, M. Symptom Reduction with Oculeve Intranasal Stimulator on Exposure to Environmental Challenge in Dry Eye: A Randomized, Controlled, Crossover Trial (E-abstract). *Optom. Vis. Sci.* **2016**, *94*, 160057.
197. Tsifetaki, N.; Kitsos, G.; Paschides, C.A.; Alamanos, Y.; Eftaxias, V.; Voulgari, P. V.; Psilas, K.; Drosos, A.A. Oral pilocarpine for the treatment of ocular symptoms in patients with Sjögren's syndrome: A randomised 12 week controlled study. *Ann. Rheum. Dis.* **2003**, *62*, 1204–1207, doi:10.1136/ARD.2002.003889.
198. Ono, M.; Takamura, E.; Shinozaki, K.; Tsumura, T.; Hamano, T.; Yagi, Y.; Tsubota, K. Therapeutic effect of cevimeline on dry eye in patients with Sjögren's syndrome: A randomized, double-blind clinical study. *Am. J. Ophthalmol.* **2004**, *138*, 6–17, doi:10.1016/J.AJO.2004.02.010.
199. McCulley, J.P.; Shine, W.E. Changing concepts in the diagnosis and management of blepharitis. *Cornea* **2000**, *19*, 650–658, doi:10.1097/00003226-200009000-00010.
200. Key, J.E. A comparative study of eyelid cleaning regimens in chronic blepharitis. *CLAO J.* **1996**, *22*, 209–212.
201. Romero, J.M.; Biser, S.A.; Perry, H.D.; Levinson, D.H.; Doshi, S.J.; Terraciano, A.; Donnenfeld, E.D. Conservative treatment of meibomian gland dysfunction. *Eye Contact Lens* **2004**, *30*, 14–19, doi:10.1097/01.ICL.0000095229.01957.89.
202. Greiner, J. V. Long-Term (3 Year) Effects of a Single Thermal Pulsation System Treatment on Meibomian Gland Function and Dry Eye Symptoms. *Eye Contact Lens* **2016**, *42*, 99–107, doi:10.1097/ICL.0000000000000166.
203. Murtaza, F.; Toameh, D.; Al-Habib, S.; Maini, R.; Chiu, H.H.; Tam, E.S.; Somani, S. Safety and Efficacy of BroadBand Intense Pulsed Light Therapy for Dry Eye Disease with Meibomian Gland Dysfunction. *Clin. Ophthalmol.* **2021**, *15*, 3983–3991, doi:10.2147/OPTH.S331289.
204. Jackson, W.B. Management of dysfunctional tear syndrome: A Canadian consensus. *Can. J. Ophthalmol.* **2009**, *44*, 385–394, doi:10.3129/I09-015.
205. Holzchuh, F.G.; Hida, R.Y.; Moscovici, B.K.; Villa Albers, M.B.; Santo, R.M.; Kara-José, N.; Holzchuh, R. Clinical treatment of ocular Demodex folliculorum by systemic ivermectin. *Am. J. Ophthalmol.* **2011**, *151*, 1030–1034, doi:10.1016/J.AJO.2010.11.024.
206. Nogueira Filho, P.A.; Hazarbassanov, R.M.; Grisolia, A.B.D.; Pazos, H.B.; Kaiserman, I.; Gomes, J.A.P. The efficacy of oral ivermectin for the treatment of chronic blepharitis in patients tested positive for Demodex spp. *Br. J. Ophthalmol.* **2011**, *95*, 893–895, doi:10.1136/BJO.2010.201194.
207. Katz, J.; Kaufman, H.E. Corneal Exposure During Sleep (Nocturnal Lagophthalmos). *Arch. Ophthalmol.* **1977**, *95*, 449–453, doi:10.1001/ARCHOPHT.1977.04450030091011.
208. Foulks, G.N.; Harvey, T.; Sundar Raj, C. V. Therapeutic contact lenses: The role of high-Dk lenses. Ophthalmol. *Clin. North Am.* **2003**, *16*, 455–461, doi:10.1016/S0896-1549(03)00045-2.
209. Sainz De La Maza Serra, M.; Simon Castellvi, C.; Kabbani, O. Nonpreserved topical steroids and lacrimal punctal occlusion for severe keratoconjunctivitis sicca. *Arch. Soc. Esp. Oftalmol.* **2000**, *75*, 751–756.
210. Avunduk, A.M.; Avunduk, M.C.; Varnell, E.D.; Kaufman, H.E. The comparison of efficacies of topical corticosteroids and nonsteroidal anti-inflammatory drops on dry eye patients: A clinical and immunocytochemical study. *Am. J. Ophthalmol.* **2003**, *136*, 593–602, doi:10.1016/S0002-9394(03)00326-X.
211. Pflugfelder, S.C.; Maskin, S.L.; Anderson, B.; Chodosh, J.; Holland, E.J.; De Paiva, C.S.; Bartels, S.P.; Micuda, T.; Proskin, H.M.; Vogel, R. A randomized, double-masked, placebo-controlled, multicenter comparison of loteprednol etabonate ophthalmic suspension, 0.5%, and placebo for treatment of keratoconjunctivitis sicca in patients with delayed tear clearance. *Am. J. Ophthalmol.* **2004**, *138*, 444–457, doi:10.1016/J.AJO.2004.04.052.
212. Lee, H.K.; Ryu, I.H.; Seo, K.Y.; Hong, S.; Kim, H.C.; Kim, E.K. Topical 0.1% Prednisolone Lowers Nerve Growth Factor Expression in Keratoconjunctivitis Sicca Patients. *Ophthalmology* **2006**, *113*, 198–205, doi:10.1016/J.OPHTHA.2005.09.033.
213. Yang, C.; Sun, W.; Gu, Y. A clinical study of the efficacy of topical corticosteroids on dry eye. *J. Zhejiang Univ. Sci. B* **2006**, *7*, 675–678, doi:10.1631/JZUS.2006.B0675.
214. Jonisch, J.; Steiner, A.; Udell, I.J. Preservative-Free Low-Dose Dexamethasone for the Treatment of Chronic Ocular Surface Disease Refractory to Standard Therapy. *Cornea* **2010**, *29*, 723–726.
215. Sheppard, J.D.; Donnenfeld, E.D.; Holland, E.J.; Slonim, C.B.; Solomon, R.; Solomon, K.D.; Mcdonald, M.B.; Perry, H.D.; Lane, S.S.; Pflugfelder, S.C.; et al. Effect of Loteprednol Etabonate 0.5% on Initiation of Dry Eye Treatment With Topical Cyclosporine 0.05%. *Eye Contact Lens Sci. Clin. Pract.* **2014**, *40*, 289–296, doi:10.1097/ICL.0000000000000049.
216. Kheirkhah, A.; Dohlman, T.H.; Amparo, F.; Arnoldner, M.A.; Jamali, A.; Hamrah, P.; Dana, R. Effects of Corneal Nerve Density on the Response to Treatment in Dry Eye Disease. *Ophthalmology* **2015**, *122*, 662–668, doi:10.1016/J.OPHTHA.2014.11.006.
217. Lin, T.; Gong, L. Topical Fluorometholone Treatment for Ocular Dryness in Patients With Sjögren Syndrome A Randomized Clinical Trial in China. *Med.* **2015**, *94*, e551, doi:10.1097/MD.0000000000000551.
218. Pinto-Fraga, J.; Lopez-Miguel, A.; Gonzalez-Garcia, M.J.; Fernandez, I.; Lopez-De-La-Rosa, A.; Enriquez-De-Salamanca, A.; Stern, M.E.; Calonge, M. Topical Fluorometholone Protects the Ocular Surface of Dry Eye Patients from Desiccating Stress: A Randomized Controlled Clinical Trial. *Ophthalmology* **2016**, *123*, 141–153, doi:10.1016/j.ophtha.2015.09.029.

219. Marsh, P.; Pflugfelder, S.C. Topical nonpreserved methylprednisolone therapy for keratoconjunctivitis sicca in Sjögren syndrome. *Ophthalmology* **1999**, *106*, 811–816, doi:10.1016/S0161-6420(99)90171-9.
220. McGhee, C.N.J.; Dean, S.; Danesh-Meyer, H. Locally Administered Ocular Corticosteroids. *Drug Saf.* **2012**, *25*, 33–55, doi:10.2165/00002018-200225010-00004.
221. LeHoang, P. The Gold Standard of Noninfectious Uveitis: Corticosteroids. *Dev. Ophthalmol.* **2012**, *51*, 7–28, doi:10.1159/000336676.
222. Mataftsi, A.; Narang, A.; Moore, W.; Nischal, K.K. Do reducing regimens of fluorometholone for paediatric ocular surface disease cause glaucoma? *Br. J. Ophthalmol.* **2011**, *95*, 1531–1533, doi:10.1136/BJO.2010.192773.
223. Bilgihan, K.; Gürelik, G.; Akata, F.; Hasanreisoglu, B. Fluorometholone-Induced Cataract after Photoref ractive Keratectomy. *Ophthalmologica* **1997**, *211*, 394–396, doi:10.1159/000310839.
224. Moawad, P.; Shamma, R.; Hassanein, D.; Ragab, G.; El Zawahry, O. Evaluation of the effect of topical tacrolimus 0.03% versus cyclosporine 0.05% in the treatment of dry eye secondary to Sjogren syndrome. *Eur. J. Ophthalmol.* **2021**, 1120672121992680, doi:10.1177/1120672121992680.
225. Barbarino, J.M.; Staatz, C.E.; Venkataramanan, R.; Klein, T.E.; Altman, R.B. PharmGKB summary: cyclosporine and tacrolimus pathways. *Pharmacogenet. Genomics* **2013**, *23*, 563–585, doi:10.1097/FPC.0b013e328364db84.
226. Donnenfeld, E.; Pflugfelder, S.C. Topical Ophthalmic Cyclosporine: Pharmacology and Clinical Uses. *Surv. Ophthalmol.* **2009**, *54*, 321–338, doi:10.1016/j.survophthal.2009.02.002.
227. Matsuda, S.; Koyasu, S. Mechanisms of action of cyclosporine. *Immunopharmacology* **2000**, *47*, 119–125, doi:10.1016/S0162-3109(00)00192-2.
228. Jewett, A.; Tseng, H.C. Immunotherapy. In *Pharmacology and Therapeutics for Dentistry: Seventh Edition*; Mosby: Maryland Heights, MO, USA, 2017; pp. 504–529 ISBN 978-0-323-39307-2.
229. Lee, T.H.; Chun, G.T.; Chang, Y.K.; Lee, J.; Agathos, S.N. Bioprocess engineering considerations in cyclosporin A fermentation by immobilized fungus *Tolypocladium inflatum*. *Prog. Biotechnol.* **1996**, *11*, 402–409, doi:10.1016/S0921-0423(96)80055-4.
230. Gao, J.; Sana, R.; Calder, V.; Calonge, M.; Lee, W.; Wheeler, L.A.; Stern, M.E. Mitochondrial permeability transition pore in inflammatory apoptosis of human conjunctival epithelial cells and T cells: effect of cyclosporin A. *Invest. Ophthalmol. Vis. Sci.* **2013**, *54*, 4717–4733, doi:10.1167/IOVS.13-11681.
231. Ambroziak, A.M.; Szaflik, J.; Szaflik, J.P.; Ambroziak, M.; Witkiewicz, J.; Skopinski, P. Immunomodulation on the ocular surface: a review. *Cent. J. Immunol.* **2016**, *41*, 195–208, doi:10.5114/CEJI.2016.60995.
232. Meyer, S.; Kohler, N.G.; Joly, A. Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-kappaB activation. *FEBS Lett.* **1997**, *413*, 354–358, doi:10.1016/S0014-5793(97)00930-7.
233. Matsuda, S.; Moriguchi, T.; Koyasu, S.; Nishida, E. T lymphocyte activation signals for interleukin-2 production involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. *J. Biol. Chem.* **1998**, *273*, 12378–12382, doi:10.1074/JBC.273.20.12378.
234. Ames, P.; Galor, A. Cyclosporine ophthalmic emulsions for the treatment of dry eye: a review of the clinical evidence. *Clin. Investig. (Lond).* **2015**, *5*, 267–285, doi:10.4155/CLI.14.135.
235. Kumar, N.S.; Khosla, R.; Makharia, G.K. Cyclosporine in steroid refractory acute severe colitis. *Trop. Gastroenterol.* **2014**, *35*, S21–S28, doi:10.7869/TG.179.
236. Gremese, E.; Ferraccioli, G. Benefit/risk of cyclosporine in rheumatoid arthritis. *Clin. Exp. Rheumatol.* **2004**, *22*, S101–S107.
237. Haddad, E.; McAlister, V.C.; Renouf, E.; Malthaner, R.; Kjaer, M.S.; Gluud, L.L. Cyclosporin versus tacrolimus for liver transplanted patients (Review). *Cochrane Database Syst Rev* **2006**, *4*, CD005161, doi:10.1002/14651858.CD005161.pub2.
238. Gorantla, V.S.; Barker, J.H.; Jones, J.W.; Prabhune, K.; Maldonado, C.; Granger, D.K. Immunosuppressive agents in transplantation: Mechanisms of action and current anti-rejection strategies. *Microsurgery* **2000**, *20*, 420–429.
239. Utine, C.A.; Stern, M.; Akpek, E.K. Clinical Review: Topical Ophthalmic Use of Cyclosporin A. *Ocul. Immunol. Inflamm.* **2010**, *18*, 352–361, doi:10.3109/09273948.2010.498657.
240. Keklikci, U.; Dursun, B.; Cingu, A.K. Topical Cyclosporine A 0.05% Eyedrops in the Treatment of Vernal Keratoconjunctivitis – Randomized Placebo-Controlled Trial. *Adv. Clin. Exp. Med.* **2014**, *23*, 455–461.
241. Gonzalez-Lopez, J.J.; Lopez-Alcalde, J.; Morcillo Laiz, R.; Fernandez Buenaga, R.; Rebolleda Fernandez, G. Topical cyclosporine for atopic keratoconjunctivitis. *Cochrane database Syst. Rev.* **2012**, doi:10.1002/14651858.CD009078.PUB2.
242. Kim, B.Y.; Riaz, K.M.; Bakhtiari, P.; Chan, C.C.; Welder, J.D.; Holland, E.J.; Basti, S.; Djalilian, A.R. Medically Reversible Limbal Stem Cell Disease: Clinical Features and Management Strategies. *Ophthalmology* **2014**, *121*, 2053–2058, doi:10.1016/J.OPHTHA.2014.04.025.
243. Sullivan, B.D.; Crews, L.A.; Baris baris sönmez, B.; De La Paz, M.F.; Comert, E.; Charoenrook, V.; De Araujo, A.L.; Pepose, J.S.; Berg, M.S.; Kosheleff, V.P.; et al. Clinical Utility of Objective Tests for Dry Eye Disease: Variability Over Time and Implications for Clinical Trials and Disease Management. *Cornea* **2012**, *31*, 1000–1008.
244. Kunert, K.S.; Tisdale, A.S.; Stern, M.E.; Smith, J.A.; Gipson, I.K. Analysis of Topical Cyclosporine Treatment of Patients With Dry Eye Syndrome: Effect on Conjunctival Lymphocytes. *Arch. Ophthalmol.* **2000**, *118*, 1489–1496, doi:10.1001/ARCHOPHT.118.11.1489.
245. Brignole, F.; Pisella, P.J.; Goldschild, M.; De Saint Jean, M.; Goguel, A.; Baudouin, C. Flow cytometric analysis of inflammatory markers in conjunctival epithelial cells of patients with dry eyes. *Investig. Ophthalmol. Vis. Sci.* **2000**, *41*, 1356–1363.
246. Sall, K.; Stevenson, O.D.; Mundorf, T.K.; Reis, B.L. Two multicenter, randomized studies of the efficacy and safety of cyclosporine ophthalmic emulsion in moderate to severe dry eye disease. *Ophthalmology* **2000**, *107*, 631–639, doi:10.1016/S0161-6420(99)00176-1.

247. Pflugfelder, S.C.; De Paiva, C.S.; Villarreal, A.L.; Stern, M.E. Effects of Sequential Artificial Tear and Cyclosporine Emulsion Therapy on Conjunctival Goblet Cell Density and Transforming Growth Factor-b2 Production. *Cornea* **2008**, *27*, 64–69.
248. Perry, H.D.; Solomon, R.; Donnenfeld, E.D.; Perry, A.R.; Wittpenn, J.R.; Greenman, H.E.; Savage, H.E. Evaluation of topical cyclosporine for the treatment of dry eye disease. *Arch. Ophthalmol.* **2008**, *126*, 1046–1050, doi:10.1001/ARCHOPHT.126.8.1046.
249. Wilson, S.E.; Perry, H.D. Long-term Resolution of Chronic Dry Eye Symptoms and Signs after Topical Cyclosporine Treatment. *Ophthalmology* **2007**, *114*, 76–79, doi:10.1016/J.OPHTHA.2006.05.077.
250. Leonardi, A.; Setten, G. Van; Amrane, M.; Ismail, D.; Garrigue, J.-S.; Figueiredo, F.C.; Baudouin, C. Efficacy and Safety of 0.1% Cyclosporine a Cationic Emulsion in the Treatment of Severe Dry Eye Disease: A Multicenter Randomized Trial. *Eur. J. Ophthalmol.* **2016**, *26*, 287–296, doi:10.5301/EJO.5000779.
251. Lallemand, F.; Felt-Baeyens, O.; Besseghir, K.; Behar-Cohen, F.; Gurny, R. Cyclosporine A delivery to the eye: A pharmaceutical challenge. *Eur. J. Pharm. Biopharm.* **2003**, *56*, 307–318, doi:10.1016/S0939-6411(03)00138-3.
252. Marlin, S.D.; Springer, T.A. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* **1987**, *51*, 813–819, doi:10.1016/0092-8674(87)90104-8.
253. Grakoui, A.; Bromley, S.K.; Sumen, C.; Davis, M.M.; Shaw, A.S.; Allen, P.M.; Dustin, M. The Immunological Synapse: A Molecular Machine Controlling T Cell Activation. *Science (80-.).* **1999**, *285*, 221–227.
254. Zhong, M.; Gadek, T.R.; Bui, M.; Shen, W.; Burnier, J.; Barr, K.J.; Hanan, E.J.; Oslob, J.D.; Yu, C.H.; Zhu, J.; et al. Discovery and Development of Potent LFA-1/ICAM-1 Antagonist SAR 1118 as an Ophthalmic Solution for Treating Dry Eye. *ACS Med. Chem. Lett.* **2012**, *3*, 203–206, doi:10.1021/ML2002482.
255. Sun, Y.; Zhang, R.; Gadek, T.R.; O'Neill, C.A.; Pearlman, E. Corneal Inflammation Is Inhibited by the LFA-1 Antagonist, Lifitegrast (SAR 1118). *J. Ocul. Pharmacol. Ther.* **2013**, *29*, 395–402, doi:10.1089/JOP.2012.0102.
256. Perez, V.L.; Pflugfelder, S.C.; Zhang, S.; Shojaei, A.; Haque, R. Lifitegrast, a Novel Integrin Antagonist for Treatment of Dry Eye Disease. *Ocul. Surf.* **2016**, *14*, 207–215, doi:10.1016/J.JTOS.2016.01.001.
257. Donnenfeld, E.D.; Karpecki, P.M.; Majmudar, P.A.; Nichols, K.K.; Raychaudhuri, A.; Roy, M.; Semba, C.P. Safety of Lifitegrast Ophthalmic Solution 5.0% in Patients With Dry Eye Disease: A 1-Year, Multicenter, Randomized, Placebo-Controlled Study. *Cornea* **2016**, *35*, 741–748.
258. Novartis Withdraws Marketing Application of Dry Eye Drug Xiidra in Europe - Eyewire+ Available online: https://eyewire.news/news/novartis-pulls-eu-filing-seeking-approval-of-xiidra-for-dry-eye-disease (accessed on Nov 11, 2021).
259. Liu, Y.; Kam, W.R.; Ding, J.; Sullivan, D.A. Can tetracycline antibiotics duplicate the ability of azithromycin to stimulate human meibomian gland epithelial cell differentiation? *Cornea* **2015**, *34*, 342, doi:10.1097/ICO.0000000000000351.
260. Bakar, Ö.; Demirçay, Z.; Gürbüz, O. Therapeutic potential of azithromycin in rosacea. *Int. J. Dermatol.* **2004**, *43*, 151–154, doi:10.1111/J.1365-4632.2004.01958.X.
261. Bakar, Ö.; Demircay, Z.; Toker, E.; Çakir, S. Ocular signs, symptoms and tear function tests of papulopustular rosacea patients receiving azithromycin. *J. Eur. Acad. Dermatology Venereol.* **2009**, *23*, 544–549, doi:10.1111/J.1468-3083.2009.03132.X.
262. Fernandez-Obregon, A. Oral use of azithromycin for the treatment of acne rosacea. *Arch. Dermatol.* **2004**, *140*, 489–490, doi:10.1001/ARCHDERM.140.4.489.
263. Voils, S.A.; Evans, M.E.; Lane, M.T.; Schosser, R.H.; Rapp, R.P. Use of Macrolides and Tetracyclines for Chronic Inflammatory Diseases. *Ann. Pharmacother.* **2005**, *39*, 86–94, doi:10.1345/APH.1E282.
264. Giamarellos-Bourboulis, E.J. Macrolides beyond the conventional antimicrobials: a class of potent immunomodulators. *Int. J. Antimicrob. Agents* **2008**, *31*, 12–20, doi:10.1016/J.IJANTIMICAG.2007.08.001.
265. Labro, M.T. Anti-inflammatory activity of macrolides: a new therapeutic potential? *J. Antimicrob. Chemother.* **1998**, *41*, 37–46, doi:10.1093/JAC/41.SUPPL_2.37.
266. Rubin, B.K. Immunomodulatory properties of macrolides: Overview and historical perspective. *Am. J. Med.* **2004**, *117*, 2S-4S.
267. Modi, S.; Harting, M.S.; Rosen, T. Azithromycin as an alternative rosacea therapy when tetracyclines prove problematic. *J. drugs dermatology* **2008**, *7*, 898–899.
268. Wladis, E.J.; Bradley, E.A.; Bilyk, J.R.; Yen, M.T.; Mawn, L.A.; Harris, J. Oral Antibiotics for Meibomian Gland-Related Ocular Surface Disease: A Report by the American Academy of Ophthalmology. *Ophthalmology* **2016**, *123*, 492–496, doi:10.1016/J.OPHTHA.2015.10.062.
269. Fadlallah, A.; El Rami, H.; Fahd, D.; Dunia, I.; Bejjani, R.; Chlela, E.; Waked, N.; Jabbour, E.; Fahed, S. Azithromycin 1.5% ophthalmic solution: efficacy and treatment modalities in chronic blepharitis. *Arq. Bras. Oftalmol.* **2012**, *75*, 178–182, doi:10.1590/S0004-27492012000300006.
270. Shine, W.E.; McCulley, J.P.; Pandya, A.G. Minocycline effect on meibomian gland lipids in meibomianitis patients. *Exp. Eye Res.* **2003**, *76*, 417–420, doi:10.1016/S0014-4835(03)00005-8.
271. Ta, C.N.; Shine, W.E.; McCulley, J.P.; Pandya, A.; Trattler, W.; Norbury, J.W. Effects of minocycline on the ocular flora of patients with acne rosacea or seborrheic blepharitis. *Cornea* **2003**, *22*, 545–548, doi:10.1097/00003226-200308000-00011.
272. Dougherty, J.; McCulley, J.; Silvany, R.; Meyer, D.R. The role of tetracycline in chronic blepharitis. Inhibition of lipase production in staphylococci. *Invest. Ophthalmol. Vis. Sci.* **1991**, *32*, 2970–2975.
273. Solomon, A.; Rosenblatt, M.; Li, D.Q.; Liu, Z.; Monroy, D.; Ji, Z.; Lokeshwar, B.L.; Pflugfelder, S.C. Doxycycline inhibition of interleukin-1 in the corneal epithelium. *Investig. Ophthalmol. Vis. Sci.* **2000**, *41*, 2544–2557, doi:10.1016/S0002-9394(00)00755-8.
274. Li, Y.; Kuang, K.; Yerxa, B.; Wen, Q.; Rosskothen, H.; Fischbarg, J. Rabbit conjunctival epithelium transports fluid, and P2Y22 receptor agonists stimulate Cl- and fluid secretion. *Am. J. Physiol. Physiol.* **2001**, *281*, C595-602, doi:10.1152/AJPCELL.2001.281.2.C595.

275. Li, D.Q.; Luo, L.; Chen, Z.; Kim, H.S.; Song, X.J.; Pflugfelder, S.C. JNK and ERK MAP kinases mediate induction of IL-1β, TNF-α and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp. Eye Res.* **2006**, *82*, 588–596, doi:10.1016/J.EXER.2005.08.019.
276. Paiva, C.S. De; Corrales, R.M.; Villarreal, A.L.; Farley, W.; Li, D.-Q.; Stern, M.E.; Pflugfelder, S.C. Apical Corneal Barrier Disruption in Experimental Murine Dry Eye Is Abrogated by Methylprednisolone and Doxycycline. *Invest. Ophthalmol. Vis. Sci.* **2006**, *47*, 2847–2856, doi:10.1167/IOVS.05-1281.
277. De Paiva, C.S.; Corrales, R.M.; Villarreal, A.L.; Farley, W.J.; Li, D.Q.; Stern, M.E.; Pflugfelder, S.C. Corticosteroid and doxycycline suppress MMP-9 and inflammatory cytokine expression, MAPK activation in the corneal epithelium in experimental dry eye. *Exp. Eye Res.* **2006**, *83*, 526–535, doi:10.1016/J.EXER.2006.02.004.
278. Gilbard, J.P. The scientific context and basis of the pharmacologic management of dry eyes. *Ophthalmol. Clin. North Am.* **2005**, *18*, 475–484, doi:10.1016/J.OHC.2005.07.003.
279. Cragg, G.M.; Newman, D.J. Natural Products: A Continuing Source of Novel Drug Leads. *Biochim. Biophys. Acta* **2013**, 1830, 3670–3695, doi:10.1016/J.BBAGEN.2013.02.008.
280. Cragg, G.M.; Newman, D.J.; Snader, K.M. Natural products in drug discovery and development. *J. Nat. Prod.* **1997**, *60*, 52–60, doi:10.1021/NP9604893.
281. Samuelsson, G. Drugs of Natural Origin: A Textbook of Pharmacognosy. 5th Edition ; Swedish Pharmaceutical Press: Stockholm, 1999.
282. Oberlies, N.H.; Kroll, D.J. Camptothecin and taxol: historic achievements in natural products research. *J. Nat. Prod.* **2004**, *67*, 129–135, doi:10.1021/NP030498T.
283. Kuphcan, S.M. Drugs from Natural Products—Plant Sources. In *Drug Discovery Science and Development in a Changing Society; American Chemical Society*: Washington, DC, 1971; Vol. 16, pp. 1–13.
284. Newman, D.J.; Cragg, G.M.; Kingston, D.G.I. Natural Products as Pharmaceuticals and Sources for Lead Structures. *Pract. Med. Chem. Fourth Ed.* **2015**, 101–139, doi:10.1016/B978-0-12-417205-0.00005-5.
285. Poulev, A.; O'Neal, J.M.; Logendra, S.; Pouleva, R.B.; Timeva, V.; Garvey, A.S.; Gleba, D.; Jenkins, I.S.; Halpern, B.T.; Kneer, R.; et al. Elicitation, a New Window into Plant Chemodiversity and Phytochemical Drug Discovery. *J. Med. Chem.* **2003**, *46*, 2542–2547, doi:10.1021/JM020359T.
286. Cordell, G.A. Biodiversity and drug discovery--a symbiotic relationship. *Phytochemistry* **2000**, *55*, 463–480, doi:10.1016/S0031-9422(00)00230-2.
287. Sorkin, B.C.; Kuszak, A.J.; Williamson, J.S.; Hopp, D.C.; Betz, J.M. The Challenge of Reproducibility and Accuracy in Nutrition Research: Resources and Pitfalls. *Adv. Nutr.* **2016**, *7*, 383–389, doi:10.3945/AN.115.010595.
288. Raskin, I.; Ribnicky, D.M.; Komarnytsky, S.; Ilic, N.; Poulev, A.; Borisjuk, N.; Brinker, A.; Moreno, D.A.; Ripoll, C.; Yakoby, N.; et al. Plants and human health in the twenty-first century. *Trends Biotechnol.* **2002**, *20*, 522–531, doi:10.1016/S0167-7799(02)02080-2.
289. Sahoo, N.; Choudhury, K.; Manchikanti, P. Manufacturing of Biodrugs: Need for Harmonization in Regulatory Standards. *BioDrugs* **2012**, *23*, 217–229, doi:10.2165/11317110-000000000-00000.
290. Koparde, A.A.; Doijad, R.C.; Magdum, C.S. Natural Products in Drug Discovery. *Pharmacogn. - Med. Plants* **2019**, doi:10.5772/INTECHOPEN.82860.
291. Kinghorn, A.D. Pharmacognosy in the 21st century. *J. Pharm. Pharmacol.* **2001**, *53*, 135–148, doi:10.1211/0022357011775334.
292. Wolfender, J.L.; Ndjoko, K.; Hostettmann, K. Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. *J. Chromatogr. A* **2003**, *1000*, 437–455, doi:10.1016/S0021-9673(03)00303-0.
293. Khan, R.A. Natural products chemistry: The emerging trends and prospective goals. *Saudi Pharm. J.* **2018**, *26*, 739–753, doi:10.1016/J.JSPS.2018.02.015.
294. Fowler, M.W. Plants, medicines and man. *J. Sci. Food Agric.* **2006**, *86*, 1797–1804, doi:10.1002/JSFA.2598.
295. Newman, D.J. Natural Products as Leads to Potential Drugs: An Old Process or the New Hope for Drug Discovery? *J. Med. Chem.* **2008**, *51*, 2589–2599, doi:10.1021/JM0704090.
296. Rates, S.M.K. Plants as source of drugs. *Toxicon* **2001**, *39*, 603–613, doi:10.1016/S0041-0101(00)00154-9.
297. Avila-Sosa, R.; Navarro-Cruz, A.R.; Vera-Lopez, O.; Hernandez-Carranza, P.; Ochoa-Velasco, C.E. Ultraviolet Light Stimulation of Bioactive Compounds with Antioxidant Capacity of Fruits and Vegetables. In *Plant Secondary Metabolites*; Apple Academic Press: Mistwell Crescent, Oakville, 2016; Vol. 2, pp. 281–306 ISBN 9781315366319.
298. Naczk, M.; Shahidi, F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1523–1542, doi:10.1016/J.JPBA.2006.04.002.
299. Ochoa-Velasco, C.E.; Avila-Sosa, R.; Navarro-Cruz, A.R.; Lopez-Malo, A.; Palou, E. Biotic and Abiotic Factors to Increase Bioactive Compounds in Fruits and Vegetables. In *Food Bioconversion*; Academic Press: London, UK, 2017; Vol. 2, pp. 317–349.
300. Roleira, F.M.F.; Varela, C.L.; Costa, S.C.; Rio, E.; Tavares-Da-Silva, J. Phenolic Derivatives From Medicinal Herbs and Plant Extracts: Anticancer Effects and Synthetic Approaches to Modulate Biological Activity. In *Studies in Natural Products Chemistry*; Atta-ur-Rahman, Ed.; Elsevier: London, UK, 2018; pp. 115–156 ISBN 9780444640574.
301. Pandey, K.B.; Rizvi, S.I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* **2009**, *2*, 270–278, doi:10.4161/OXIM.2.5.9498.
302. Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006**, *99*, 191–203, doi:10.1016/J.FOODCHEM.2005.07.042.

303. Ozcan, T.; Akpinar-Bayizit, A.; Yilmaz-Ersan, L.; Delikanli, B. Phenolics in Human Health. *Int. J. Chem. Eng. Appl.* **2014**, *5*, 393–396, doi:10.7763/IJCEA.2014.V5.416.
304. Quideau, S.; Deffieux, D.; Douat-Casassus, C.; Pouységu, L. Plant polyphenols: Chemical properties, biological activities, and synthesis. *Angew. Chemie - Int. Ed.* **2011**, *50*, 586–621, doi:10.1002/ANIE.201000044.
305. Besnard, G.; Rubio De Casas, R.; Christin, P.A.; Vargas, P. Phylogenetics of Olea (Oleaceae) based on plastid and nuclear ribosomal DNA sequences: Tertiary climatic shifts and lineage differentiation times. *Ann. Bot.* **2009**, *104*, 143–160, doi:10.1093/AOB/MCP105.
306. Green, P.S. A revision of *olea* l. (Oleaceae). *Kew Bull.* **2002**, *57*, 91–140, doi:10.2307/4110824.
307. Galili, E.; Weinstein-Evron, M.; Zohary, D. Appearance of Olives in Submerged Neolithic Sites Along the Carmel Coast. *J. Isr. Prehist. Soc.* **1989**, *22*, 95–97.
308. Kapellakis, I.E.; Tsagarakis, K.P.; Crowther, J.C. Olive oil history, production and by-product management. *Rev. Environ. Sci. Biotechnol.* **2008**, *7*, 1–26, doi:10.1007/s11157-007-9120-9.
309. Polymerou-Kamilakis, A. The Culture of the Olive Tree (Mediterranean World). In *Olive Oil: Chemistry and Technology*; Boskou, D., Ed.; AOCS Press: IL, USA, 2006; pp. 1–12.
310. Ray, N.B.; Lam, N.T.; Luc, R.; Bonvino, N.P.; Karagiannis, T.C. Cellular and Molecular Effects of Bioactive Phenolic Compounds in Olives and Olive Oil. In *Olive and Olive Oil Bioactive Constituents*; Boskou, D., Ed.; AOCS Press: IL, USA, 2015; pp. 53–91.
311. Alarcon de la Lastra, C.; Barranco, M.D.; Motilva, V.; Herrerias, J.M. Mediterranean diet and health: biological importance of olive oil. *Curr. Pharm. Des.* **2001**, *7*, 933–950, doi:10.2174/1381612013397654.
312. Hertog, M.G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med.* **1995**, *155*, 381–386, doi:10.1001/ARCHINTE.1995.00430040053006.
313. Visioli, F.; Galli, C. Biological properties of olive oil phytochemicals. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 209–221, doi:10.1080/10408690290825529.
314. Martin-Moreno, J. The role of olive oil in lowering cancer risk: Is this real gold or simply pinchbeck? *J. Epidemiol. Community Health* **2000**, *54*, 726, doi:10.1136/JECH.54.10.726.
315. Singer, P.; Jaeger, W.; Berger, I.; Barleben, H.; Wirth, M.; Richter-Heinrich, E.; Voigt, S.; Gödicke, W. Effects of dietary oleic, linoleic and alpha-linolenic acids on blood pressure, serum lipids, lipoproteins and the formation of eicosanoid precursors in patients with mild essential hypertension. *J. Hum. Hypertens.* **1990**, *4*, 227–233.
316. Seppänen-Laakso, T.; Vanhanen, H.; Laakso, I.; Kohtamäki, H.; Viikari, J. Replacement of Margarine on Bread by Rapeseed and Olive Oils: Effects on Plasma Fatty Acid Composition and Serum Cholesterol. *Ann. Nutr. Metab.* **1993**, *37*, 161–174, doi:10.1159/000177765.
317. Aviram, M.; Eias, K. Dietary Olive Oil Reduces Low-Density Lipoprotein Uptake by Macrophages and Decreases the Susceptibility of the Lipoprotein to Undergo Lipid Peroxidation. *Ann. Nutr. Metab.* **1993**, *37*, 75–84, doi:10.1159/000177753.
318. Blekas, G.; Psomiadou, E.; Tsimidou, M.; Boskou, D. On the importance of total polar phenols to monitor the stability of Greek virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 340–346.
319. Trovato, A.; Forestieri, A.M.; Iauk, L.; Barbera, R.; Mnoforte, M.T.; Galati, E.M. Hypoglycemic activity of different extracts of Olea europaea L. in the rats. *Plantes Med. Phyther.* **1993**, *26*, 300–308.
320. Petroni, A.; Blasevich, M.; Papini, N.; Salami, M.; Sala, A.; Galli, C. Inhibition of leukocyte leukotriene B4 production by an olive oil-derived phenol identified by mass-spectrometry. *Thromb. Res.* **1997**, *87*, 315–322.
321. Visioli, F.; Bellosta, S.; Galli, C. Cardioprotective properties of olive oil-derived polyphenols. *Atherosclerosis* **1997**, *134*, 336.
322. Owen, R.W.; Giacosa, A.; Hull, W.E.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer* **2000**, *36*, 1235–1247, doi:10.1016/S0959-8049(00)00103-9.
323. Owen, R.W.; Giacosa, A.; Hull, W.E.; Haubner, R.; Würtele, G.; Spiegelhalder, B.; Bartsch, H. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol.* **2000**, *1*, 107–112, doi:10.1016/S1470-2045(00)00015-2.
324. Visioli, F.; Caruso, D.; Galli, C.; Viappiani, S.; Galli, G.; Sala, A. Olive oils rich in natural catecholic phenols decrease isoprostane excretion in humans. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 797–799, doi:10.1006/BBRC.2000.3879.
325. Visioli, F.; Galli, C.; Bornet, F.; Mattei, A.; Patelli, R.; Galli, G.; Caruso, D. Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett.* **2000**, *468*, 159–160, doi:10.1016/S0014-5793(00)01216-3.
326. Vissers, M.N.; Zock, P.L.; Wiseman, S.A.; Meyboom, S.; Katan, M.B. Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers. *Eur. J. Clin. Nutr.* **2001**, *55*, 334–341, doi:10.1038/SJ.EJCN.1601161.
327. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance . *EFSA J.* **2011**, *9*, 2033.
328. Bonvino, N.P.; Liang, J.; Mccord, E.D.; Zafiris, E.; Benetti, N.; Ray, N.B.; Hung, A.; Boskou, D.; Karagiannis, T.C. OliveNet TM : a comprehensive library of compounds from Olea europaea. *Database* **2018**, *2018*, 1–9, doi:10.1093/database/bay016.
329. Kougan, G.B.; Tabopda, T.; Kuete, V.; Verpoorte, R. Simple Phenols, Phenolic Acids, and Related Esters from the Medicinal Plants of Africa. In *Medicinal Plant Research in Africa: Pharmacology and Chemistry*; Elsevier: London, UK, 2013; pp. 225–249.
330. Murkovic, M. Phenolic compounds. In *Encyclopedia of Food Sciences and Nutrition*; Elsevier: London, UK, 2003; pp. 4507–4514.
331. Alam, M.A.; Subhan, N.; Hossain, H.; Hossain, M.; Reza, H.M.; Rahman, M.M.; Ullah, M.O. Hydroxycinnamic acid derivatives: a potential class of natural compounds for the management of lipid metabolism and obesity. *Nutr. Metab. (Lond).* **2016**, *13*, 1–13, doi:10.1186/S12986-016-0080-3.

332. Brito Arias, M. Synthesis and Characterization of Glycosides; Springer: Singapore, Singapore, 2007; ISBN 978-0-387-26251-2.
333. Evans, W.C. Miscellaneous isoprenoids. In *Trease and Evans' Pharmacognosy*: Sixteenth Edition; W.B. Saunders: London, UK, 2009; pp. 333–346 ISBN 978-0-7020-2933-2.
334. secoiridoid - Wiktionary Available online: https://en.wiktionary.org/wiki/secoiridoid (accessed on Oct 19, 2021).
335. Wu, H.; Guo, J.; Chen, S.; Liu, X.; Zhou, Y.; Zhang, X.; Xu, X. Recent developments in qualitative and quantitative analysis of phytochemical constituents and their metabolites using liquid chromatography–mass spectrometry. *J. Pharm. Biomed. Anal.* **2013**, *72*, 267–291, doi:10.1016/J.JPBA.2012.09.004.
336. de Souza Farias, S.A.; da Costa, K.S.; Martins, J.B.L. Analysis of Conformational, Structural, Magnetic, and Electronic Properties Related to Antioxidant Activity: Revisiting Flavan, Anthocyanidin, Flavanone, Flavonol, Isoflavone, Flavone, and Flavan-3-ol. *ACS Omega* **2021**, *6*, 8918, doi:10.1021/ACSOMEGA.0C06156.
337. Zitterman, A. Dietary Fiber | Bran. In *Encyclopedia of Food Sciences and Nutrition*; Academic Press, Elsevier: London, UK, 2003; pp. 1844–1850.
338. Pragst, F.; Yegles, M. Determination of fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) in hair: A promising way for retrospective detection of alcohol abuse during pregnancy? *Ther. Drug Monit.* **2008**, *30*, 255–263, doi:10.1097/FTD.0B013E318167D602.
339. Chanioti, S.; Tzia, C. Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: Oil recovery, unsaponifiable matter, total phenol content and antioxidant activity. *LWT - Food Sci. Technol.* **2017**, *79*, 178–189, doi:10.1016/J.LWT.2017.01.029.
340. Rodrigues, F.; Pimentel, F.B.; Oliveira, M.B.P.P. Olive by-products: Challenge application in cosmetic industry. *Ind. Crops Prod.* **2015**, *70*, 116–124, doi:10.1016/j.indcrop.2015.03.027.
341. Romero-Garcia, J.M.; Nino, L.; Martinez-Patino, C.; Alvarez, C.; Castro, E.; Negro, M.J. Biorefinery based on olive biomass. State of the art and future trends. *Bioresour. Technol.* **2014**, *159*, 421–432, doi:10.1016/j.biortech.2014.03.062.
342. *British Pharmacopoeia*; Medicines and Healthcare products Regulatory Agency: London, UK, 2021;
343. *The United States Pharmacopeia*; United States Pharmacopeial Convention: Rockville (MD), 2019;
344. *European Pharmacopeia (10th edition)*; European Pharmacopoeia Commission.; European Directorate for the Quality of Medicines & Healthcare: Strasbourg, 2020;
345. Rasouli, H.; Hosseini Mazinani, M.; Haghbeen, K. Benefits and challenges of olive biophenols: a perspective. In *Olives and Olive Oil in Health and Disease Prevention (Second Edition)*; Academic Press: London, UK, 2021; pp. 489–503.
346. Nardini, M.; Pisu, P.; Gentili, V.; Natella, F.; Di Felice, M.; Piccolella, E.; Scaccini, C. Effect of caffeic acid on tert-butyl hydroperoxide-induced oxidative stress in U937. *Free Radic. Biol. Med.* **1998**, *25*, 1098–1105, doi:10.1016/S0891-5849(98)00180-4.
347. Abdel-Wahab, M.H.; El-Mahdy, M.A.; Abd-Ellah, M.F.; Helal, G.K.; Khalifa, F.; Hamada, F.M.A. Influence of p-coumaric acid on doxorubicin-induced oxidative stress in rat's heart. *Pharmacol. Res.* **2003**, *48*, 461–465, doi:10.1016/S1043-6618(03)00214-7.
348. Sawa, T.; Nakao, M.; Akaike, T.; Ono, K.; Maeda, H. Alkylperoxyl Radical-Scavenging Activity of Various Flavonoids and Other Phenolic Compounds: Implications for the Anti-Tumor-Promoter Effect of Vegetables. *J. Agric. Food Chem.* **1999**, *47*, 397–402, doi:10.1021/jf980765e.
349. Daels-Rakotoarison, D.A.; Seidel, V.; Gressier, B.; Brunet, C.; Tillequin, F.; Bailleul, F.; Luyckx, M.; Dine, T.; Cazin, M.; Cazin, J.-C. Neurosedative and Antioxidant Activities of Phenylpropanoids from Ballota nigra. *Arzneimittelforschung* **2011**, *50*, 16–23.
350. Janbaz, K.H.; Saeed, S.A.; Gilani, A.H. Protective effect of rutin on paracetamol- and CCl4-induced hepatotoxicity in rodents. *Fitoterapia* **2002**, *73*, 557–563, doi:10.1016/S0367-326X(02)00217-4.
351. De La Puerta, R.; Gutierrez, V.R.; Hoult, J.R.S. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* **1999**, *57*, 445–449, doi:10.1016/S0006-2952(98)00320-7.
352. Rotelli, A.E.; Guardia, T.; Juarez, A.O.; De La Rocha, N.E.; Pelzer, L.E. Comparative study of flavonoids in experimental models of inflammation. *Pharmacol. Res.* **2003**, *48*, 601–606, doi:10.1016/S1043-6618(03)00225-1.
353. Diaz, A.M.; Abad, M.J.; Fernandez, L.; Silvan, A.M.; De Santos, J.; Bermejo, P. Phenylpropanoid glycosides from Scrophularia scorodonia: *In vitro* anti-inflammatory activity. *Life Sci.* **2004**, *74*, 2515–2526, doi:10.1016/J.LFS.2003.10.008.
354. Aziz, N.; Farag, S.; Mousa, L.A.; Abo-Zaid, M.A. Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios* **1998**, *93*, 43–54.
355. Fleming, H.P.; Walter, W.M.; Etchells, J.L. Antimicrobial Properties of Oleuropein and Products of Its Hydrolysis from Green Olives. *Appl. Microbiol.* **1973**, *26*, 777–782, doi:10.1128/AEM.26.5.777-782.1973.
356. Renis, H. *In vitro* antiviral activity of calcium elenolate. *Antimicrob. Agents Chemother.* **1969**, *9*, 167–172.
357. Quiles, J.L.; Farquharson, A.J.; Simpson, D.K.; Grant, I.; Wahle, K.W.J. Olive oil phenolics: effects on DNA oxidation and redox enzyme mRNA in prostate cells. *Br. J. Nutr.* **2002**, *88*, 225–234, doi:10.1079/BJN2002620.
358. Li, J.; Zheng, Y.; Zhou, H.; Su, B.; Zheng, R. Differentiation of human gastric adenocarcinoma cell line MGc80-3 induced by verbascoside. *Planta Med.* **1997**, *63*, 499–502, doi:10.1055/S-2002-20255.
359. Hirose, M.; Takahashi, S.; Ogawa, K.; Futakuchi, M.; Shirai, T. Phenolics: blocking agents for heterocyclic amine-induced carcinogenesis. *Food Chem. Toxicol.* **1999**, *37*, 985–992, doi:10.1016/S0278-6915(99)00092-7.
360. Meyer, A.S.; Heinonen, M.; Frankel, E.N. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chem.* **1998**, *61*, 71–75, doi:10.1016/S0308-8146(97)00100-3.
361. Pennacchio, M.; Alexander, E.; Syah, Y.M.; Ghisalberti, E.L. The effect of verbascoside on cyclic 3',5'-adenosine monophosphate levels in isolated rat heart. *Eur. J. Pharmacol.* **1996**, *305*, 169–171, doi:10.1016/0014-2999(96)00153-7.

362. Kang, D.G.; Lee, Y.S.; Kim, H.J.; Lee, Y.M.; Lee, H.S. Angiotensin converting enzyme inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. *J. Ethnopharmacol.* **2003**, *89*, 151–154, doi:10.1016/S0378-8741(03)00274-5.
363. Liu, M.J.; Li, J.X.; Guo, H.Z.; Lee, K.M.; Qin, L.; Chan, K.M. The effects of verbascoside on plasma lipid peroxidation level and erythrocyte membrane fluidity during immobilization in rabbits: A time course study. *Life Sci.* **2003**, *73*, 883–892, doi:10.1016/S0024-3205(03)00354-0.
364. Hou, L.; Zhou, B.; Yang, L.; Liu, Z.L. Inhibition of human low density lipoprotein oxidation by flavonols and their glycosides. *Chem. Phys. Lipids* **2004**, *129*, 209–219, doi:10.1016/J.CHEMPHYSLIP.2004.02.001.
365. Takeda, H.; Tsuji, M.; Inazu, M.; Egashira, T.; Matsumiya, T. Rosmarinic acid and caffeic acid produce antidepressive-like effect in the forced swimming test in mice. *Eur. J. Pharmacol.* **2002**, *449*, 261–267, doi:10.1016/S0014-2999(02)02037-X.
366. Speroni, E.; Guerra, M.C.; Minghetti, A.; Crespi-Perellino, N.; Pasini, P.; Piazza, F.; Roda, A. Oleuropein Evaluated *In Vitro* and *In Vivo* as an Antioxidant. *Phytother. Res* **1998**, *12*, 98–100, doi:10.1002/(SICI)1099-1573(1998)12:1.
367. Saija, A.; Trombetta, D.; Tomaino, A.; Lo Cascio, R.; Princi, P.; Uccella, N.; Bonina, F.; Castelli, F. "*In vitro*" evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. *Int. J. Pharm.* **1998**, *166*, 123–133, doi:10.1016/S0378-5173(98)00018-0.
368. Al-Qarawi, A.A.; Al-Damegh, M.A.; ElMougy, S.A. Effect of freeze dried extract of *Olea europaea* on the pituitary-thyroid axis in rats. *Phyther. Res.* **2002**, *16*, 286–287, doi:10.1002/PTR.855.
369. Saenz, M.T.; Garcia, M.D.; Ahumada, M.C.; Ruiz, V. Cytostatic activity of some compounds from the unsaponifiable fraction obtained from virgin olive oil. *Farmaco* **1998**, *53*, 448–449, doi:10.1016/S0014-827X(98)00043-3.
370. Kubo, I.; Matsumoto, A. Molluscicides from olive Olea europaea and their efficient isolation by counter-current chromatographies. *J. Agric. Food Chem.* **2002**, *32*, 687–688, doi:10.1021/JF00123A067.
371. Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G.F.; Galli, C. Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thromb. Res.* **1995**, *78*, 151–160, doi:10.1016/0049-3848(95)00043-7.
372. Andrikopoulos, N.K.; Antonopoulou, S.; Kaliora, A.C. Oleuropein Inhibits LDL Oxidation Induced by Cooking Oil Frying By-products and Platelet Aggregation Induced by Platelet-Activating Factor. *LWT - Food Sci. Technol.* **2002**, *35*, 479–484, doi:10.1006/FSTL.2002.0893.
373. Ruiz-Gutiérrez, V.; Muriana, F.J.G.; Maestro, R.; Graciani, E. Oleuropein on lipid and fatty acid composition of rat heart. *Nutr. Res.* **1995**, *15*, 37–51, doi:10.1016/0271-5317(95)91651-R.
374. Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life Sci.* **1998**, *62*, 541–546, doi:10.1016/S0024-3205(97)01150-8.
375. Duarte, J.; Pérez, O.; Zarzuelo, A.; Jiménez, J.; Pérez-Vizcaino, F.; Tamargo, J. Effects of oleuropeoside in isolated guinea-pig atria. *Planta Med.* **1993**, *59*, 318–322, doi:10.1055/S-2006-959690.
376. Polzonetti, V.; Egidi, D.; Vita, A.; Vincenzetti, S.; Natalini, P. Involvement of oleuropein in (some) digestive metabolic pathways. *Food Chem.* **2004**, *88*, 11–15, doi:10.1016/J.FOODCHEM.2004.01.029.
377. Furneri, P.M.; Marino, A.; Saija, A.; Uccella, N.; Bisignano, G. *In vitro* antimycoplasmal activity of oleuropein. *Int. J. Antimicrob. Agents* **2002**, *20*, 293–296, doi:10.1016/S0924-8579(02)00181-4.
378. Bisignano, G.; Tomaino, A.; Cascio, R. Lo; Crisafi, G.; Uccella, N.; Saija, A. On the *In-vitro* Antimicrobial Activity of Oleuropein and Hydroxytyrosol. *J. Pharm. Pharmacol.* **2010**, *51*, 971–974, doi:10.1211/0022357991773258.
379. Lee-Huang, S.; Zhang, L.; Huang, P.L.; Chang, Y.T.; Huang, P.L. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 1029–1037, doi:10.1016/S0006-291X(03)01292-0.
380. Czerwinska, M.; Kiss, A.K.; Naruszewicz, M. A comparison of antioxidant activities of oleuropein and its dialdehydic derivative from olive oil, oleacein. *Food Chem.* **2012**, *131*, 940–947, doi:10.1016/j.foodchem.2011.09.082.
381. Filipek, A.; Czerwinska, M.E.; Kiss, A.K.; Wrzosek, M.; Naruszewicz, M. Oleacein enhances anti-inflammatory activity of human macrophages by increasing CD163 receptor expression. *Phytomedicine* **2015**, *22*, 1255–1261, doi:10.1016/j.phymed.2015.10.005.
382. Cirmi, S.; Celano, M.; Lombardo, G.E.; Maggisano, V.; Procopio, A.; Russo, D.; Navarra, M. Oleacein inhibits STAT3, activates the apoptotic machinery, and exerts anti-metastatic effects in the SH-SY5Y human neuroblastoma cells. *Food Funct.* **2020**, *11*, 3271–3279, doi:10.1039/d0fo00089b.
383. Lepore, S.M.; Maggisano, V.; Bulotta, S.; Mignogna, C.; Arcidiacono, B.; Procopio, A.; Brunetti, A.; Russo, D.; Celano, M. Oleacein Prevents High Fat Diet-Induced Adiposity and Ameliorates Some Biochemical Parameters of Insulin Sensitivity in Mice. *Nutrients* **2019**, *11*, 1829, doi:10.3390/NU11081829.
384. Visioli, F.; Galli, C.; Galli, G.; Caruso, D. Biological activities and metabolic fate of olive oil phenols. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 677–684, doi:10.1002/1438-9312(200210)104:9/10<677::AID-EJLT677>3.0.CO;2-M.
385. Manna, C.; Galletti, P.; Maisto, G.; Cucciolla, V.; D'Angelo, S.; Zappia, V. Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cells. *FEBS Lett.* **2000**, *470*, 341–344, doi:10.1016/S0014-5793(00)01350-8.
386. Léger, C.L.; Kadiri-Hassani, N.; Descomps, B. Decreased Superoxide Anion Production in Cultured Human Promonocyte Cells (THP-1) Due to Polyphenol Mixtures from Olive Oil Processing Wastewaters. *J. Agric. Food Chem.* **2000**, *48*, 5061–5067, doi:10.1021/JF991349C.
387. Damiani, E.; Belaid, C.; Carloni, P.; Greci, L. Comparison of antioxidant activity between aromatic indolinonic nitroxides and natural and synthetic antioxidants. *Free Radic. Res.* **2003**, *37*, 731–741, doi:10.1080/1071576031000102169.

388. Giovannini, C.; Straface, E.; Modesti, D.; Coni, E.; Cantafora, A.; De Vincenzi, M.; Malorni, W.; Masella, R. Tyrosol, the major olive oil biophenol, protects against oxidized-LDL- induced injury in Caco-2 cells. *J. Nutr.* **1999**, *129*, 1269–1277, doi:10.1093/JN/129.7.1269.
389. Soler-Rivas, C.; Espin, J.C.; Wichers, H.J. Review Oleuropein and related compounds. *J. Sci. Food Agric.* **2000**, *80*, 1013–1023, doi:10.1002/(SICI)1097-0010(20000515)80:7.
390. Visioli, F.; Vinceri, F.F.; Galli, C. "Waste waters" from olive oil production are rich in natural antioxidants. *Experientia* **1995**, *51*, 32–34.
391. Casalino, E.; Calzaretti, G.; Sblano, C.; Landriscina, V.; Tecce, M.F.; Landriscina, C. Antioxidant effect of hydroxytyrosol (DPE) and Mn2+ in liver of cadmium-intoxicated rats. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2002**, *133*, 625–632, doi:10.1016/S1532-0456(02)00180-1.
392. Visioli, F.; Caruso, D.; Plasmati, E.; Patelli, R.; Mulinacci, N.; Romani, A.; Galli, G.; Galli, C. Hydroxytyrosol, as a component of olive mill waste water, is dose-dependently absorbed and increases the antioxidant capacity of rat plasma. *Free Radic. Res.* **2001**, *34*, 301–305, doi:10.1080/10715760100300271.
393. Marrugat, J.; Covas, M.I.; Fito, M.; Schröder, H.; Miro-Casas, E.; Gimeno, E.; Lopez-Sabater, M.C.; De La Torre, R.; Farré, M. Effects of differing phenolic content in dietary olive oil on lipids and LDL oxidation: A randomized controlled trial. *Eur. J. Nutr.* **2004**, *43*, 140–147, doi:10.1007/S00394-004-0452-8.
394. Deiana, M.; Aruoma, O.I.; Bianchi, M.D.L.P.; Spencer, J.P.E.; Kaur, H.; Halliwell, B.; Aeschbach, R.; Banni, S.; Dessi, M.A.; Corongiu, F.P. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radic. Biol. Med.* **1999**, *26*, 762–769, doi:10.1016/S0891-5849(98)00231-7.
395. Fabiani, R.; De Bartolomeo, A.; Rosignoli, P.; Servili, M.; Montedoro, G.F.; Morozzi, G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G1 cell cycle arrest and apoptosis. *Eur. J. Cancer Prev.* **2002**, *11*, 351–358, doi:10.1097/00008469-200208000-00006.
396. Della Ragione, F.; Cucciolla, V.; Borriello, A.; Pietra, V. Della; Pontoni, G.; Racioppi, L.; Manna, C.; Galletti, P.; Zappia, V. Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome c-dependent apoptosis. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 733–739, doi:10.1006/BBRC.2000.3875.
397. Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M.A.; Cristinzio, G. Antibacterial polyphenols from olive oil mill waste waters. *J. Appl. Bacteriol.* **1995**, *79*, 393–398, doi:10.1111/J.1365-2672.1995.TB03153.X.
398. Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A Highly Convenient Synthesis of Hydroxytyrosol and Its Recovery from Agricultural Waste Waters. *J. Agric. Food Chem.* **1999**, *47*, 1745–1748, doi:10.1021/JF9809030.
399. Kumar, N.; Gorai, B.; Gupta, S.; Shiva; Goel, N. Extrapolation of hydroxytyrosol and its analogues as potential anti-inflammatory agents. *J. Biomol. Struct. Dyn.* **2021**, *39*, 5588–5599, doi:10.1080/07391102.2020.1792990.
400. Granner, T.; Maloney, S.; Antecka, E.; Correa, J.A.; Burnier, M.N. 3,4 Dihydroxyphenyl Ethanol Reduces Secretion of Angiogenin in Human Retinal Pigment Epithelial Cells. *Br. J. Ophthalmol.* **2013**, *97*, 371–374, doi:10.1136/bjophthalmol-2012-302002.
401. Liu, Z.; Sun, L.; Zhu, L.; Jia, X.; Li, X.; Jia, H.; Wang, Y.; Weber, P.; Long, J.; Liu, J. Hydroxytyrosol protects retinal pigment epithelial cells from acrolein-induced oxidative stress and mitochondrial dysfunction. *J. Neurochem.* **2007**, *103*, 2690–2700, doi:10.1111/j.1471-4159.2007.04954.x.
402. Zou, X.; Feng, Z.; Li, Y.; Wang, Y.; Wertz, K.; Weber, P.; Fu, Y.; Liu, J. Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: Activation of Nrf2 and JNK-p62/SQSTM1 pathways. *J. Nutr. Biochem.* **2012**, *23*, 994–1006, doi:10.1016/j.jnutbio.2011.05.006.
403. Zhu, L.; Liu, Z.; Feng, Z.; Hao, J.; Shen, W.; Li, X.; Sun, L.; Sharman, E.; Wang, Y.; Wertz, K.; et al. Hydroxytyrosol protects against oxidative damage by simultaneous activation of mitochondrial biogenesis and phase II detoxifying enzyme systems in retinal pigment epithelial cells. *J. Nutr. Biochem.* **2010**, *21*, 1089–1098, doi:10.1016/j.jnutbio.2009.09.006.
404. Gonzalez-Correa, J.A.; Rodriguez-Pérez, M.D.; Marquez-Estrada, L.; Lopez-Villodres, J.A.; Reyes, J.J.; Rodriguez-Gutierrez, G.; Fernandez-Bolanos, J.; De La Cruz, J.P. Neuroprotective Effect of Hydroxytyrosol in Experimental Diabetic Retinopathy: Relationship with Cardiovascular Biomarkers. *J. Agric. Food Chem.* **2018**, *66*, 637–644, doi:10.1021/acs.jafc.7b05063.
405. Liu, J.; Schalch, W.; Wang-Schmidt, Y.; Wertz, K. US2010130620A1 - Novel use of hydroxytyrosol and olive extracts/concentrates containing it 2010.
406. Hao, C.; Zhou, Z.; Bin, W.; Peng, C.; Xianggen, W. CN105902486 - Hydroxytyrosol eye drops for effectively targeting trigeminal ganglion and preparation method thereof 2016.
407. Xiao, L.; Shende, J.; Xinyan, G. CN103316032 (A) - A hydroxytyrosol composition and applications thereof 2013.
408. Simsek, T.; Altinisik, U.; Ersan, I.; Sahin, H.; Altinisik, B.; Erbas, M.; Pala, Ç. Prevention of intraocular pressure elevation with oleuropein rich diet in rabbits, during the general anaesthesia. *Springerplus* **2016**, *5*, 952, doi:10.1186/s40064-016-2402-3.
409. FAOSTAT. Food and Agriculture Organization Database Available online: http://www.fao.org/home/search/en/?q=Olive mill wastes production (accessed on May 7, 2021).
410. Paraskeva, P.; Diamadopoulos, E. Technologies for olive mill wastewater (OMW) treatment: a review. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 1475–1485, doi:10.1002/JCTB.1553.
411. Balatsouras, G. *I Elaiourgia (The Olive Mill)*; Idiotiki Ekdosi: Athens, Greece, 1999; Vol. 4; ISBN 9789609028721.
412. Gimeno, E.; Castellote, A.I.; Lamuela-Raventos, R.M.; La Torre, M.C.D.; Lopez-Sabater, M.C. The effects of harvest and extraction methods on the antioxidant content (phenolics, α-tocopherol, and β-carotene) in virgin olive oil. *Food Chem.* **2002**, *78*, 207–211, doi:10.1016/S0308-8146(01)00399-5.

413. Kiritsakis, A.; Nanos, G.D.; Polymenopulos, Z.; Thomai, T.; Sfakiotakis, E.M. Effect of fruit storage conditions on olive oil quality. *J. Am. Oil Chem. Soc.* **1998**, *75*, 721–724, doi:10.1007/S11746-998-0212-7.
414. Kiritsakis, A.K. *Olive Oil: From the Tree to the Table*; Ackman, R., Ed.; 2nd ed.; Food and Nutrition Press: CT, USA, 1999;
415. Souilem, S.; El-Abbassi, A.; Kiai, H.; Hafidi, A.; Sayadi, S.; Galanakis, C.M. Olive oil production sector: environmental effects and sustainability challenges. In *Olive Mill Waste: Recent Advances for Sustainable Management*; Academic Press, 2017; pp. 1–28.
416. Vlyssides, A.G.; Loizides, M.; Karlis, P.K. Integrated strategic approach for reusing olive oil extraction by-products. *J. Clean. Prod.* **2004**, *12*, 603–611, doi:10.1016/S0959-6526(03)00078-7.
417. Demicheli, M.C.; Bontoux, L. Novel technologies for olive oil manufacturing and their incidence on the environment. *Fresenius Environ. Bull.* **1997**, *6*, 240–247.
418. *Analisis de la consideracion como subproducto del orujo graso humedo procedente de la elaboracion de aceite de oliva y destinado a la extraccion de aceite de orujo de oliva*; **2019**. Available online: https://www.miteco.gob.es/es/calidad-y-evaluacion-ambiental/participacion-publica/estudiosubproductoalperujo_191120_tcm30-506764.pdf (accessed on November 5, 2021)
419. Gomez-Munoz, B.; J., D.; Bol, R.; Garca-Ruiz, R. The Compost of Olive Mill Pomace: From a Waste to a Resource - Environmental Benefits of Its Application in Olive Oil Groves. In *Sustainable Development - Authoritative and Leading Edge Content for Environmental Management*; IntechOpen: London, UK, 2012.
420. Seçmeler, Ö.; Güçlü Üstündag, Ö.; Fernandez-Bolanos, J.; Rodriguez-Gutiérrez, G. Effect of subcritical water and steam explosion pretreatments on the recovery of sterols, phenols and oil from olive pomace. *Food Chem.* **2018**, *265*, 298–307, doi:10.1016/J.FOODCHEM.2018.05.088.
421. Roig, A.; Cayuela, M.L.; Sanchez-Monedero, M.A. An overview on olive mill wastes and their valorisation methods. *Waste Manag.* **2006**, *26*, 960–969, doi:10.1016/J.WASMAN.2005.07.024.
422. Sanchez Moral, P.; Ruiz Méndez, M.V. Production of pomace olive oil. *Grasas y Aceites* **2006**, *57*, doi:10.3989/gya.2006.v57.i1.21.
423. Tirado, D.F.; Fuente, E. de la; Calvo, L. A selective extraction of hydroxytyrosol rich olive oil from alperujo. *J. Food Eng.* **2019**, *263*, 409–416, doi:10.1016/j.jfoodeng.2019.07.030.
424. Cayuela, M.L.; Millner, P.; Slovin, J.; Roig, A. Duckweed (Lemna gibba) growth inhibition bioassay for evaluating the toxicity of olive mill wastes before and during composting. *Chemosphere* **2007**, *68*, 1985–1991, doi:10.1016/J.CHEMOSPHERE.2007.02.064.
425. Gullon, P.; Gullon, B.; Astray, G.; Carpena, M.; Fraga-Corral, M.; Prieto, M.A.; Simal-Gandara, J. Valorization of by-products from olive oil industry and added-value applications for innovative functional foods. *Food Res. Int.* **2020**, *137*, 109683, doi:10.1016/J.FOODRES.2020.109683.
426. Dermeche, S.; Nadour, M.; Larroche, C.; Moulti-Mati, F.; Michaud, P. Olive mill wastes: Biochemical characterizations and valorization strategies. *Process Biochem.* **2013**, *48*, 1532–1552, doi:10.1016/j.procbio.2013.07.010.
427. Cioffi, G.; Pesca, M.S.; De Caprariis, P.; Braca, A.; Severino, L.; De Tommasi, N. Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity. *Food Chem.* **2010**, *121*, 105–111, doi:10.1016/J.FOODCHEM.2009.12.013.
428. Chanioti, S.; Tzia, C. Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. *Innov. Food Sci. Emerg. Technol.* **2018**, *48*, 228–239, doi:10.1016/J.IFSET.2018.07.001.
429. Lozano-Sanchez, J.; Giambanelli, E.; Quirantes-Piné, R.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernandez-Gutiérrez, A. Wastes generated during the storage of extra virgin olive oil as a natural source of phenolic compounds. *J. Agric. Food Chem.* **2011**, *59*, 11491–11500, doi:10.1021/jf202596q.
430. Obied, H.K.; Bedgood, D.R.; Prenzler, P.D.; Robards, K. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Anal. Chim. Acta* **2007**, *603*, 176–189, doi:10.1016/j.aca.2007.09.044.
431. Peralbo-Molina, A.; Priego-Capote, F.; Luque De Castro, M.D. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography-tandem mass spectrometry with a quadrupole- quadrupole-time-of-flight mass detector. *J. Agric. Food Chem.* **2012**, *60*, 11542–11550, doi:10.1021/jf302896m.
432. Cardoso, S.M.; Guyot, S.; Marnet, N.; Lopes-da-Silva, J.A.; Renard, C.M.; Coimbra, M.A. Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J. Sci. Food Agric.* **2005**, *85*, 21–32, doi:10.1002/jsfa.1925.
433. Rigane, G.; Ben Salem, R.; Sayadi, S.; M, B. Phenolic composition, isolation, and structure of a new deoxyloganic acid derivative from Dhokar and Gemri-Dhokar olive cultivars. *J. Food Sci.* **2011**, *76*, C965–C973, doi:10.1111/J.1750-3841.2011.02290.X.
434. Alu'datt, M.H.; Alli, I.; Ereifej, K.; Alhamad, M.; Al-Tawaha, A.R.; Rababah, T. Optimisation, characterisation and quantification of phenolic compounds in olive cake. *Food Chem.* **2010**, *123*, 117–122, doi:10.1016/J.FOODCHEM.2010.04.011.
435. Suarez, M.; Romero, M.-P.; Motilva, M.-J. Development of a Phenol-Enriched Olive Oil with Phenolic Compounds from Olive Cake. *J. Agric. Food Chem.* **2010**, *58*, 10396–10403, doi:10.1021/JF102203X.
436. Panja, P. Green extraction methods of food polyphenols from vegetable materials. *Curr. Opin. Food Sci.* **2018**, *23*, 173–182, doi:10.1016/j.cofs.2017.11.012.
437. Meneses, N.G.T.; Martins, S.; Teixeira, J.A.; Mussatto, S.I. Influence of extraction solvents on the recovery of antioxidant phenolic compounds from brewer's spent grains. *Sep. Purif. Technol.* **2013**, *108*, 152–158, doi:10.1016/J.SEPPUR.2013.02.015.
438. Mussatto, S.I.; Ballesteros, L.F.; Martins, S.; Teixeira, J.A. Extraction of antioxidant phenolic compounds from spent coffee grounds. *Sep. Purif. Technol.* **2011**, *83*, 173–179, doi:10.1016/J.SEPPUR.2011.09.036.
439. Chirinos, R.; Rogez, H.; Campos, D.; Pedreschi, R.; Larondelle, Y. Optimization of extraction conditions of antioxidant phenolic compounds from mashua (Tropaeolum tuberosum Ruiz & Pavon) tubers. *Sep. Purif. Technol.* **2007**, *55*, 217–225, doi:10.1016/J.SEPPUR.2006.12.005.

440. Haminiuk, C.W.I.; Plata-Oviedo, M.S.V.; de Mattos, G.; Carpes, S.T.; Branco, I.G. Extraction and quantification of phenolic acids and flavonols from Eugenia pyriformis using different solvents. *J. Food Sci. Technol.* **2014**, *51*, 2866, doi:10.1007/S13197-012-0759-Z.
441. Ashurst, J. V.; Nappe, T.M. Methanol Toxicity. In *StatPearls [Internet]*; StatPearls Publishing: Treasure Island, FL, USA, 2021.
442. Tekin, K.; Hao, N.; Karagoz, S.; Ragauskas, A.J. Ethanol: A Promising Green Solvent for the Deconstruction of Lignocellulose. *ChemSusChem* **2018**, *11*, 3559–3575, doi:10.1002/CSSC.201801291.
443. Sant'Anna, V.; Brandelli, A.; Marczak, L.D.F.; Tessaro, I.C. Kinetic modeling of total polyphenol extraction from grape marc and characterization of the extracts. *Sep. Purif. Technol.* **2012**, *100*, 82–87, doi:10.1016/J.SEPPUR.2012.09.004.
444. Cacace, J.E.; Mazza, G. Optimization of Extraction of Anthocyanins from Black Currants with Aqueous Ethanol. *J. Food Sci.* **2003**, *68*, 240–248, doi:10.1111/J.1365-2621.2003.TB14146.X.
445. Pasrija, D.; Anandharamakrishnan, C. Techniques for Extraction of Green Tea Polyphenols: A Review. *Food Bioprocess Technol.* **2015**, *8*, 935–950, doi:10.1007/S11947-015-1479-Y.
446. Hossain, M.B.; Rawson, A.; Aguilo-Aguayo, I.; Brunton, N.P.; Rai, D.K. Recovery of Steroidal Alkaloids from Potato Peels Using Pressurized Liquid Extraction. *Molecules* **2015**, *20*, 8560–8573, doi:10.3390/MOLECULES20058560.
447. Kohli, R. Removal of Surface Contaminants Using Ionic Liquids. In *Developments in Surface Contamination and Cleaning: Methods of Cleaning and Cleanliness Verification*; William Andrew Publishing, 2013; Vol. 6, pp. 1–63 ISBN 9781437778809.
448. Smith, E.L.; Abbott, A.P.; Ryder, K.S. Deep Eutectic Solvents (DESs) and Their Applications. *Chem. Rev.* **2014**, *114*, 11060–11082, doi:10.1021/CR300162P.
449. Rodrigues, L.A.; Radojcic Redovnikovic, I.; Duarte, A.R.C.; Matias, A.A.; Paiva, A. Low-Phytotoxic Deep Eutectic Systems as Alternative Extraction Media for the Recovery of Chitin from Brown Crab Shells. *ACS Omega* **2021**, *6*, 28729–28741, doi:10.1021/ACSOMEGA.1C03402/SUPPL_FILE/A01C03402_SI_001.PDF.
450. Soxhlet, F. Soxhlet, über gewichtsanalytische Bestimmung des Milchfettes. *Polytech. J.* **1879**, *232*, 461–465.
451. Harwood, L.M.; Moody, C.J. *Experimental organic chemistry*; Wiley-Blackwell Scientific: NJ, USA, 1989; ISBN 0632026677.
452. Zhang, Q.W.; Lin, L.G.; Ye, W.C. Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese Med. (United Kingdom)* **2018**, *13*, 1–26, doi:10.1186/S13020-018-0177-X/FIGURES/13.
453. Teh, S.-S.; Niven, B.E.; Bekhit, A.E.-D.A.; Carne, A.; Birch, E.J. Microwave and pulsed electric field assisted extractions of polyphenols from defatted canola seed cake. *Int. J. Food Sci. Technol.* **2015**, *50*, 1109–1115, doi:10.1111/IJFS.12749.
454. Puértolas, E.; Martinez De Maranon, I. Olive oil pilot-production assisted by pulsed electric field: Impact on extraction yield, chemical parameters and sensory properties. *Food Chem.* **2015**, *167*, 497–502, doi:10.1016/J.FOODCHEM.2014.07.029.
455. Andreou, V.; Psarianos, M.; Dimopoulos, G.; Tsimogiannis, D.; Taoukis, P. Effect of pulsed electric fields and high pressure on improved recovery of high-added-value compounds from olive pomace. *J. Food Sci.* **2020**, *85*, 1500–1512, doi:10.1111/1750-3841.15122.
456. Chemat, F.; Zill-E-Huma; Khan, M.K. Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrason. Sonochem.* **2011**, *18*, 813–835, doi:10.1016/J.ULTSONCH.2010.11.023.
457. Goldsmith, C.D.; Vuong, Q. V.; Stathopoulos, C.E.; Roach, P.D.; Scarlett, C.J. Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace. *LWT* **2018**, *89*, 284–290, doi:10.1016/J.LWT.2017.10.065.
458. Sahin, S.; Samli, R. Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology. *Ultrason. Sonochem.* **2013**, *20*, 595–602, doi:10.1016/J.ULTSONCH.2012.07.029.
459. Wang, Z.; Wang, C.; Zhang, C.; Li, W. Ultrasound-assisted enzyme catalyzed hydrolysis of olive waste and recovery of antioxidant phenolic compounds. *Innov. Food Sci. Emerg. Technol.* **2017**, *44*, 224–234, doi:10.1016/J.IFSET.2017.02.013.
460. Mustapa, A.N.; Martin, A.; Gallego, J.R.; Mato, R.B.; Cocero, M.J. Microwave-assisted extraction of polyphenols from Clinacanthus nutans Lindau medicinal plant: Energy perspective and kinetics modeling. *Chem. Eng. Process. Process Intensif.* **2015**, *97*, 66–74, doi:10.1016/J.CEP.2015.08.013.
461. Alvarez, A. Development of a microwave pretreatment for the extraction of active compounds from natural by-products (PhD Thesis) - Chapter 6, University of Valladolid (UVa), Valladolid, Spain, 2018.
462. Macedo, G.A.; Santana, A.L.; Crawford, L.M.; Wang, S.C.; Dias, F.F.G.; de Mour Bell, J.M.L.N. Integrated microwave- and enzyme-assisted extraction of phenolic compounds from olive pomace. *LWT* **2021**, *138*, 110621, doi:10.1016/J.LWT.2020.110621.
463. Xie, P.; Huang, L.; Zhang, C.; Deng, Y.; Wang, X.; Cheng, J. Enhanced extraction of hydroxytyrosol, maslinic acid and oleanolic acid from olive pomace: Process parameters, kinetics and thermodynamics, and greenness assessment. *Food Chem.* **2019**, *276*, 662–674, doi:10.1016/j.foodchem.2018.10.079.
464. Attard, T.M.; Bukhanko, N.; Eriksson, D.; Arshadi, M.; Geladi, P.; Bergsten, U.; Budarin, V.L.; Clark, J.H.; Hunt, A.J. Supercritical extraction of waxes and lipids from biomass: A valuable first step towards an integrated biorefinery. *J. Clean. Prod.* **2018**, *177*, 684–698, doi:10.1016/j.jclepro.2017.12.155.
465. Belbaki, A.; Louaer, W.; Meniai, A.H. Supercritical CO2 extraction of oil from Crushed Algerian olives. *J. Supercrit. Fluids* **2017**, *130*, 165–171, doi:10.1016/j.supflu.2017.08.005.
466. Tirado, D.F.; Fuente, E. de la; Calvo, L. A selective extraction of hydroxytyrosol rich olive oil from alperujo. *J. Food Eng.* **2019**, *263*, 409–416, doi:10.1016/j.jfoodeng.2019.07.030.
467. Attard, T.M.; Theeuwes, E.; Gomez, L.D.; Johansson, E.; Dimitriou, I.; Wright, P.C.; Clark, J.H.; McQueen-Mason, S.J.; Hunt, A.J. Supercritical extraction as an effective first-step in a maize stover biorefinery. *RSC Adv.* **2015**, *5*, 43831–43838, doi:10.1039/C5RA07485A.
468. Budarin, V.L.; Shuttleworth, P.S.; Dodson, J.R.; Hunt, A.J.; Lanigan, B.; Marriott, R.; Milkowski, K.J.; Wilson, A.J.; Breeden, S.W.; Fan, J.; et al. Use of green chemical technologies in an integrated biorefinery. *Energy Environ. Sci.* **2011**, *4*, 471–479, doi:10.1039/C0EE00184H.

469. Brunner, G. *Topics in Physical Chemistry - Gas extraction*; Steinkopff: Heidelberg, Germany, 1994; ISBN 978-3-662-07382-7.
470. Difonzo, G.; Aresta, A.; Cotugno, P.; Ragni, R.; Squeo, G.; Summo, C.; Massari, F.; Pasqualone, A.; Faccia, M.; Zambonin, C.; et al. Supercritical CO_2 Extraction of Phytocompounds from Olive Pomace Subjected to Different Drying Methods. *Molecules* **2021**, *26*, 598, doi:10.3390/MOLECULES26030598.
471. Mustapa, A.N.; Martin, A.; Mato, R.B.; Cocero, M.J. Extraction of phytocompounds from the medicinal plant Clinacanthus nutans Lindau by microwave-assisted extraction and supercritical carbon dioxide extraction. *Ind. Crops Prod.* **2015**, *74*, 83–94, doi:10.1016/j.indcrop.2015.04.035.
472. Cacace, J.E.; Mazza, G. Pressurized Low-Polarity Water Extraction of Biologically Active Compounds from Plant Products. In *Functional Food Ingredients and Nutraceuticals: Processing Technologies*; CRC Press: FL, USA, 2015; pp. 177–198 ISBN 9780429154416.
473. Mukhopadhyay, M.; Panja, P. Pressurised Hot Water as a Novel Extractant of Natural Products: A Review. *Indian Chem. Eng.* **2010**, *51*, 311–324, doi:10.1080/00194500903430655.
474. Herrero, M.; Castro-Puyana, M.; Mendiola, J.A.; Ibanez, E. Compressed fluids for the extraction of bioactive compounds. *TrAC Trends Anal. Chem.* **2013**, *43*, 67–83, doi:10.1016/J.TRAC.2012.12.008.
475. Herrero, M.; Temirzoda, T.N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibanez, E. New possibilities for the valorization of olive oil by-products. *J. Chromatogr. A* **2011**, *1218*, 7511–7520, doi:10.1016/j.chroma.2011.04.053.
476. Rosa, A.D.; Junges, A.; Fernandes, I.A.; Cansian, R.L.; Corazza, M.L.; Franceschi, E.; Backes, G.T.; Valduga, E. High pressure extraction of olive leaves (*Olea europaea*): bioactive compounds, bioactivity and kinetic modelling. *J. Food Sci. Technol.* **2019**, *56*, 3864–3876, doi:10.1007/S13197-019-03856-W.
477. Xynos, N.; Papaefstathiou, G.; Gikas, E.; Argyropoulou, A.; Aligiannis, N.; Skaltsounis, A.L. Design optimization study of the extraction of olive leaves performed with pressurized liquid extraction using response surface methodology. *Sep. Purif. Technol.* **2014**, *122*, 323–330, doi:10.1016/j.seppur.2013.10.040.
478. Lozano-Sanchez, J.; Castro-Puyana, M.; Mendiola, J.A.; Segura-Carretero, A.; Cifuentes, A.; Ibanez, E. Recovering bioactive compounds from olive oil filter cake by advanced extraction techniques. *Int. J. Mol. Sci.* **2014**, *15*, 16270–16283, doi:10.3390/ijms150916270.
479. Tapia-Quiros, P.; Montenegro-Landivar, M.F.; Reig, M.; Vecino, X.; Alvarino, T.; Cortina, J.L.; Saurina, J.; Granados, M. Olive Mill and Winery Wastes as Viable Sources of Bioactive Compounds: A Study on Polyphenols Recovery. *Antioxidants* **2020**, *9*, 1074, doi:10.3390/antiox9111074.
480. Pavez, I.C.; Lozano-Sanchez, J.; Borras-Linares, I.; Nunez, H.; Robert, P.; Segura-Carretero, A. Obtaining an extract rich in phenolic compounds from olive pomace by pressurized liquid extraction. *Molecules* **2019**, *24*, doi:10.3390/MOLECULES24173108.
481. Kruse, S.O.; Sultan, K. Stability Testing of Herbal Medicinal Products. *Innov. Pharm. Technol.* **2005**, 64–68.
482. Food and Drug Administration *Botanical Drug Development - Guidance for industry*; 2016;
483. Farrés-Cebrian, M.; Sero, R.; Saurina, J.; Nunez, O. HPLC-UV Polyphenolic Profiles in the Classification of Olive Oils and Other Vegetable Oils via Principal Component Analysis. *Separations* **2016**, *3*, 33, doi:10.3390/separations3040033.
484. Carranco, N.; Farrés-Cebrian, M.; Saurina, J.; Nunez, O. Authentication and Quantitation of Fraud in Extra Virgin Olive Oils Based on HPLC-UV Fingerprinting and Multivariate Calibration. *Foods* **2018**, *7*, 44, doi:10.3390/foods7040044.
485. Ghanbari Shendi, E.; Sivri Ozay, D.; Ozkaya, M.T.; Ustunel, N.F. Changes occurring in chemical composition and oxidative stability of virgin olive oil during storage. *OCL - Oilseeds fats, Crop. Lipids* **2018**, *25*, A602, doi:10.1051/ocl/2018052.
486. Krichene, D.; Allalout, A.; Mancebo-Campos, V.; Salvador, M.D.; Zarrouk, M.; Fregapane, G. Stability of virgin olive oil and behaviour of its natural antioxidants under medium temperature accelerated storage conditions. *Food Chem.* **2010**, *121*, 171–177, doi:10.1016/j.foodchem.2009.12.026.
487. Krichene, D.; Salvador, M.D.; Fregapane, G. Stability of Virgin Olive Oil Phenolic Compounds during Long-Term Storage (18 Months) at Temperatures of 5-50°C. *J. Agric. Food Chem.* **2015**, *63*, 6779–6786, doi:10.1021/acs.jafc.5b02187. 488. Alonso-Salces, R.M.; Gallo, B.; Collado, M.I.; Sasia-Arriba, A.; Viacava, G.E.; Garcia-Gonzalez, D.L.; Gallina Toschi, T.; Servili, M.; Berrueta, L.A. ^{1}H–NMR fingerprinting and supervised pattern recognition to evaluate the stability of virgin olive oil during storage. *Food Control* **2021**, *123*, doi:10.1016/j.foodcont.2020.107831.
489. Ahmad-Qasem, M.H.; Ahmad-Qasem, B.H.; Barrajon-Catalan, E.; Micol, V.; Carcel, J.A.; Garcia-Pérez, J. V. Drying and storage of olive leaf extracts. Influence on polyphenols stability. *Ind. Crops Prod.* **2016**, *79*, 232–239, doi:10.1016/j.indcrop.2015.11.006.
490. European Medicines Agency *ICH Topic Q 1 A (R2) Stability Testing of new Drug Substances and Products Step 5 NOTE FOR GUIDANCE ON STABILITY TESTING: STABILITY TESTING OF NEW DRUG SUBSTANCES AND PRODUCTS*; 2003;
491. European Medicines Agency *ICH guideline S2 (R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use*; 2012;
492. Tomasello, B.; Malfa, G.; Galvano, F.; Renis, M. DNA damage in normal-weight obese syndrome measured by Comet assay. *Med. J. Nutrition Metab.* **2011**, *4*, 99–104, doi:10.1007/s12349-010-0035-6.

Hypothesis

It is possible to use phenolic compounds and extracts present in the by-products of the olive oil industry, such as olive pomace, with proven antioxidant and anti-inflammatory activity, as therapeutic agents for immune-based inflammatory diseases of the ocular surface, such as dry eye disease or allergies.

Objectives

The general and specific objectives to test the hypothesis of this thesis are:

General objective

Optimal obtention, evaluation of the topical ocular use, and evaluation of the stability and safety of the phenolic compounds of the olive pomace for their future use as treatment for dry eye disease.

Specific objectives

1. To establish the most efficient pretreatment/storage method for the olive pomace that leads to the greater stability of its main phenolic compounds (OL and HT) in the extract obtained.

2. To establish the most efficient extraction conditions for each of the phenolic compounds selected from the olive pomace, obtaining an extract with the highest concentration of the active compounds of interest.

3. To establish *in vitro* the cytotoxicity of the pure compounds and the selected extracts in two cell lines from the human corneal and conjunctival epithelia.

4. To determine *in vitro* the anti-inflammatory effect of extracts derived from olive pomace (choosing the extract(s) of interest) and their main pure phenolic compounds (OL, HT, and their combination) in two cell lines from the human corneal and conjunctival epithelia stimulated with tumor necrosis factor (TNF)-α.

5. To determine *in vitro* the antioxidant effect of extracts derived from olive pomace (choosing the extract(s) of interest) and their main pure phenolic compounds (OL, HT, and their combination), in two cell lines from the human corneal and conjunctival epithelia stimulated with UV-B light.

6. To determine the immunoregulatory activity of the chosen extract and its main pure phenolic compounds (HT), on CD4+ T lymphocytes isolated from human peripheral blood.

7. To determine *in vivo* the therapeutic effect of the selected extracts and compounds in a murine model of dry eye.

8. To establish *in vitro* the safety (cellular genotoxicity) of the pure compounds and the selected extracts in two cell lines from the human corneal and conjunctival epithelia, assays necessary for their approval as future pharmaceutical products.

9. To determine the long-term stability of aqueous solutions of the pure compounds and selected extracts, as a requirement for their approval as future ophthalmic products.

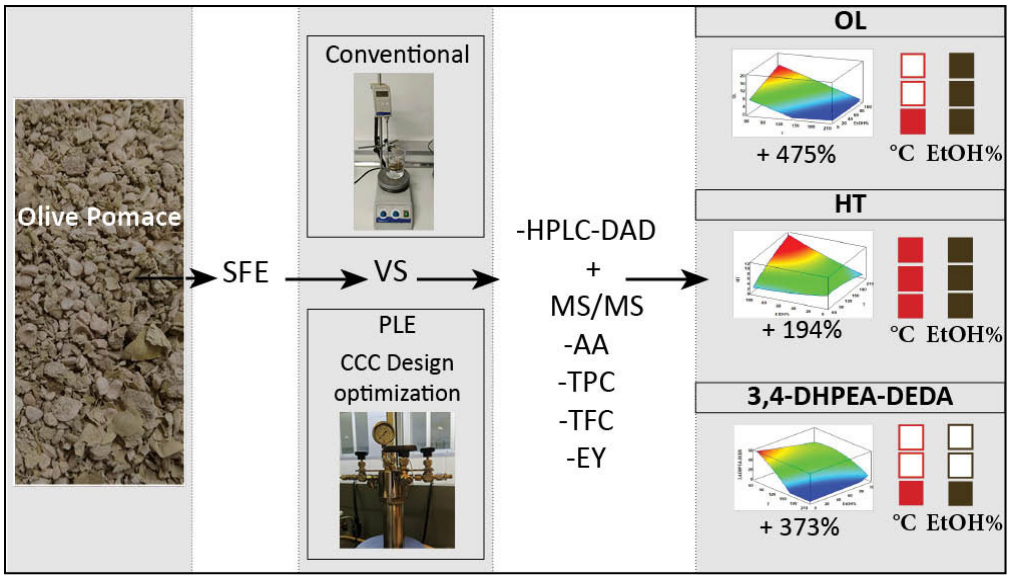
Chapter 1

Citation

Katsinas, N.; da Silva, A.B.; Enríquez-de-Salamanca, A.; Fernández, N.; Bronze, M.R.; Rodríguez-Rojo, S. Pressurized Liquid Extraction Optimization from Supercritical Defatted Olive Pomace: A Green and Selective Phenolic Extraction Process. ***ACS Sustain. Chem. Eng.*** **2021**, *9*, 5590–5602, doi:10.1021/acssuschemeng.0c09426.

Abstract

Olive pomace (OP) is the main by-product of the olive oil industry produced in large quantities. Its valorization as a source of phenolic bioactive compounds is paramount for the sustainable growth of related industries. This work proposes an intensified process to maximize the recovery of phenolic compounds in dry extracts using hydroalcoholic mixtures. Supercritical carbon dioxide defatting pre-treatment was performed. Following this, pressurized liquid extraction was optimized through a circumscribed central composite design. The factors consisted of temperature (65.0–185.0 °C), ethanol percentage (8.0–92.0%), and solid/liquid ratio (0.2–0.8 $g_{OP}/mL_{SOLVENT}$). Besides the total phenolic content (TPC) and the total flavonoid content (TFC), the major phenolic compounds of OP [hydroxytyrosol (HT), tyrosol (TY), and oleuropein (OL)] were evaluated. Further, decarboxymethyl OL aglycone dialdehyde (3,4-DHPEA-DEDA) was identified by HPLC-DAD-MS/MS as the most abundant polyphenol and was studied for the first time for OP. Different conditions were found to optimize each key compound. In 67% shorter extraction time and 38% less solvent consumption compared to conventional extraction, an increase of 475% for OL, 428% for HT, 194% for TY, 373% for 3,4-DHPEADEDA, 89% for TPC, and 158% for TFC was observed. The antioxidant activity by oxygen radical absorbance capacity (ORAC) assay increased 89% (optimal conditions) and correlated with TPC, 3,4-DHPEA-DEDA, and TFC. Thus, an efficient, selective, scalable, and green extraction process was established.



KEYWORDS: olive pomace phenolic compounds antioxidant capacity pressurized fluid extraction oleuropein hydroxytyrosol decarboxymethyl oleuropein aglycone dialdehyde (oleacein).

Chapter 2

Citation

Katsinas, N.; Rodríguez-Rojo, S; Enríquez-de-Salamanca, A. Olive Pomace Phenolic Compounds and Extracts Can Inhibit Inflammatory- and Oxidative-Related Diseases of Human Ocular Surface Epithelium. ***Antioxidants*** **2021**, ***10***, **1150, doi:10.3390/antiox10071150.**

Abstract

Oxidative- and inflammatory-related ocular surface diseases have high prevalence and are an emerging issue in ophthalmology. Olive pomace (OP) is the olive oil's industry main by-product and is potentially environmentally hazardous. Nevertheless, it contains phenolic compounds with important bioactivities, like oleuropein (OL) and hydroxytyrosol (HT). The antioxidant and anti-inflammatory effects of four OP extracts (CONV, OPT(1–3)), pure OL and HT, and mixtures thereof were screened on human corneal (HCE) and conjunctival epithelial (IM-ConjEpi) cells. CONV was conventionally extracted, while OPT(1–3) were produced by pressurized liquid extraction. Thanks to their improved activity, CONV and OPT3 (HT-enriched) were selected for dose-dependent studies. Cells were stimulated with tumor necrosis factor-α or ultraviolet-B radiation, measuring interleukin (IL)-1β, IL-6, IL-8, and IL-17A, as well as interferon γ-induced protein (IP)-10 secretion or intracellular ROS production, respectively. On HCE, both extracts and HT inhibited the secretion of most measured ILs, demonstrating a strong anti-inflammatory effect; while in IM-ConjEpi, all samples decreased IP- 10 secretion. Moreover, HT, OL, and both extracts showed strong dose-dependent antioxidant activity in both cell lines. Compared with CONV, OPT3 was active at lower concentrations, demonstrating that intensified extraction techniques are selective towards targeted biomarkers. Hence, a high-value application as potential ocular surface therapy was proposed for the OP valorization.

KEYWORDS: agro-industrial by-product; sustainability; cornea and conjunctiva; ocular surface; olive pomace phenolic extracts; oleuropein; hydroxytyrosol; inflammation; dry eye; cellular antioxidant activity.

Chapter 3

Olive Pomace Phenolic Compounds: From an Agro-industrial By-product to a Promising Ocular Surface Protection for Dry Eye Disease

Manuscript submitted to *International Journal of Molecular Sciences*

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1. Abstract

Purpose

To determine the anti-inflammatory and immunomodulatory effect of an olive pomace extract (OPT3) and its major compound, Hydroxytyrosol (HT), on human CD4+ T cells (hTCD4+) and in a dry eye disease (DED) animal model.

Methods

hTCD4+ isolated from peripheral blood were activated with phytohemagglutinin-M, treated for 48h with OPT3 (0.10-0.40 mg/mL) and HT (25-100 µM), and evaluated for their cell viability, using inactivated cells as control. Regarding the DED animal model, 100 µM HT, 0.20 mg/mL OPT3, or vehicle (borate buffer) were topically administered to C57BL/6 mice exposed for 14 days to desiccating stress (constant airflow/scopolamine administration). Tear volume, corneal fluorescein staining (CFS), CD4+ and CD8+ T cell count in lymph nodes (flow cytometry), and *IP-10* and *TNF-a* gene expression (qRT-PCR) in the cornea, conjunctiva, and lacrimal glands were evaluated.

Results

OPT3 (0.2-0.4 mg/mL) and HT (100 µM) significantly reduced the proliferation of stimulated hTCD4+. In mice, both treatments reduced CFS and lacrimal gland *IP-10* gene expression. In cervical lymph nodes, HT decreased CD3+ count, while OPT3 CD8+ count. OPT3 also reduced conjunctival *IP-10* and corneal *TNF-a* gene expression.

Conclusion

The anti-inflammatory and immunomodulatory effects of OPT3 and HT *in vitro* and *in vivo* suggest their use as topical DED protection.

KEYWORDS: desiccating stress; dry eye syndrome; inflammation; olive pomace phenolic extract; hydroxytyrosol.

Dry eye disease (DED) is a very common disorder, with a considerably high prevalence (ranging from 5 to 50 % depending on the population[1]) and a big impact on the quality of life of the patients.[2] A remarkable economic burden is caused by DED, not only by direct costs (medical or treatment) but also by indirect ones (e.g., loss of work productivity) which form the biggest proportion.[2] Immune-mediated inflammation has been considered the principal pathophysiological mechanism for DED, leading to lacrimal functional unit dysfunction, tear fluid alterations, and epitheliopathy in cornea and conjunctiva.[3-5] Further, elevated inflammatory cell infiltration (e.g., CD4+ T cells) in the conjunctiva and lacrimal gland has also been observed,[6,7] being significantly related to increased tear cytokine/chemokine levels.[8]

Mild cases of DED are usually treated with artificial tears, while the treatment approaches for more severe DED are currently based on anti-inflammatory drugs like topical corticosteroids, antibiotics administration, serum eye drops, or immunosuppressants, such as cyclosporine A.[9] However, side effects have been reported for most of these treatments, such as a strong burning sensation during application or infections related to long-term use.[10-14] Also, most corticosteroids cannot be used long-term, as elevation of intraocular pressure and cataract formation may occur.[15,16] Therefore, their use is restricted and there is still a scarcity of effective therapy. Cyclosporine A is one of the few drugs approved with the indication of DED treatment. It is a calcineurin inhibitor, modifying T cell response by inhibiting Interleukin (IL)-2 activation. It increases tear production, visual acuity, and goblet cell density, while it decreases the damage of the ocular surface epithelium and levels of inflammatory biomarkers in tear film.[17-20] However, in many countries like Australia and China, cyclosporine A eye drops are not commercially available or can be administered only to more severe DED cases,[21,22] while in Europe cyclosporine A is only approved for severe keratitis.[21] In addition, it has very low solubility in water[23] and therefore, oil or surfactants-based formulations were produced, which usually cause irritations, visual problems, etc.[14,24] Another treatment available is lifitegrast, which acts by inhibiting intercellular adhesion molecule – 1, a molecule expressed in DED inflamed epithelium and T cells.[25] Although it was found to be effective in improving the eye dryness score and ocular discomfort, no effect in corneal staining was reported after 84 days of treatment. Also, side effects similar to cyclosporine A were observed, including instillation site burning and reaction, decreased visual acuity, and dysgeusia,[26] while Xiidra (lifitegrast ophthalmic solution 5.0%) is only available in the US.[27-29]

Nowadays, there is increasing interest in the use of natural phenolic compounds as promising therapeutic agents especially in multifactorial diseases (such as DED), due to their versatile biological activities, including neuroprotective, anticancer, anti-microbial, anti-inflammatory, and antioxidant, among others.[30] They are normally consumed in the regular diet through fruits and vegetables and can have big potential applicability in the pharmaceutical industry.[30,31] More recently, their use as a treatment for ocular surface inflammatory diseases was proposed, based on their anti-inflammatory and immunomodulatory activities.[32,33] Several phenolic compounds, such as quercetin and resveratrol[34,35] or epigallocatechin gallate,[36-40] have been proved to be a potential DED therapy based on promising *in vitro* and *in vivo* results.

Among the most highlighted phenolic compounds, olive polyphenols are increasingly getting attention, due to their numerous biological activities reported, such as anti-inflammatory, antioxidant, antiviral, and cardioprotective, among others.[41] These compounds are present not only in olive oil but also in olive pomace (OP).[42,43] In fact, during olive oil production, only 1-2% of the polyphenols of the olive fruit end

up in olive oil, as the rest 98% remain in OP.[44] OP is the solid by-product, a mix of olive pulp, skin, and pit, generated after crushing and spinning out the olive oil during olive oil production.[45] It is the principal by-product and is produced in huge quantities (between 7 and 30 million m^3 per year only in the Mediterranean area).[46] Its high organic/phenolic load increases its biological and chemical demand for oxygen, which together with its phytotoxic properties and storage in open-air spaces, generates a major environmental concern, being a relevant pollutant of atmosphere, water, and soil.[47-49] Hence, there is increasing awareness worldwide for the valorization of this by-product, including biotechnological transformations into biofuel or biogas, use as fertilizers on soils, or applications in composting industry,[50] as well as applications in food and cosmetic industries.[31] However, its valorization as a source of high-value phenolic bioactive compounds is of utmost importance for a sustainable development of the olive industry, as the removal of phenolic compounds from OP would offer a by-product more easily processed further, with less oxygen demand and thus, less environmental impact.[49-51]

Hydroxytyrosol (HT) is one of the most representative olive phenolic compounds.[52] It is a simple phenol, mainly found in olive oil,[53] fruit,[54] and by-products (OP,[55] mill wastewaters,[56] and leaves[57]), but also in red and white wines.[58] HT has attracted remarkable scientific interest due to its anti-inflammatory, antiatherogenic, cardioprotective, chemoprotective, antioxidant, and antimicrobial activities.[59-64] It has also been found to protect from neovascular age-related macular degeneration, diabetic retinopathy, and retinal apoptosis.[65-70]

The effect of HT and extracts derived from olive by-products on the maintenance of the ocular surface has also been reported. The anti-inflammatory and antioxidant activity of the olive mill wastewaters on rabbit corneal epithelial cells has already been proved.[71] More recently, our group demonstrated the strong antioxidant and anti-inflammatory effect of OP-derived extracts, together with their major compound HT, on human corneal and conjunctival epithelial cells.[72] Thus, the purpose of the present study was to investigate the anti-inflammatory and immunomodulatory activity of HT and a selected OP extract enriched in HT (OPT3) for the protection of DED, *in vitro* on human CD4+ T cells (hTCD4+) and *in vivo* in a well-characterized desiccating stress mouse model.[73,74]

3.1 Plant Material and Extract Preparation

OP of 2018 crop was kindly provided by Oliduero (Medina del Campo, Spain). It was the solid by-product generated during the olive oil production using Arbequina variety of olives, which is mostly grown in Spain, but also California, Argentina, Australia, Chile, and Azerbaijan.[75] Storage conditions and characterization of the material, as well as the extraction procedure and the extract (OPT3) characterization, are all described in detail elsewhere.[55] The extraction conditions were based on the same study, which determined the selective optimal extraction conditions using freeze-dried OP defatted with supercritical carbon CO_2. Three different optimal extracts (OPT) were produced by pressurized liquid extraction at a distinct percentage of ethanol (EtOH, non-denaturalized – purity: 99.9%, Dávila Villalobos S.L., Valladolid, Spain) in water, temperature, and solid/liquid ratio. OPT3 was produced using a hydroalcoholic solvent with 90.0% v/v of EtOH and a solid/liquid ratio of 0.8 $g_{RAW\ OP}/mL_{SOLVENT}$ at 184.0 °C and 10 MPa under N_2 atmosphere (99.996% purity from Linde Gas, Puçol, Valencia, Spain). An extraction time of 20 min was applied, using freeze-dried OP defatted with supercritical carbon CO_2 (CO_2 purity: 99.95% – Carburos Metálicos, Barcelona, Spain). The conditions were selected to produce an extract with the maximum concentration in HT (7.7 ± 0.7 mg/g of dry extract).

3.2 *In vitro* Immunosuppressive Effect

3.2.1 Cell Isolation and Culture

hTCD4+ cells were isolated from the peripheral blood of healthy volunteers. This study followed the Tenets of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the University of Valladolid. Briefly, up to 9 mL of peripheral blood were obtained from 3 healthy male donors with an average age of 28 ± 3 years old, after signing informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque PLUS density (GE Healthcare, Chicago, IL, USA) gradient centrifugation at 400*g* for 30 min, at room temperature. PBMCs were collected, washed with Dulbecco's phosphate-buffered saline (DPBS – Thermo Fisher Scientific, Rockford, IL, USA), and used to isolate hTCD4+ cells. hTCD4+ cells were isolated from PBMCs by magnetic separation using a QuadroMACS™ separator (Miltenyi Biotech, Bergisch Gladbach, North Rhine-Westphalia, Germany). Untouched hTCD4+ cells were negatively selected using the CD4+ T Cell Isolation Kit human and LS columns (Miltenyi Biotech, Bergisch Gladbach, North Rhine-Westphalia, Germany), following the manufacturer's protocol. Isolated hTCD4+ cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 + L-glutamine cell culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS – Thermo Fisher Scientific, Rockford, IL, USA) and penicillin/streptomycin (Thermo Fisher Scientific, Rockford, IL, USA).

3.2.2 Preparation of Phenolic Solutions

HT (≥98% purity, Extrasynthese, Genay, France) and OPT3 were dissolved in DPBS at concentrations of 400 µM (0.062 mg/mL) and 1.6 mg/mL respectively, aliquoted in tubes, and stored at -20 °C. On the day of the experiment, an aliquot of HT or OPT3 was left at room temperature until thawing, and then diluted with cell culture medium to reach the final desired concentrations: 25-100 µM (0.004-0.015 mg/mL) for HT and 0.1-0.4 mg/mL for OPT3. At the end of each day, the remaining quantity was discarded, to ensure that no degradation occurred in the compounds.[76]

3.2.3 hTCD4+ Cell Proliferation Inhibition Study

hTCD4+ cells were seeded in 96-well plates at a density of 10^5 cells/well. All cells were activated with 1% phytohemagglutinin – M form (PHA-M – GIBCO, Grand Island, NY, USA), except for the inactivated control. Then, cells were treated for 48 h with 0.1, 0.2, and 0.4 mg/mL of OPT3, and 25, 50, and 100 µM (0.004, 0.008, and 0.015 mg/mL, respectively) of HT. After that time, 10% alamarBlue HS Cell Viability reagent (Invitrogen, Waltham, MA, USA) was added to each well and incubated for 3 h, before reading fluorescence at 560 nm excitation and 590 nm emission wavelengths on a Spectra Max M5 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). Three independent experiments were performed in duplicates.

3.3 Desiccating Stress (DS) Mouse Model

3.3.1 Animals and DS Conditions

All animal experiments followed the University of Cologne regulations, the German animal protection law (LANUV), and the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. They were also approved by the Decentral animal facility (EURL 2010/63) of the Medical Faculty of the University of Cologne.

Female C57BL/6 mice, 10–12 weeks old were obtained from Charles River Laboratories (Sulzfeld, Germany) and screened for ocular abnormalities (lid edema, corneal opacity, scarring, etc). DS protocol was applied as previously described.[73,74] Briefly, mice were exposed to DS for 14 days, by placing the cages in a controlled environment chamber (conditions: humidity 30 ± 5 %, forced airflow 18 h/day, and temperature 25 ± 1 °C). Scopolamine hydrobromide (Sigma-Aldrich, St. Louis, MO, USA) was administered continuously (0.1 mg/day) by subcutaneous implanted osmotic pumps (Alzet, Cupertino, CA, USA). The experiments ended at day 14 and regional lymph nodes, whole eyes with conjunctiva, as well as lacrimal glands, were dissected and processed for further analysis as described below. Two experimental sets of 14 days each were performed.

3.3.2 Topical Treatments

HT (100 µM – 0.015 mg/mL – Biomol, Hamburg, Germany) and OPT3 (0.2 mg/mL) were dissolved in borate buffer (vehicle – pH=8.2, Sigma-Aldrich, St. Louis, MO, USA) at the aforementioned concentrations, aliquoted in tubes, and stored at –20 °C. Each treatment day, an aliquot of HT or OPT3 was used and then discarded, to ensure that no degradation occurred in the compounds.[76] Mice were distributed randomly in three groups (n = 5 mice/group): (1) HT, (2) OPT3, and (3) vehicle (control). All groups were exposed to the same DS and housing conditions. The treatments started the same day with the setup of the DS (day 1) and were administered topically three times a day (8 AM, 12 AM, and 4 PM) in both eyes (5 µL/eye).

3.3.3 Assessment of Clinical DED Signs

Corneal damage (corneal fluorescein staining – CFS) and tear volume were assessed at baseline-day 0 and day 11 of the DS, as previously described.[74] To evaluate corneal damage, fluorescein staining was used, applying 5 µL/eye of 5% fluorescein sodium salt in DPBS (Thermo Fisher Scientific, Rockford, IL, USA).

The solution was carefully wiped off after 1 min and grading under blue light was performed with a stereomicroscope. A modified Oxford grading scheme was used with severities varying from grade 0 to 5.[77] For tear volume, a phenol red thread (Zone Quick Thread – Oasis Medical, San Dimas, CA, USA) was placed in the lateral cantus of each eye for 10 sec and a change of color from yellow to red in the wet part (absorption of tears) was measured in millimeters.

3.3.4 Flow Cytometry Analysis (FACS): % Count of CD3+, CD4+ and CD8+ in Lymph Nodes

Lymph nodes of all mice in each of the 3 groups (vehicle, HT, and OPT3 -treated) were mashed through a cell strainer and single-cell suspensions were transferred to FACS buffer (0.5% N,O-bis(trimethylsilyl)acetamide – BSA, 1% FBS, and Ethylenediaminetetraacetic acid – EDTA 1:50 in DPBS – Thermo Fisher Scientific, Rockford, IL, USA). After blocking with 0.5 mg/mL Fc block anti-mouse CD16/32 (eBioscience, San Diego, CA, USA) for 15 min, the samples were stained with fluorescent-labelled anti-mouse CD3, CD4, and CD8a antibodies (Table 1 – also including their concentrations), for 30 min at 4 °C and protected from light, following the manufacturer's instructions. Subsequently, cells were washed and resuspended in DPBS, and eFluor450fixable viability dye staining (eBioscience, San Diego, CA, USA) was performed. After washing the cells again, a stabilizing fixative (eBioscience, San Diego, CA, USA) was added. Stained samples were analyzed on a FACS Canto (BD, Germany) and results were extracted using FlowJo Software (FlowJo LLC, Tree Star Inbc., Ashland, OR, USA).

Table 1. Antibody-panel used for FACS analysis

Antibody (clone)	Target	Conjugation	Manufacturer	Catalog-no.	Concentration (mg/mL)
Anti-mouse CD3 (17A2)	CD3+ T cells	allophycocyanin-cyanine 7 (APC-Cy7)	Biolegend (San Diego, CA, USA)	100221	0.10
Anti-mouse CD4 (GK1.5)	CD4+T cells	fluorescein isothiocyanate (FITC)		100405	0.125
Anti-mouse CD8a (53-6.7)	CD8+T cells	APC		100711	0.05

3.3.5 Cytokine/Chemokine Gene Expression

Interferon γ-induced protein (IP)-10 (also known as CXCL10) and *tumor necrosis factor (TNF)-α* gene expression in the cornea, conjunctiva, and lacrimal gland tissues was studied by quantitative real-time polymerase chain reaction with retrotranscription (qRT-PCR). All procedures were performed as previously described.[78] The corneas and conjunctivas of two eyes per mouse were pooled in the same Eppendorf tube, while for lacrimal glands one sample per mouse was used. All samples were placed in RLT buffer (Qiagen, Hilden, Germany), with 10 µL/mL β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA) and the isolation of the RNA was performed using a RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription was performed using a Revert Aid First-Stand cDNA Synthesis Kit (Thermo Fisher Scientific, Rockford, IL, USA), following the manufacturer's instructions. To quantify the transcripts of *IP-10* and *TNF-α*, an SYBR Green-based qRT-PCR was performed on the cDNA samples. For the qRT-PCR reactions, a 20 µL volume was used with 20 ng cDNA and 0.75 µM of each

forward and reverse primer (Thermo Fisher Scientific, Rockford, IL, USA), together with SsoFast EvaGreen Supermix (Bio-Rad, Bonn, Germany). The incubation included 2 min at 95 °C followed by 45 cycles of 5 sec at 95 °C and 30 sec at 60 °C, using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Bonn, Germany). Results of *IP-10* and *TNF-α* were calculated by the comparative threshold method using the *hypoxanthine-guanine phosphoribosyl-transferase* (*HPRT* – Thermo Fisher Scientific, Rockford, IL, USA) as housekeeping gene. All samples were performed in triplicates, and water control, as well as primer controls, were included to detect possible contamination. The primers were designed according to the bibliography.[79,80] Table 2 presents the primer sequences, together with their annealing temperatures.

Table 2. Selected primers for qRT-PCR

mRNA	Sequence	Annealing Temperature
HPRT	F: 5′-TTGGATACAGGCCAGACTTTGTTG-3′ R: 5′-GATTCAACTTGCGCTCATCTTAGGC-3′	60 °C
IP-10	F: 5′-ATATACGCGTTGACATTGATTATTGACTAG-3′ R: 5′-ATTGCTAG-CAGCTGGTTCTTTCCGCCTC-3′	60 °C
TNF-α	F: 5′- AGGACTCAAATGGGCTTTCC-3′ R: 5′-CAGAGGCAACCTGACCACTC-3′	63 °C

3.4 Statistical Analysis

In vitro hTCD4+ cell proliferation data are presented as mean of fold change with respect to PHA-M-activated cells ± standard error of the mean (SEM). Results of CFS score, tear volume (in millimeters), and % IL gene expression in the cornea, conjunctiva, and lacrimal glands are presented as minimum to maximum value, in a box & whiskers diagram. Results of % CD3+, CD4+, or CD8+ T cell count in lymph nodes (FACS analysis) are presented as mean of % count ± SEM.

Normality tests were performed on all data, testing their Gaussian distribution using the Kolmogorov-Smirnov test. Based on the results, student's *t*-test or *t*-test with Welch's correction was performed between the groups, to analyze statistically significant differences. Two-tailed ρ-values lower than 0.05 were considered statistically significant. For the statistical analyses, the SPSS software (SPSS 15.0; SPSS, Inc., Chicago, IL, USA) was used.

4.1 *In vitro* Effect of HT and OPT3 on hTCD4+ Cell Proliferation

PHA-M-activated hTCD4+ cells isolated from PBMCs were used to test the immunosuppressive activity of the OPT3 extract and its major compound, HT. The proliferation of hTCD4+ cells was significantly stimulated at 48h by 1% PHA-M (p-value < 0.001) (Figure 1). Both treatments significantly reduced PHA-M-activated hTCD4+ cell proliferation at 48h (Figure 1). In particular, 0.2 mg/mL of OPT3 decreased it by 81 ± 3 % (p-value < 0.001), 0.4 mg/ml of OPT3 by 99 ± 5 % (p-value < 0.001), and 100 µM of HT by 41 ± 2 % (p-value < 0.001). For OPT3, the reduction in cell proliferation at 0.2 and 0.4 mg/mL was also significant compared to the basal levels (control/non-activated cells).

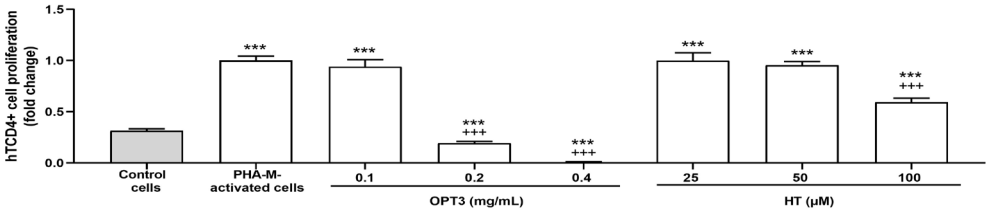

Figure 1. Effect of a selected crude OP extract (OPT3) and HT, on the proliferation of PHA-M–activated hTCD4+. Cells were activated with 1% PHA-M, except for the control, and treated for 48h with OPT3 (0.1-0.4 mg/mL), HT (25-100 µM / 0.004-0.015 mg/mL) or vehicle (cell culture medium). Cell proliferation was measured with alamarBlue HS assay (n = 3). *** p-value < 0.001, compared to control (non-stimulated) cells; +++ p-value < 0.001, compared to vehicle-treated-PHA-M-activated cells.

4.2 Effect of HT and OPT3 on the DS-Induced DED Animal Model

4.2.1 CFS

After 11 days of DS, all groups demonstrated a statistically significant increase in CFS score (p-value < 0.001) compared to baseline-day 0 (Figure 2 – A). In contrast, topical application of HT and OPT3 led to a significant decrease in CFS (p-value < 0.001, in both cases), compared to the vehicle-treated group. In the case of OPT3, the maximum CFS score observed was 2, being in some cases even 0 (no staining – no corneal alterations).

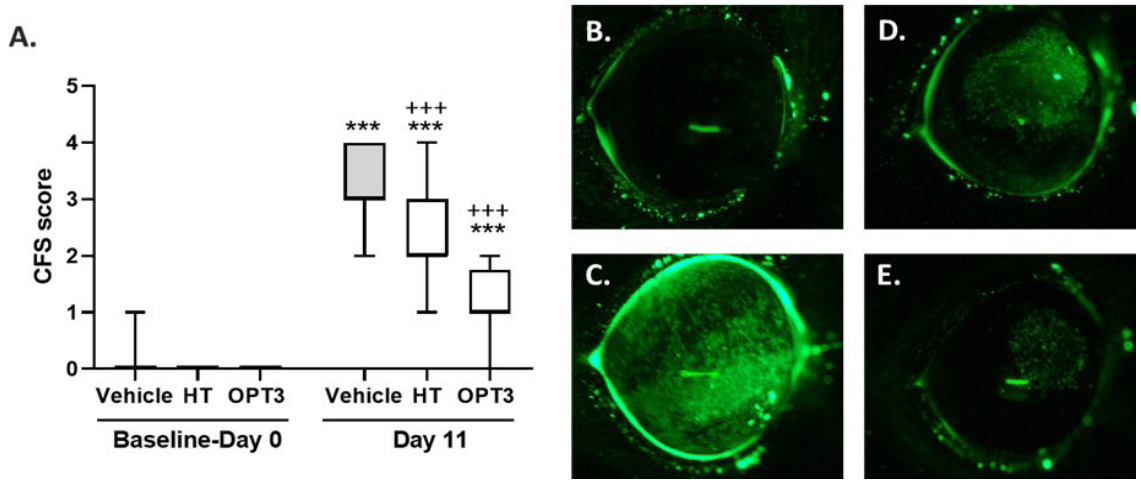

Figure 2. Effect of a selected crude OP extract (OPT3) (0.20 mg/mL) and HT (100 µM / 0.015 mg/mL), on CFS score of DS-exposed mice after topical application. **A.** Corneal damage: *** p-value < 0.001, compared to baseline-day 0 intragroup values, +++ p-value < 0.001, compared to vehicle-treated mice (borate buffer) at day 11 of the DS. Representative photos of the murine cornea, in which the epithelial damage is stained in green for: **B.** Baseline-day 0 grading, **C.** Vehicle group after DS, **D.** HT group after DS, and **E.** OPT3 group after DS. Score grading varied from 0 to 5, depending on the extent of corneal alterations.

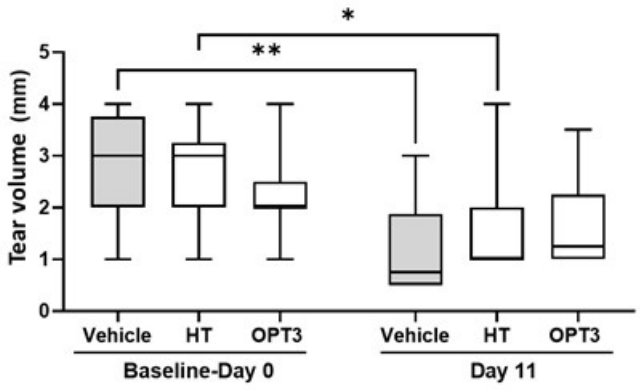

Figure 3. Effect of a selected crude OP extract (OPT3) (0.20 mg/mL) and HT (100 µM / 0.015 mg/mL), on tear volume of DS-exposed mice after topical application. ** ρ-value < 0.01, * ρ-value < 0.05.

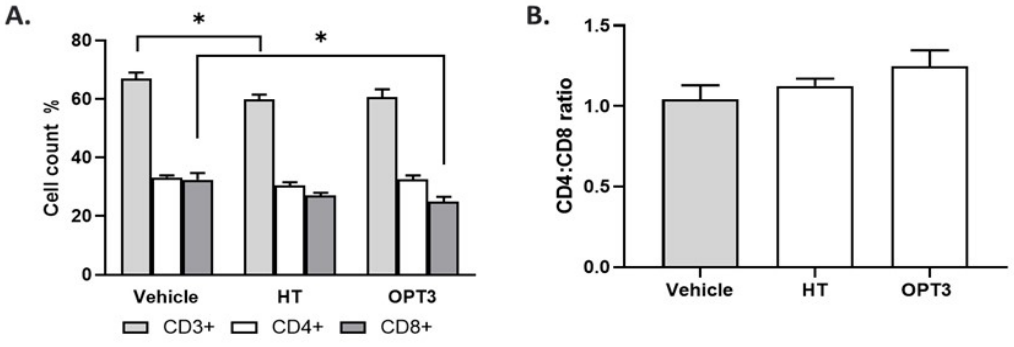

Figure 4. FACS analysis of CD3+, CD4+, and CD8+ T cells of cervical lymph nodes of mice exposed to DS for 14 days and received topical treatment of a selected crude OP extract (OPT3) (0.20 mg/mL) and HT (100 µM / 0.015 mg/mL). **A.** Percentages of CD3+, CD4+, and CD8+ T cells in terms of total live cells. HT significantly decreased the total number of CD3+, and OPT3 the total number of CD8+, compared to control (vehicle-treated mice). **B.** Calculated CD4:CD8 ratio. * ρ-value < 0.05.

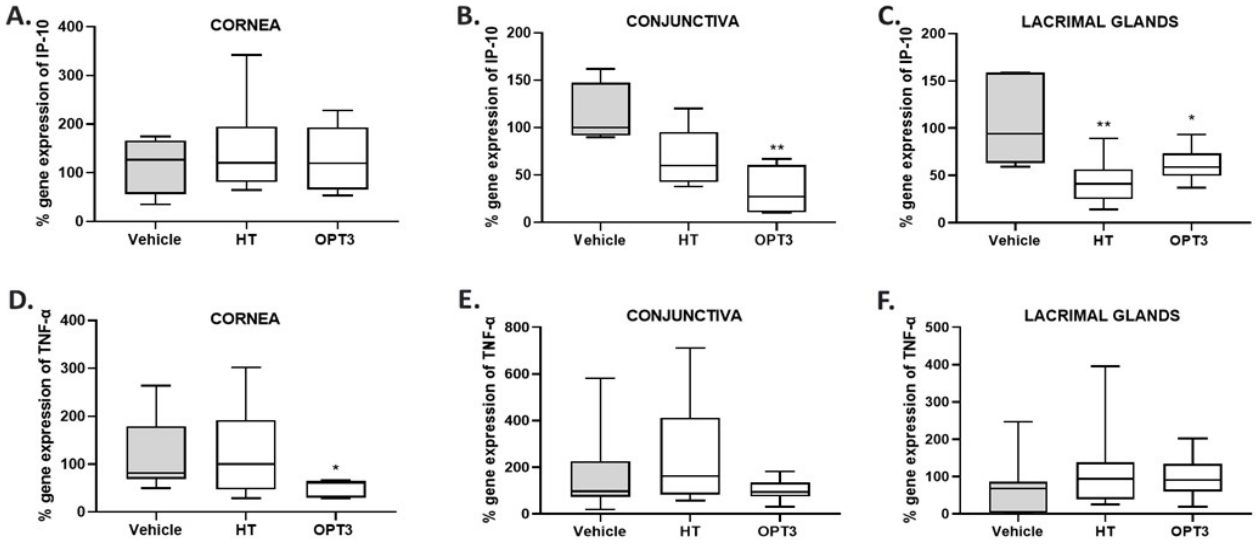

Figure 5. Effect of a selected crude OP extract (OPT3) (0.20 mg/mL) and HT (100 µM / 0.015 mg/mL), on *IP-10* (**A-C**) and *TNF-α* (**D-F**) gene expression in the cornea (**A**, **D**), conjunctiva (**B**, **E**), and lacrimal glands (**C**, **F**) of 14-days-DS-exposed mice after topical application. *IP-10* gene expression was reduced in conjunctiva by OPT3 (**B**) and in lacrimal glands by both treatments (**C**). *TNF-α* gene expression was decreased only in the cornea by OPT3 (**D**). ** ρ-value < 0.01, * ρ-value < 0.05, compared to vehicle-treated mice (borate buffer).

4.2.2 Tear Volume

DS and scopolamine administration caused a significant decrease in tear volume compared to baseline levels (p-value < 0.01 for the vehicle group and p-value < 0.05 for the HT group) (Figure 3). For the OPT3 group, the reduction of tear volume was not statistically significant. However, tear volume was not increased by any of the treatments significantly after 11 days of topical administration under DS conditions.

4.2.3 FACS Analysis: % Count of CD3+, CD4+ and CD8+ in Cervical Lymph Nodes

The number of T cells (CD3, CD4, and CD8) was analyzed in the regional lymph nodes at the end of each experimental set, comparing the vehicle-treated with the HT- and OPT3-treated groups (Figure 4). Regarding the total number of CD3+ lymphocytes (Fig. 4 – A), HT demonstrated a significant decrease compared to the vehicle-treated group (p-value < 0.05), while OPT3 did not show any significant effect. Regarding the percentage of CD8+ T cells (Fig. 4 – A), a significant reduction was observed by OPT3 (p-value < 0.05). Although CD8+ reduction by HT was not considered statistically significant, a trend (p-value = 0.0511) was detected compared to vehicle-treated mice. In terms of total CD4+ number (Fig. 4 – A) and CD4:CD8 ratio (Fig. 4 – B), no significant variations were detected between the vehicle-treated group and the treatment groups (neither for HT nor for OPT3). However, for CD4+, HT demonstrated a tendency for decrease (p-value = 0.0895).

4.2.4 *IP-10* and *TNF-α* Gene Expression in Cornea, Conjunctiva, and Lacrimal Glands

Gene expression of *IP-10* and *TNF-α* was analyzed in corneal, conjunctival, and lacrimal gland tissues from naïve and DS-exposed mice. A reduction of the expression of these genes related to DED was observed in all tissues by either both or one of the treatments, compared to the control (vehicle-treated) group (Figure 5). % *IP-10* gene expression (Fig. 5 – A, 5 – B and 5 – C) was significantly decreased by OPT3 in the conjunctiva (p-value < 0.01) and by both treatments in the lacrimal glands (p-value < 0.01 for HT, p-value < 0.05 for OPT3), compared to vehicle-treated mice. In the conjunctiva, there was also a trend for decrease of *IP-10* expression by HT (p-value = 0.0741) (Fig. 5 – B). In the cornea, none of the treatments affected it significantly. Regarding % *TNF-α* gene expression (Fig. 5 – D, 5 – E, and 5 – F), it was significantly reduced (p-value < 0.05) by OPT3 in the cornea, while no significant changes were observed in the conjunctiva and lacrimal glands neither by OPT3 nor by HT.

5. Discussion

This work proposes the use of an agro-industrial by-product that is potentially hazardous for the environment as a potential treatment for DED. The results presented prove that a crude OP extract enriched in HT, namely OPT3, and its major phenolic compound HT, can inhibit hTCD4+ proliferation and reduce clinical signs in a DED animal model. The results from the present work are also in agreement with previous results of our group, in which strong *in vitro* antioxidant and anti-inflammatory activities of OPT3 and HT were shown on human corneal and conjunctival epithelial cells.[72]

Immunomodulatory and anti-inflammatory effects of plant phenolic compounds have widely been studied, including HT and other principal olive compounds.[81-83] In our study, OPT3 and HT were able to significantly decrease *in vitro* the PHA-M-activated hTCD4+ cell proliferation. The tested concentrations were selected based on the maximum allowable concentration of both treatments on human ocular surface epithelial cells.[72] The pathophysiological mechanism of inflammation of DED has been demonstrated to be immune-mediated and is highly related to the activation and increase of CD4+ and Th17 T cells.[84,85] Activated T cells are the inflammatory mediators at the ocular surface, reinforcing the damage of goblet cell and epithelial cell loss and leading to ocular surface epitheliopathy and tear film instability.[86] They have been found in the conjunctiva in experimental DED studies[87] and in patients with DED and Sjögren's syndrome.[88-92] In addition, the severity of conjunctiva inflammation depends on T cell activation.[92]

Mechanistically, for HT, a high affinity with the CD4 cellular receptor has been shown, e.g., preventing human immunodeficiency virus (HIV) from bonding during viral infection.[93] The anti-inflammatory effect of HT observed in this study is comparable to that observed by other natural phenolic compounds like isorhamnetin, curcumin, resveratrol, and vanillic acid, which reduced the secretion of pro-inflammatory cytokines *in vitro* on Jurkat CD4+ T cells, suggesting also a synergistic activity on cytokine modulation.[94] Resveratrol has also been found to inhibit CD4+ activation *in vitro* and *in vivo*,[95] while cirsilineol, another phenolic compound, was proved to be a potential treatment for T-cell-mediated inflammatory bowel diseases, due to its targeted activity on CD4+ cells.[96] In addition, enzymatically polymerized polyphenols derived from caffeic acid, *p*-coumaric acid, and ferulic acid have been found to bind with a recombinant CD4 protein and CD4 molecules on the cell surface.[97]

In our study, the protective and anti-inflammatory effect of OPT3 and HT in the ocular surface was also demonstrated *in vivo* for the first time. The doses of HT (100 μM – 0.015 mg/mL) and OPT3 (0.2 mg/mL) were selected based on our previous *in vitro* experimental work,[72] the current *in vitro* results on hTCD4+, and the existing bibliography of same or similar compounds/extracts.[66,98] Both compounds decreased dramatically the CFS score in DS-exposed mice. These results demonstrated that topical application of HT and OPT3 improve corneal integrity in mice exposed to DS and thus, protect the ocular surface. The protective effect of other polyphenols on corneal integrity has already been studied. Abengózar-Vela et al.[34] proved that 0.1 mg/mL of quercetin and a mixture of 0.1 mg/mL quercetin with 1 mg/mL resveratrol reduced the CFS score when applied topically in a similar DED animal model. Epigallocatechin gallate also decreased CFS *in vivo* either as a pure solution at 0.1 mg/mL and 1 mg/mL after 4 and 9 days of topical application, respectively,[38] or formulated in gelatin-g-poly(N-isopropylacrylamide) copolymers after 3 days of treatment.[99] Similarly, catechin in a 14 days DED rabbit model demonstrated the reversion of corneal damage.[100] Topical application of phenolic extracts was also found to reduce CFS, i.e. 1 mg/mL of a

mixture of ethanolic extracts from different medicinal plants (*Schizonepeta tenuifolia*, *Angelica dahurica*, *Rehmanniag lutinosa Liboschitz*, *Makino*, and *Cassia tora* L) after 10 days of treatment[101] and 1 mg/mL of *Chamaecyparis obtusa* extract after 7 days of treatment in mouse models of DED.[102] However, in our study, not only an improvement of the corneal integrity is demonstrated, but also the valorization of an agro-industrial by-product is performed, aiming to add an extra value to the related industries and contributing to the emerging solutions of environmental pollution. Also, it should be highlighted that at the concentration tested (0.20 mg/mL), OPT3 comprised 0.0015 mg/mL (10.0 μM) of HT (i.e., 10 times less compared to the pure HT tested). Thus, there is a strong synergistic effect in the OP extract.

Our results also showed that OPT3 and HT did not affect tear volume. Apart from the exposure to DS conditions, this can be attributed to the subcutaneous scopolamine administration, which blocks the already shown muscarinic activity of polyphenols.[103,104] In accordance with our results, quercetin and resveratrol (alone or in combination) did not either improve tear volume after topical administration in DS-exposed mice.[34] In contrast, while using the same DS-model as in our study (including the use of scopolamine), Oh et al.[105] presented increases in tear volume after quercetin topical administration, probably because of the different types of mice used and the different concentrations and daily application times. Thus, more experiments are needed to evaluate the effect of the treatments on lacrimal glands and tears. However, as previously described, corneal integrity was maintained by both treatments, even when the tear film was not restored. Thus, the treatments can be effective in the case of aqueous deficient DED (in which lacrimal secretion is decreased in conditions of normal evaporation of the tear fluid from the eye), probably by protecting the cornea.[86]

In accordance with the *in vitro* results on hTCD4+ cells, the *in vivo* data also indicate the modulatory effect of OPT3 and HT on immune cells and their possible preventive effect on lymph nodes in the early phase of the DED animal model. The important role of T cells for the development and progression of the DED has already been described.[88-92] Activated T cells are highly infiltrated into the lacrimal functional unit tissues due to DS conditions.[3] Also, Cyclosporine A, a T cell inhibitor, acts on T cells by suppressing their lymphokines (IL-2) secretion and is proved to be highly effective regarding symptoms and signs on DED patients.[106] Lymph nodes are the reservoir of immune cells and are the place where adaptive immunity is regulated and autoreactive CD4+ and CD8+ T cells are activated.[107,108] Apart from CD4+, a significant increase of CD8+ has also been observed for draining lymph nodes of DS-exposed mice.[109] Therefore, in this study, the effect of the OP phenolic compounds on the CD4+ and CD8+ ratio in the lymph nodes was studied. This effect was comparable to another study, where a significant decrease of CD4+ T cell infiltration was observed in the conjunctiva of recipient nude mice, after being adoptively transferred with CD4+ isolated from resveratrol- and quercetin-treated mice exposed to DS for 10 days, compared to vehicle-treated mice.[34] Thus, more detailed studies are needed to fully conclude the effect of HT and OPT3 on the local tissues and lacrimal glands.

We also demonstrated that both HT and OPT3 phenolic treatments significantly reduced the gene expression of cytokines/chemokines in the lacrimal functional unit tissues. In particular, OPT3 reduced *TNF-a* gene expression in the cornea and *IP-10* gene expression in the conjunctiva and lacrimal glands, while HT decreased *IP-10* gene expression in the lacrimal glands. Increased levels of these molecules have been related to the pathophysiology of the DED and have been observed in conjunctiva and tears of DED patients.[8,110] High levels of *IP-10* and increased *TNF-a* transcripts were also detected in the corneal and

conjunctival epithelium of mice exposed to DS.[111,112] Previous *in vitro* data from our group already demonstrated the strong inhibition of *IP-10* secretion by OPT3 and HT on human corneal and conjunctival epithelial cells.[72] In accordance with our results, the reduction of levels or expression of cytokines/chemokines related to the DED in the lacrimal functional unit of a DED animal model has already been reported for phenolic compounds or phenolic-rich extracts. Topical application of several polyphenols has been proved to significantly downregulate the cytokine levels in the cornea (0.2 mg/mL of epigallocatechin gallate[40]) and lacrimal glands (30 mg/mL of 7-carboxymethyloxy-3′,4′,5-trimethoxy flavone[113]) of DED rabbits, as well as lacrimal glands (10 mg/mL of catechin[114]) and tear fluid (0.1 mg/mL quercetin and 1 mg/mL resveratrol – alone or in combination[34]) of DED mice. In addition, topical application of phenolic-rich extracts in the conjunctiva of DS-induced mice inhibited the expression (1 mg/mL of a mixture of ethanolic extracts of pharmaceutical plants: *Schizonepeta tenuifolia* var. *japonica Kitagawa*, *Angelica dahurica* Bentham et Hooker, *Rehmannia glutinosa Liboschitz* var. *purpurea*, *Makino*, and *Cassia tora* L. [101]) and the levels (0.1 mg/mL of a *Chamaecyparis obtusa* leaves extract[102] and 1 mg/mL of *Camellia japonica* leaves extract[115]) of several cytokines.

This work has some limitations that should be addressed in future studies. First, the application of the treatments was performed in parallel with the development of the DED under DS conditions and scopolamine administration, demonstrating that our treatments are effective in inhibiting the development of the disease. As patients visit the ophthalmologist with already established symptoms, additional studies starting the treatment after the 14 day of DS conditions, would explore the possible treating capacity and downregulating effect of the olive phenolic compounds on established DED conditions. Second, only one concentration of HT and OPT3 was tested, demonstrating strong protective immunomodulatory and anti-inflammatory effects; however, more concentrations should be tested to detect any further possible effectiveness, but also safety issues. Third, since a clear synergistic effect was detected for OPT3 compared to the pure HT, different OP-derived phenolic compounds present in the OPT3 extract[76] should be tested as a pure solution, alone or in combination with HT, to identify more potential bio-active olive secondary metabolites. And fourth, regarding the *in vitro* results, both treatments demonstrated a strong immunosuppressive effect on hTCD4+ cells; however, their effect on the cytokine/chemokine secretion by hTCD4+ should also be examined.

6. Conclusions

This work demonstrates that a selected extract derived from OP and its major phenolic compound, HT, can be a promising topical protection for the DED, despite the limitations. Both treatments not only demonstrated an immunosuppressive effect *in vitro* on hTCD4+ cells but also reduced clinical signs (corneal damage), together with an inflammatory response (cytokine gene expression) in the lacrimal functional unit of a DED animal model. This work is paramount for the sustainable development of olive industries, proposing a high-value alternative use of an environmentally hazardous agro-industrial by-product as therapy of immune-based ocular surface diseases.

References

1. Stapleton, F.; Alves, M.; Bunya, V.Y.; Jalbert, I.; Lekhanont, K.; Malet, F.; Na, K.-S.; Schaumberg, D.; Od, S.D.; Uchino, M.; et al. TFOS DEWS II Epidemiology Report. *Ocul. Surf.* **2017**, *15*, 334–365, doi:10.1016/j.jtos.2017.05.003.
2. McDonald, M.; Patel, D.A.; Keith, M.S.; Snedecor, S.J. Economic and Humanistic Burden of Dry Eye Disease in Europe, North America, and Asia: A Systematic Literature Review. *Ocul. Surf.* **2016**, *14*, 144–167, doi:10.1016/j.jtos.2015.11.002.
3. Stern, M.E.; Schaumburg, C.S.; Pflugfelder, S.C. Dry eye as a mucosal autoimmune disease. *Int. Rev. Immunol.* **2013**, *32*, 19–41, doi :10.3109/08830185.2012.748052.
4. Stern, M.E.; Gao, J.; Siemasko, K.F.; Beuerman, R.W.; Pflugfelder, S.C. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp. Eye Res.* **2004**, *78*, 409–416, doi:10.1016/j.exer.2003.09.003.
5. Barabino, S.; Chen, Y.; Chauhan, S.; Dana, R. Ocular Surface Immunity: Homeostatic Mechanisms and Their Disruption in Dry Eye Disease. *Prog. Retin. Eye Res.* **2012**, *31*, 271–285, doi:10.1016/j.preteyeres.2012.02.003.
6. Stern, M.E.; Beuerman, R.W.; Fox, R.I.; Gao, J.; Mircheff, A.K.; Pflugfelder, S.C. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. *Cornea* **1998**, *17*, 584–589, doi:10.1097/00003226-199811000-00002.
7. Pflugfelder, S.C.; Solomon, A.; Stern, M.E. The diagnosis and management of dry eye: a twenty-five-year review. *Cornea* **2000**, *19*, 644–649, doi:10.1097/00003226-200009000-00009.
8. Enríquez-de-Salamanca, A.; Castellanos, E.; Stern, M.E.; Fernández, I.; Carreño, E.; García-Vázquez, C.; Herreras, J.M.; Calonge, M. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol. Vis.* **2010**, *16*, 862–873.
9. Jones, L.; Downie, L.E.; Korb, D.; Benitez-del-Castillo, J.M.; Dana, R.; Deng, S.X.; Dong, P.N.; Geerling, G.; Hida, R.Y.; Liu, Y.; et al. TFOS DEWS II Management and Therapy Report. *Ocul. Surf.* **2017**, *15*, 575–628, doi:10.1016/J.JTOS.2017.05.006.
10. Pinto-Fraga, J.; Lopez-Miguel, A.; Gonzalez-Garcia, M.J.; Fernandez, I.; Lopez-De-La-Rosa, A.; Enriquez-De-Salamanca, A.; Stern, M.E.; Calonge, M. Topical Fluorometholone Protects the Ocular Surface of Dry Eye Patients from Desiccating Stress: A Randomized Controlled Clinical Trial. *Ophthalmology* **2016**, *123*, 141–153, doi:10.1016/j.ophtha.2015.09.029.
11. Garrott, H.M.; Walland, M.J. Glaucoma from topical corticosteroids to the eyelids. *Clin. Experiment. Ophthalmol.* **2004**, *32*, 224–226, doi:10.1111/J.1442-9071.2004.00787.X.
12. Avunduk, A.M.; Avunduk, M.C.; Varnell, E.D.; Kaufman, H.E. The comparison of efficacies of topical corticosteroids and nonsteroidal anti-inflammatory drops on dry eye patients: A clinical and immunocytochemical study. *Am. J. Ophthalmol.* **2003**, *136*, 593–602, doi:10.1016/S0002-9394(03)00326-X.
13. Calonge, M. The Treatment of Dry Eye. *Surv. Ophthalmol.* **2001**, *45*, S227–S239, doi:10.1016/S0039-6257(00)00205-8.
14. Steven, P.; Cursiefen, C. Anti-inflammatory treatment in dry eye disease. *Klin. Monbl. Augenheilkd.* **2012**, *229*, 500–505, doi:10.1055/S-0031-1299519.
15. Cutolo, C.A.; Barabino, S.; Bonzano, C.; Traverso, C.E. The Use of Topical Corticosteroids for Treatment of Dry Eye Syndrome. *Ocul. Immunol. Inflamm.* **2019**, *27*, 266–275, doi:10.1080/09273948.2017.1341988.
16. Rice, M.L.; Wong, B.; Horn, P.S.; Yang, M.B. Cataract development associated with long-term glucocorticoid therapy in Duchenne muscular dystrophy patients. *J. Am. Assoc. Pediatr. Ophthalmol. Strabismus {JAAPOS}* **2018**, *22*, 192–196, doi:10.1016/J.JAAPOS.2018.01.017.
17. Kunert, K.S.; Tisdale, A.S.; Stern, M.E.; Smith, J.A.; Gipson, I.K. Analysis of Topical Cyclosporine Treatment of Patients With Dry Eye Syndrome: Effect on Conjunctival Lymphocytes. *Arch. Ophthalmol.* **2000**, *118*, 1489–1496, doi:10.1001/archopht.118.11.1489.
18. Strong, B.; Farley, W.; Stern, M.E.; Pflugfelder, S.C. Topical cyclosporine inhibits conjunctival epithelial apoptosis in experimental murine keratoconjunctivitis sicca. *Cornea* **2005**, *24*, 80–85, doi:10.1097/01.ico.0000133994.22392.47.
19. Sall, K.; Stevenson, O.D.; Mundorf, T.K.; Reis, B.L. Two multicenter, randomized studies of the efficacy and safety of cyclosporine ophthalmic emulsion in moderate to severe dry eye disease. *Ophthalmology* **2000**, *107*, 631–639, doi:10.1016/S0161-6420(99)00176-1.
20. Donnenfeld, E.; Pflugfelder, S.C. Topical Ophthalmic Cyclosporine: Pharmacology and Clinical Uses. *Surv. Ophthalmol.* **2009**, *54*, 321–338, doi:10.1016/j.survophthal.2009.02.002.
21. Lallemand, F.; Schmitt, M.; Bourges, J.L.; Gurny, R.; Benita, S.; Garrigue, J.S. Cyclosporine A delivery to the eye: A comprehensive review of academic and industrial efforts. *Eur. J. Pharm. Biopharm.* **2017**, *117*, 14–28, doi:10.1016/J.EJPB.2017.03.006.
22. *Business Wire, Novaliq Announces Positive Topline Results of Phase 2 Clinical Trial Evaluating CyclASol® in Adults with Moderate to Severe Dry Eye Disease*; 2016.
23. Berton, P.; Mishra, M.K.; Choudhary, H.; Myerson, A.S.; Rogers, R.D. Solubility Studies of Cyclosporine Using Ionic Liquids *ACS Omega* **2019**, *4*, 7938–7943, doi:10.1021/acsomega.9b00603.
24. Sheppard, J.D.; Scoper, S. V.; Samudre, S. Topical Loteprednol Pretreatment Reduces Cyclosporine Stinging in Chronic Dry Eye Disease. *J. Ocul. Pharmacol. Ther.* **2011**, *27*, 23–27, doi:10.1089/jop.2010.0085.
25. Perez, V.L.; Pflugfelder, S.C.; Zhang, S.; Shojaei, A.; Haque, R. Lifitegrast, a Novel Integrin Antagonist for Treatment of Dry Eye Disease. *Ocul. Surf.* **2016**, *14*, 207–215, doi:10.1016/J.JTOS.2016.01.001.
26. Donnenfeld, E.D.; Karpecki, P.M.; Majmudar, P.A.; Nichols, K.K.; Raychaudhuri, A.; Roy, M.; Semba, C.P. Safety of Lifitegrast Ophthalmic Solution 5.0% in Patients With Dry Eye Disease: A 1-Year, Multicenter, Randomized, Placebo-Controlled Study. *Cornea* **2016**, *35*, 741–748.
27. *Xiidra (lifitegrast) [package insert] - Shire US Inc.*; Lexington, MA, 2016.
28. U.S. Food and Drug Administration. FDA approves new medication for dry eye disease. Available online: https://www.fda.gov/news-events/press-announcements/fda-approves-new-medication-dry-eye-disease (accessed on Nov 10, 2021).
29. Novartis Withdraws Marketing Application of Dry Eye Drug Xiidra in Europe - Eyewire+ Available online: https://eyewire.news/news/novartis-pulls-eu-filing-seeking-approval-of-xiidra-for-dry-eye-disease (accessed on Nov 11, 2021).
30. Kumar, N.; Goel, N. Phenolic acids: Natural versatile molecules with promising therapeutic applications. *Biotechnol. Reports* **2019**, *24*, e00370, doi:10.1016/J.BTRE.2019.E00370.
31. Albuquerque, B.R.; Heleno, S.A. 8.; Oliveira, M.B.P.P.; Barros, L.; Ferreira, I.C.F.R. Phenolic compounds: Current industrial applications, limitations and future challenges. *Food Funct.* **2021**, *12*, 14–29, doi:10.1039/D0FO02324H.

32. González, R.; Ballester, I.; López-Posadas, R.; Suárez, M.D.; Zarzuelo, A.; Martínez-Augustin, O.; Sánchez de Medina, F. Effects of flavonoids and other polyphenols on inflammation. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 331–362, doi:10.1080/10408390903584094.
33. Shakoor, H.; Feehan, J.; Apostolopoulos, V.; Platat, C.; Dhaheri, A.S. Al; Ali, H.I.; Ismail, L.C.; Bosevski, M.; Stojanovska, L. Immunomodulatory Effects of Dietary Polyphenols. *Nutr.* **2021**, *13*, 728, doi:10.3390/NU13030728.
34. Abengózar-Vela, A.; Schaumburg, C.S.; Stern, M.E.; Calonge, M.; Enríquez-de-Salamanca, A.; González-García, M.J. Topical Quercetin and Resveratrol Protect the Ocular Surface in Experimental Dry Eye Disease. *Ocul. Immunol. Inflamm.* **2019**, *27*, 1023–1032, doi:10.1080/09273948.2018.1497664.
35. Abengózar-Vela, A.; Calonge, M.; Stern, M.E.; González-García, M.J.; Enríquez-De-Salamanca, A. Quercetin and resveratrol decrease the inflammatory and oxidative responses in human ocular surface epithelial cells. *Investig. Ophthalmol. Vis. Sci.* **2015**, *56*, 2709–2719, doi:10.1167/iovs.15-16595.
36. Luo, L.-J.; Lai, J.-Y. Epigallocatechin Gallate-Loaded Gelatin-g-Poly(N-Isopropylacrylamide) as a New Ophthalmic Pharmaceutical Formulation for Topical Use in the Treatment of Dry Eye Syndrome. *Sci. Reports* **2017**, *7*, 1–14, doi:10.1038/s41598-017-09913-8.
37. Cavet, M.E.; Harrington, K.L.; Vollmer, T.R.; Ward, K.W.; Zhang, J.Z. Anti-inflammatory and anti-oxidative effects of the green tea polyphenol epigallocatechin gallate in human corneal epithelial cells. *Mol. Vis.* **2011**, *17*, 533–542.
38. Soo Lee, H.; Chauhan, S.K.; Okanobo, A.; Nallasamy, N.; Dana, R. Therapeutic Efficacy of Topical Epigallocatechin Gallate in Murine Dry Eye. *Cornea* **2011**, *30*, 1465–1472, doi:10.1097/ICO.0b013e31821c9b5a.
39. Tseng, C.-L.; Hung, Y.-J.; Chen, Z.-Y.; Fang, H.-W.; Chen, K.-H. Synergistic Effect of Artificial Tears Containing Epigallocatechin Gallate and Hyaluronic Acid for the Treatment of Rabbits with Dry Eye Syndrome. *PLoS One* **2016**, *11*, e0157982, doi:10.1371/journal.pone.0157982.
40. Huang, H.Y.; Wang, M.C.; Chen, Z.Y.; Chiu, W.Y.; Chen, K.H.; Lin, I.C.; Yang, W.C.V.; Wu, C.C.; Tseng, C.L. Gelatin–epigallocatechin gallate nanoparticles with hyaluronic acid decoration as eye drops can treat rabbit dry-eye syndrome effectively via inflammatory relief. *Int. J. Nanomedicine* **2018**, *13*, 7251–7273, doi:10.2147/IJN.S173198.
41. Obied, H.K.; Allen, M.S.; Bedgood, D.R.; Prenzler, P.D.; Robards, K.; Stockmann, R. Bioactivity and analysis of biophenols recovered from olive mill waste. *J. Agric. Food Chem.* **2005**, *53*, 823–837, doi:10.1021/jf048569x.
42. Lozano-Sánchez, J.; Giambanelli, E.; Quirantes-Piné, R.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Wastes generated during the storage of extra virgin olive oil as a natural source of phenolic compounds. *J. Agric. Food Chem.* **2011**, *59*, 11491–11500, doi:10.1021/jf202596q.
43. Obied, H.K.; Bedgood, D.R.; Prenzler, P.D.; Robards, K. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Anal. Chim. Acta* **2007**, *603*, 176–189, doi:10.1016/j.aca.2007.09.044.
44. Chanioti, S.; Tzia, C. Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: Oil recovery, unsaponifiable matter, total phenol content and antioxidant activity. *LWT - Food Sci. Technol.* **2017**, *79*, 178–189, doi:10.1016/J.LWT.2017.01.029.
45. Kapellakis, I.E.; Tsagarakis, K.P.; Crowther, J.C. Olive oil history, production and by-product management. *Rev. Environ. Sci. Biotechnol.* **2008**, *7*, 1–26, doi:10.1007/s11157-007-9120-9.
46. Gómez-Muñoz, B.; J., D.; Bol, R.; Garca-Ruiz, R. The Compost of Olive Mill Pomace: From a Waste to a Resource - Environmental Benefits of Its Application in Olive Oil Groves. In *Sustainable Development - Authoritative and Leading Edge Content for Environmental Management*; IntechOpen: London, UK, 2012.
47. Rodrigues, F.; Pimentel, F.B.; Oliveira, M.B.P.P. Olive by-products: Challenge application in cosmetic industry. *Ind. Crops Prod.* **2015**, *70*, 116–124, doi:10.1016/j.indcrop.2015.03.027.
48. Souilem, S.; El-Abbassi, A.; Kiai, H.; Hafidi, A.; Sayadi, S.; Galanakis, C.M. Olive oil production sector: environmental effects and sustainability challenges. In *Olive Mill Waste: Recent Advances for Sustainable Management*; Academic Press, 2017; pp. 1–28.
49. Romero-García, J.M.; Niño, L.; Martínez-Patiño, C.; Álvarez, C.; Castro, E.; Negro, M.J. Biorefinery based on olive biomass. State of the art and future trends. *Bioresour. Technol.* **2014**, *159*, 421–432, doi:10.1016/j.biortech.2014.03.062.
50. Roig, A.; Cayuela, M.L.; Sánchez-Monedero, M.A. An overview on olive mill wastes and their valorisation methods. *Waste Manag.* **2006**, *26*, 960–969, doi:10.1016/J.WASMAN.2005.07.024.
51. Dermeche, S.; Nadour, M.; Larroche, C.; Moulti-Mati, F.; Michaud, P. Olive mill wastes: Biochemical characterizations and valorization strategies. *Process Biochem.* **2013**, *48*, 1532–1552, doi:10.1016/j.procbio.2013.07.010.
52. Napolitano, A.; De Lucia, M.; Panzella, L.; d'Ischia, M. The Chemistry of Tyrosol and Hydroxytyrosol: Implications for Oxidative Stress. In *Olives and Olive Oil in Health and Disease Prevention*; Elsevier Inc., 2010; pp. 1225–1232 ISBN 9780123744203.
53. Dierkes, G.; Krieger, S.; Dück, R.; Bongartz, A.; Schmitz, O.J.; Hayen, H. High-Performance Liquid Chromatography–Mass Spectrometry Profiling of Phenolic Compounds for Evaluation of Olive Oil Bitterness and Pungency. *J. Agric. Food Chem.* **2012**, *60*, 7597–7606, doi:10.1021/JF3020574.
54. Savarese, M.; De Marco, E.; Sacchi, R. Characterization of phenolic extracts from olives (Olea europaea cv. Pisciottana) by electrospray ionization mass spectrometry. *Food Chem.* **2007**, *105*, 761–770, doi:10.1016/j.foodchem.2007.01.037.
55. Katsinas, N.; Bento da Silva, A.; Enríquez-de-Salamanca, A.; Fernández, N.; Bronze, M.R.; Rodríguez-Rojo, S. Pressurized Liquid Extraction Optimization from Supercritical Defatted Olive Pomace: A Green and Selective Phenolic Extraction Process. *ACS Sustain. Chem. Eng.* **2021**, *9*, 5590–5602, doi:10.1021/acssuschemeng.0c09426.
56. De Marco, E.; Savarese, M.; Paduano, A.; Sacchi, R. Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. *Food Chem.* **2007**, *104*, 858–867, doi:10.1016/j.foodchem.2006.10.005.
57. De Leonardis, A.; Aretini, A.; Alfano, G.; Macciola, V.; Ranalli, G. Isolation of a hydroxytyrosol-rich extract from olive leaves (Olea Europaea L.) and evaluation of its antioxidant properties and bioactivity. *Eur. Food Res. Technol.* **2007**, *226*, 653–659, doi:10.1007/S00217-007-0574-3.
58. Fernández-Mar, M.I.; Mateos, R.; García-Parrilla, M.C.; Puertas, B.; Cantos-Villar, E. Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. *Food Chem.* **2012**, *130*, 797–813, doi:10.1016/J.FOODCHEM.2011.08.023.
59. Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F.F.; Galli, C. Antioxidant and other biological activities of olive mill waste waters. *J. Agric. Food Chem.* **1999**, *47*, 3397–3401, doi:10.1021/jf9900534.
60. Ceccon, L.; Saccù, D.; Procida, G.; Cardinali, S. Liquid Chromatographic Determination of Simple Phenolic Compounds in Waste Wa-

ters from Olive Oil Production Plants. *J. AOAC Int.* **2001**, *84*, 1739–1744, doi:10.1093/jaoac/84.6.1739.
61. Fernández-Bolaños, J.; Rodríguez, G.; Rodríguez, R.; Heredia, A.; Guillén, R.; Jimínez, A. Production in large quantities of highly purified hydroxytyrosol from liquid-solid waste of two-phase olive oil processing or "alperujo." *J. Agric. Food Chem.* **2002**, *50*, 6804–6811, doi:10.1021/jf011712r.
62. Capasso, R.; Evidente, A.; Visca, C. Production of hydroxytyrosol from olive oil vegetation waters. *Agrochimica* **1994**, *38*, 165–172.
63. Vazquez-Roncero, A.; Maestro-Duran, R.; Graciani-Constante, E. Phenolic compounds in olive fruits. II. Polyphenols in vegetation water. *Grasas y aceites* **1974**, *25*, 341–345.
64. Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D- and 2D-nuclear magnetic resonance characterization. *JAOCS, J. Am. Oil Chem. Soc.* **1999**, *76*, 873–882, doi:10.1007/s11746-999-0079-2.
65. Granner, T.; Maloney, S.; Antecka, E.; Correa, J.A.; Burnier, M.N. 3,4 Dihydroxyphenyl Ethanol Reduces Secretion of Angiogenin in Human Retinal Pigment Epithelial Cells. *Br. J. Ophthalmol.* **2013**, *97*, 371–374, doi:10.1136/bjophthalmol-2012-302002.
66. Zou, X.; Feng, Z.; Li, Y.; Wang, Y.; Wertz, K.; Weber, P.; Fu, Y.; Liu, J. Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: Activation of Nrf2 and JNK-p62/SQSTM1 pathways. *J. Nutr. Biochem.* **2012**, *23*, 994–1006, doi:10.1016/j.jnutbio.2011.05.006.
67. Zhu, L.; Liu, Z.; Feng, Z.; Hao, J.; Shen, W.; Li, X.; Sun, L.; Sharman, E.; Wang, Y.; Wertz, K.; et al. Hydroxytyrosol protects against oxidative damage by simultaneous activation of mitochondrial biogenesis and phase II detoxifying enzyme systems in retinal pigment epithelial cells. *J. Nutr. Biochem.* **2010**, *21*, 1089–1098, doi:10.1016/j.jnutbio.2009.09.006.
68. Liu, Z.; Sun, L.; Zhu, L.; Jia, X.; Li, X.; Jia, H.; Wang, Y.; Weber, P.; Long, J.; Liu, J. Hydroxytyrosol protects retinal pigment epithelial cells from acrolein-induced oxidative stress and mitochondrial dysfunction. *J. Neurochem.* **2007**, *103*, 2690–2700, doi:10.1111/j.1471-4159.2007.04954.x.
69. Deiana, M.; Aruoma, O.I.; Bianchi, M.D.L.P.; Spencer, J.P.E.; Kaur, H.; Halliwell, B.; Aeschbach, R.; Banni, S.; Dessi, M.A.; Corongiu, F.P. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radic. Biol. Med.* **1999**, *26*, 762–769, doi:10.1016/S0891-5849(98)00231-7.
70. González-Correa, J.A.; Rodríguez-Pérez, M.D.; Márquez-Estrada, L.; López-Villodres, J.A.; Reyes, J.J.; Rodriguez-Gutierrez, G.; Fernández-Bolaños, J.; De La Cruz, J.P. Neuroprotective Effect of Hydroxytyrosol in Experimental Diabetic Retinopathy: Relationship with Cardiovascular Biomarkers. *J. Agric. Food Chem.* **2018**, *66*, 637–644, doi:10.1021/acs.jafc.7b05063.
71. Di Mauro, M.D.; Fava, G.; Spampinato, M.; Aleo, D.; Melilli, B.; Saita, M.G.; Centonze, G.; Maggiore, R.; D'antona, N. Polyphenolic fraction from olive mill wastewater: Scale-up and in vitro studies for ophthalmic nutraceutical applications. *Antioxidants* **2019**, *8*, 1–21, doi:10.3390/antiox8100462.
72. Katsinas, N.; Rodríguez-Rojo, S.; Enríquez-de-Salamanca, A. Olive Pomace Phenolic Compounds and Extracts Can Inhibit Inflammatory- and Oxidative-Related Diseases of Human Ocular Surface Epithelium. *Antioxidants* **2021**, *10*, 1150, doi:10.3390/ANTIOX10071150.
73. Dursun, D.; Wang, M.; Monroy, D.; Li, D.Q.; Lokeshwar, B.L.; Stern, M.E.; Pflugfelder, S.C. A mouse model of keratoconjunctivitis sicca. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 632–638.
74. Gehlsen, U.; Braun, T.; Notara, M.; Krösser, S.; Steven, P. A semifluorinated alkane (F4H5) as novel carrier for cyclosporine A: a promising therapeutic and prophylactic option for topical treatment of dry eye. *Graefe's Arch. Clin. Exp. Ophthalmol.* **2017**, *255*, 767–775, doi:10.1007/S00417-016-3572-Y.
75. Jain, S.M.; Priyadarshan, P.M. *Breeding plantation tree crops: Tropical species*; Springer : New York, NY, USA, 2009; ISBN 9780387712031.
76. Katsinas, N.; Enríquez-de-Salamanca, A.; Silva, A.B. da; Bronze, M.R.; Rodríguez-Rojo, S. Olive Pomace Phenolic Compounds Stability and Safety Evaluation: From Raw Material to Future Ophthalmic Applications. *Molecules* **2021**, *26*, 6002, doi:10.3390/MOLECULES26196002.
77. Bron, A.J.; Evans, V.E.; Smith, J.A. Grading Of Corneal and Conjunctival Staining in the Context of Other Dry Eye Tests. *Cornea* **2003**, *22*, 640–650, doi:10.1097/00003226-200310000-00008.
78. Gehlsen, U.; Stary, D.; Maass, M.; Riesner, K.; Musial, G.; Stern, M.E.; Penack, O.; Steven, P. Ocular Graft-versus-Host Disease in a Chemotherapy-Based Minor-Mismatch Mouse Model Features Corneal (Lymph-) Angiogenesis. *Int. J. Mol. Sci.* **2021**, *22*, 6191, doi:10.3390/IJMS22126191.
79. Mirzaei, H.; Salehi, H.; Oskuee, R.K.; Mohammadpour, A.; Mirzaei, H.R.; Sharifi, M.R.; Salarinia, R.; Darani, H.Y.; Mokhtari, M.; Masoudifar, A.; et al. The therapeutic potential of human adipose-derived mesenchymal stem cells producing CXCL10 in a mouse melanoma lung metastasis model. *Cancer Lett.* **2018**, *419*, 30–39, doi:10.1016/J.CANLET.2018.01.029.
80. Salabarria, A.-C.; Koch, M.; Schönberg, A.; Zinser, E.; Hos, D.; Hamdorf, M.; Imhof, T.; Braun, G.; Cursiefen, C.; Bock, F. Topical VEGF-C/D Inhibition Prevents Lymphatic Vessel Ingrowth into Cornea but Does Not Improve Corneal Graft Survival. *J. Clin. Med.* **2020**, *9*, 1270, doi:10.3390/jcm9051270.
81. Grigore, A. Plant Phenolic Compounds as Immunomodulatory Agents. In *Phenolic Compounds - Biological Activity*; IntechOpen: London, UK, 2017; pp. 75–98 ISBN 978-953-51-2960-8.
82. Marković, A.K.; Torić, J.; Barbarić, M.; Brala, C.J. Hydroxytyrosol, Tyrosol and Derivatives and Their Potential Effects on Human Health. *Molecules* **2019**, *24*, 2001, doi:10.3390/MOLECULES24102001.
83. Alvarez-Laderas, I.; Ramos, T.L.; Medrano, M.; Caracuel-García, R.; Barbado, M.V.; Sánchez-Hidalgo, M.; Zamora, R.; Alarcón-de-la-Lastra, C.; Hidalgo, F.J.; Piruat, J.I.; et al. Polyphenolic Extract (PE) from Olive Oil Exerts a Potent Immunomodulatory Effect and Prevents Graft-versus-Host Disease in a Mouse Model. *Biol. Blood Marrow Transplant.* **2020**, *26*, 615–624, doi:10.1016/J.BBMT.2019.11.019.
84. Chen, Y.; Dana, R. Autoimmunity in dry eye disease – An updated review of evidence on effector and memory Th17 cells in disease pathogenicity. *Autoimmun. Rev.* **2021**, *20*, 102933, doi:10.1016/J.AUTREV.2021.102933.
85. Pflugfelder, S.C.; Corrales, R.M.; de Paiva, C.S. T helper cytokines in dry eye disease. *Exp. Eye Res.* **2013**, *117*, 118–125, doi:10.1016/J.EXER.2013.08.013.
86. Craig, J.P.; Nelson, J.D.; Azar, D.T.; Belmonte, C.; Bron, A.J.; Chauhan, S.K.; de Paiva, C.S.; Gomes, J.A.P.; Hammitt, K.M.; Jones, L.; et al. TFOS DEWS II Report Executive Summary. *Ocul. Surf.* **2017**, *15*, 802–812, doi:10.1016/j.jtos.2017.08.003.
87. Paiva, C.S. De; Villarreal, A.L.; Corrales, R.M.; Rahman, H.T.; Chang, V.Y.; Farley, W.J.; Stern, M.E.; Niederkorn, J.Y.; Li, D.-Q.; Pflugfelder,

S.C. Dry Eye–Induced Conjunctival Epithelial Squamous Metaplasia Is Modulated by Interferon-γ. *Invest. Ophthalmol. Vis. Sci.* **2007**, *48*, 2553–2560, doi:10.1167/IOVS.07-0069.
88. Solomon, A.; Dursun, D.; Liu, Z.; Xie, Y.; Macri, A.; Pflugfelder, S.C. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. *Investig. Ophthalmol. Vis. Sci.* **2001**, *42*, 2283–2292.
89. Jones, D.; Monroy, D.; Ji, Z.; Atherton, S.; Pflugfelder, S. Sjögren's syndrome: Cytokine and Epstein-Barr viral gene expression within the conjunctival epithelium. *Invest. Ophthalmol. Vis. Sci.* **1994**, *35*, 3493–3504.
90. Pflugfelder, S.C.; Jones, D.; Ji, Z.; Afonso, A.; Monroy, D. Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjogren's syndrome keratoconjunctivitis sicca. *Curr. Eye Res.* **1999**, *19*, 201–211, doi:10.1076/ceyr.19.3.201.5309.
91. Calonge, M. The Treatment of Dry Eye. *Surv. Ophthalmol.* **2001**, *45*, S227–S239, doi:10.1016/S0039-6257(00)00205-8.
92. Stern, M.E.; Gao, J.; Schwalb, T.A.; Ngo, M.; Tieu, D.D.; Chan, C.C.; Reis, B.L.; Whitcup, S.M.; Thompson, D.; Smith, J.A. Conjunctival T-cell subpopulations in Sjögren's and non-Sjögren's patients with dry eye. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 2609–2614.
93. Lee-Huang, S.; Huang, P.L.; Zhang, D.; Lee, J.W.; Bao, J.; Sun, Y.; Chang, Y.-T.; Zhang, J.; Huang, P.L. Discovery of small-molecule HIV-1 fusion and integrase inhibitors oleuropein and hydroxytyrosol: Part I. Integrase inhibition. *Biochem. Biophys. Res. Commun.* **2007**, *354*, 872, doi:10.1016/J.BBRC.2007.01.071.
94. Ford, C.T.; Richardson, S.; McArdle, F.; Lotito, S.B.; Crozier, A.; McArdle, A.; Jackson, M.J. Identification of (poly)phenol treatments that modulate the release of pro-inflammatory cytokines by human lymphocytes. *Br. J. Nutr.* **2016**, *115*, 1699–1710, doi:10.1017/S0007114516000805.
95. Zou, T.; Yang, Y.; Xia, F.; Huang, A.; Gao, X.; Fang, D.; Xiong, S.; Zhang, J. Resveratrol Inhibits CD4+ T cell activation by enhancing the expression and activity of Sirt1. *PLoS One* **2013**, *8*, doi:10.1371/JOURNAL.PONE.0075139.
96. Sun, Y.; Wu, X.X.; Yin, Y.; Gong, F.Y.; Shen, Y.; Cai, T.T.; Zhou, X. Bin; Wu, X.F.; Xu, Q. Novel immunomodulatory properties of cirsilineol through selective inhibition of IFN-γ signaling in a murine model of inflammatory bowel disease. *Biochem. Pharmacol.* **2010**, *79*, 229–238, doi:10.1016/J.BCP.2009.08.014.
97. Yamanaka, D.; Tamiya, Y.; Motoi, M.; Ishibashi, K.; Miura, N.N.; Adachi, Y.; Ohno, N. The Effect of Enzymatically Polymerised Polyphenols on CD4 Binding and Cytokine Production in Murine Splenocytes. *PLoS One* **2012**, *7*, e36025, doi:10.1371/JOURNAL.PONE.0036025.
98. Park, B.; Lee, I.S.; Hyun, S.W.; Jo, K.; Lee, T.G.; Kim, J.S.; Kim, C.S. The protective effect of Polygonum cuspidatum (PCE) aqueous extract in a dry eye model. *Nutrients* **2018**, *10*, doi:10.3390/nu10101550.
99. Luo, L.J.; Lai, J.Y. Epigallocatechin Gallate-Loaded Gelatin-g-Poly(N-Isopropylacrylamide) as a New Ophthalmic Pharmaceutical Formulation for Topical Use in the Treatment of Dry Eye Syndrome. *Sci. Rep.* **2017**, *7*, 1–14, doi:10.1038/s41598-017-09913-8.
100. Li, Y.J.; Luo, L.J.; Harroun, S.G.; Wei, S.C.; Unnikrishnan, B.; Chang, H.T.; Huang, Y.F.; Lai, J.Y.; Huang, C.C. Synergistically dual-functional nano eye-drops for simultaneous anti-inflammatory and anti-oxidative treatment of dry eye disease. *Nanoscale* **2019**, *11*, 5580–5594, doi:10.1039/c9nr00376b.
101. Choi, W.; Lee, J.B.; Cui, L.; Li, Y.; Li, Z.; Choi, J.S.; Lee, H.S.; Yoon, K.C. Therapeutic Efficacy of Topically Applied Antioxidant Medicinal Plant Extracts in a Mouse Model of Experimental Dry Eye. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 1-10, doi:10.1155/2016/4727415.
102. Cui, L.; Lee, H.S.; Li, Y.; Choi, J.H.; Yun, J.J.; Jung, J.E.; Choi, W.; Yoon, K.C. Experimental and clinical applications of chamaecyparis obtusa extracts in dry eye disease. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 1-12, doi:10.1155/2017/4523673.
103. Saito, K.; Mori, S.; Date, F.; Hong, G. Epigallocatechin gallate stimulates the neuroreactive salivary secretomotor system in autoimmune sialadenitis of MRL-Faslpr mice via activation of cAMP-dependent protein kinase A and inactivation of nuclear factor κb. *Autoimmunity* **2015**, *48*, 379–388, doi:10.3109/08916934.2015.1030617.
104. Takahashi, A.; Inoue, H.; Mishima, K.; Ide, F.; Nakayama, R.; Hasaka, A.; Ryo, K.; Ito, Y.; Sakurai, T.; Hasegawa, Y.; et al. Evaluation of the Effects of Quercetin on Damaged Salivary Secretion. *PLoS One* **2015**, *10*, doi:10.1371/JOURNAL.PONE.0116008.
105. Oh, H.N.; Kim, C.E.; Lee, J.H.; Yang, J.W. Effects of Quercetin in a Mouse Model of Experimental Dry Eye. *Cornea* **2015**, *34*, 1130–1136, doi:10.1097/ICO.0000000000000543.
106. de Paiva, C.; Pflugfelder, S.; Ng, S.; Akpek, E. Topical cyclosporine A therapy for dry eye syndrome. *Cochrane database Syst. Rev.* **2019**, *9*, doi:10.1002/14651858.CD010051.PUB2.
107. Schaumburg, C.S.; Siemasko, K.F.; Paiva, C.S. De; Wheeler, L.A.; Niederkorn, J.Y.; Pflugfelder, S.C.; Stern, M.E. Ocular Surface APCs Are Necessary for Autoreactive T Cell-Mediated Experimental Autoimmune Lacrimal Keratoconjunctivitis. J. Immunol. 2011, 187, 3653–3662, doi:10.4049/JIMMUNOL.1101442.
108. Gasteiger, G.; Ataide, M.; Kastenmüller, W. Lymph node – an organ for T-cell activation and pathogen defense. *Immunol. Rev.* **2016**, *271*, 200–220, doi:10.1111/IMR.12399.
109. Annan, J. El; Chauhan, S.K.; Ecoiffier, T.; Zhang, Q.; Saban, D.R.; Dana, R. Characterization of Effector T Cells in Dry Eye Disease. *Invest. Ophthalmol. Vis. Sci.* **2009**, *50*, 3802–3807, doi:10.1167/IOVS.08-2417.
110. Yoon, K.C.; Park, C.S.; You, I.C.; Choi, H.J.; Lee, K.H.; Im, S.K.; Park, H.Y.; Pflugfelder, S.C. Expression of CXCL9, -10, -11, and CXCR3 in the tear film and ocular surface of patients with dry eye syndrome. *Investig. Ophthalmol. Vis. Sci.* **2010**, *51*, 643–650, doi:10.1167/iovs.09-3425.
111. Corrales, R.M.; Villarreal, A.; Farley, W.; Stern, M.E.; Li, D.Q.; Pflugfelder, S.C. Strain-related cytokine profiles on the murine ocular surface in response to desiccating stress. *Cornea* **2007**, *26*, 579–584, doi:10.1097/ICO.0b013e318033a729.
112. Yoon, K.C.; De Paiva, C.S.; Qi, H.; Chen, Z.; Farley, W.J.; Li, D.Q.; Pflugfelder, S.C. Expression of Th-1 chemokines and chemokine receptors on the ocular surface of C57BL/6 mice: Effects of desiccating stress. *Investig. Ophthalmol. Vis. Sci.* **2007**, *48*, 2561–2569, doi:10.1167/iovs.07-0002.
113. Seo, M.J.; Kim, J.M.; Lee, M.J.; Sohn, Y.S.; Kang, K.K.; Yoo, M. The therapeutic effect of DA-6034 on ocular inflammation via suppression of MMP-9 and inflammatory cytokines and activation of the MAPK signaling pathway in an experimental dry eye model. *Curr. Eye Res.* **2010**, *35*, 165–175, doi:10.3109/02713680903453494.
114. Lee, H.; Shim, W.; Kim, C.E.; Choi, S.Y.; Lee, H.; Yang, J. Therapeutic efficacy of nanocomplex of poly(ethylene glycol) and catechin for dry eye disease in a mouse model. *Investig. Ophthalmol. Vis. Sci.* **2017**, *58*, 1682–1691, doi:10.1167/iovs.16-20843.
115. Lee, H.S.; Choi, J.H.; Cui, L.; Li, Y.; Yang, J.M.; Yun, J.J.; Jung, J.E.; Choi, W.; Yoon, K.C. Anti-inflammatory and antioxidative effects of Camellia japonica on human corneal epithelial cells and experimental dry eye: In vivo and in vitro study. *Investig. Ophthalmol. Vis. Sci.* **2017**, *58*, 1196–1207, doi:10.1167/iovs.16-20634.

Chapter 4

Citation

Katsinas, N.; Enríquez-de-Salamanca, A.; da Silva, A.B.; Bronze, M.R.; Rodríguez-Rojo, S. Olive Pomace Phenolic Compounds Stability and Safety Evaluation: From Raw Material to Future Ophthalmic Applications. ***Molecules*** **2021,** ***26***, 6002, doi:10.3390/molecules26196002.

Abstract

Nowadays, increasing interest in olive pomace (OP) valorization aims to improve olive's industry sustainability. Interestingly, several studies propose a high-value application for OP extracts containing its main phenolic compounds, hydroxytyrosol and oleuropein, as therapy for ocular surface diseases. In this work, the stability and accessibility of OP total phenolic and flavonoid content, main representative compounds, and antioxidant activity were assessed under different pretreatment conditions. Among them, lyophilization and supercritical CO_2 extraction were found to increase significantly most responses measured in the produced extracts. Two selected extracts (CONV and OPT3) were obtained by different techniques (conventional and pressurized liquid extraction); Their aqueous solutions were characterized by HPLC-DAD-MS/MS. Additionally, their safety and stability were evaluated according to EMA requirements towards their approval as ophthalmic products: their genotoxic effect on ocular surface cells and their 6-months storage stability at 4 different temperature/moisture conditions (CPMP/ICH/2736/99), together with pure hydroxytyrosol and oleuropein solutions. The concentration of hydroxytyrosol and oleuropein in pure or extract solutions was tracked, and possible degradation products were putatively identified by HPLC-DAD-MS/MS. Hydroxytyrosol and oleuropein had different stability as standard or extract solutions, with oleuropein also showing different degradation profile. All compounds/extracts were safe for ophthalmic use at the concentrations tested.

KEYWORDS: olive pomace; phenolic extracts; oleuropein; hydroxytyrosol; storage stability; genotoxicity assay.

Conclusions

This thesis is a contribution to the search for new therapies for the treatment of dry eye disease, using bioactive compounds of natural origin. Specifically, the use of phenolic extracts derived from olive pomace is proposed because of their proven anti-inflammatory and antioxidant activity. This application of high added value for this by-product of the olive industry, potentially hazardous for the environment, is of great importance for the sustainable development of this industry. Also, the results of the present study illustrate how the sustainable and intensified extraction techniques prove to be competitive compared to conventional ones, also establishing a high selectivity towards compounds with high biological activity for the treatment of dry eye disease. The main conclusions that respond to the specific objectives set are presented below.

1. Freeze-drying followed by a defatting process with supercritical carbon dioxide is the most suitable pretreatment process condition for olive pomace, obtaining a hydroalcoholic extract with a higher concentration in its main phenolic compounds (increments between 90-224%, depending on the compound), as well as a higher chemical antioxidant activity (22% increase), compared to fresh material.

2. The use of pressurized liquid extraction allows the establishment of different optimal extraction conditions, which maximize the richness of the dry extract in the different compounds of interest (oleuropein, hydroxytyrosol, and oleacein), as well as in the chemical antioxidant activity, reducing solvent consumption (hydroalcoholic mixtures) by 1.6 times and the extraction time by 1/3, and increasing the richness by 89-475% (depending on the compound) with respect to the conventional process.

3. Olive pomace extracts, as well as the pure phenolic compounds (oleuropein, hydroxytyrosol, and their combination), do not present cytotoxicity problems in human conjunctival and corneal epithelial cells at concentrations below 0.80 mg/mL for the conventional extract (CONV) and the extract enriched in oleacein and with the highest chemical antioxidant activity (OPT1), 0.40 mg/mL for the extract enriched in oleuropein (OPT2) and the extract enriched in hydroxytyrosol (OPT3), 100 µM for hydroxytyrosol, 300 µM for oleuropein and 5 +50 µM for oleuropein + hydroxytyrosol mixture. Thus, all treatments can be used in the aforementioned doses safely.

4. The use of hydroxytyrosol and the CONV and OPT3 extracts could be useful to alleviate the innate inflammatory response of the corneal and/or conjunctival epithelium in pathologies of the human ocular surface.

5. The use of hydroxytyrosol, oleuropein, alone or in combination at low doses, as well as the CONV and OPT3 extracts, could be useful as antioxidant therapies to alleviate the innate oxidative stress response of the corneal and/or conjunctival epithelium in pathologies of the human ocular surface.

6. The immunosuppressive activity on CD4+ T lymphocytes of hydroxytyrosol, as well as the OPT3 extract, suggests that both treatments could be useful as therapies in immune-based inflammatory pathologies of the human ocular surface.

7. The OPT3 extract and hydroxytyrosol are proved to be promising therapeutic agents for the treatment of dry eye disease, based on the immunoregulatory and anti-inflammatory properties shown *in vivo* in the cervical lymph nodes, the lacrimal gland, the cornea, and the conjunctiva of mice exposed to desiccating stress (dry eye animal model).

8. The CONV and OPT3 olive pomace extracts, as well as the pure phenolic compounds (oleuropein, hydroxytyrosol, and their combination), could be safely used as topical ophthalmic products on the human ocular surface based on their non-genotoxic effect at concentrations of 0.80 mg/mL for CONV, 0.40 mg/mL for OPT3, 100 µM for hydroxytyrosol, 300 µM for oleuropein and 5 + 50 µM for oleuropein + hydroxytyrosol, a necessary assay for the approval of the treatments as future pharmaceutical products.

9. The establishment for the first time of the interactions between oleuropein and hydroxytyrosol and of the degradation profile of the aqueous solutions of oleuropein through the stability studies establishes a necessary evaluation baseline for the future approval of olive pomace extracts and their major compounds as ophthalmic products.

Limitations and Future Studies

This work has some limitations that should be addressed in future studies.

Olive pomace was valorized as a potential source of phenolic bioactive compounds, that could be used as a treatment for dry eye. The material of this study was produced in mid-north Spain (Medina del Campo, Valladolid, Castilla y León) in 2018 and its variety was arbequina, found to be rich in the phenolic compounds of interest. However, no different varieties or different production countries or crops of the same variety were compared. According to the bibliography, climatic, varietal, and geographical factors highly affect the material's richness in polyphenols. In addition, for the phenolic recovery, pressurized liquid extraction was selected as the principal extraction method to be optimized through design of experiments. Although it was compared to conventional methods, however, no comparison was performed with other intensified techniques widely cited in the bibliography, such as ultrasounds, pulsed electric field extraction, etc. Furthermore, the sequential extraction process established was potentially scalable. However, all experiments were performed at a laboratory scale and no scale-up studies were designed, possibly adapting the experimental parameters. Thus, for future studies, it is proposed to:

i) use different varieties, or different production countries and crops of the same variety to compare the variance of the phenolic richness in the raw material;

ii) compare the pressurized liquid extraction efficiency with other intensified techniques, to possibly increase the recovery of phenolic compounds; and

iii) perform semi-continuous experiments to advance in the scale-up of the process.

The antioxidant and anti-inflammatory activity of the treatments were tested *in vitro* on two ocular surface cell lines, the human corneal epithelial (HCE) and the immortalized human conjunctival epithelial (IM-ConjEpi). This type of cell lines is commonly used to study the pathophysiological mechanisms involved in inflammation and oxidative stress of the ocular surface, as well as to test new potential treatments. HCE is a fully characterized cell line, demonstrating high correspondence to the human tissues of origin. However, IM-ConjEpi, a commercial immortalized cell line derived from primary human conjunctival epithelium, is yet not fully characterized, being relatively new. Based on the manufacturer's information, it has a very high purity (ca. 99%) and good correspondence to the human conjunctival epithelium, expressing specific biomarkers, such as CK18 and CK19. Regarding this point, a complete characterization study for this cell line has been performed by the Ocular Surface group of IOBA, and the manuscript is currently under preparation (publication Nº 5 in section: Diffusion/dissemination: Publications/congresses related to this thesis). Moreover, it is true that *in vitro* models do not represent the complexity of a living organism, i.e., human ocular surface epithelium. However, they offer the possibility to study the cellular and biological mechanism of action of the treatments in a short time and at a low cost. They are also necessary before any *in vivo* experiment, as they contribute to the "3Rs" guideline (replacement, reduction, and refinement) for ethical animal research. Another important limitation is that our *in vitro* studies were performed using monolayer cell culture, rather than stratified, considering the 2-7 layers of stratified squamous epithelial cells of the ocular surface epithelia.

Also, although TNF-α acted good as stimulus for inflammatory response, some inflammatory pathways (e.g., MAPK) were not studied, and the stimulation of some cytokines/chemokines (i.e., IL-17A or TNF-α itself) was not achieved. Regarding antioxidant activity, it was studied on both cell lines using as stimulus UV-B radiation and measuring intracellular reactive oxygen species. However, reactive oxygen species are also involved in cellular apoptosis, whereas UV-B is a common inductor of cellular apoptosis in the ocular surface epithelium, which is continuously exposed to the UV rays of light. Hence, further studies should be performed to address the effect of the proposed treatments in ocular surface epithelial cell apoptosis.

Finally, comparing the active concentrations between the pure phenolic compounds and the solutions of the olive pomace extracts, the extracts achieved the same or even higher activity, comprising 2.5 to 40 times less concentration of the major active ingredients. Thus, a strong synergistic effect occurs between the major olive compounds (hydroxytyrosol and/or oleuropein) and others present in the extract. However, it is not clear which are the compounds that play a crucial role in the synergistic activity of the extract.

Hence, for further *in vitro* studies on ocular surface cells, it is suggested to:

i) use stratified human epithelial ocular surface cell cultures, primary human corneal or conjunctival cell cultures, or 3D culture models to study the anti-inflammatory and antioxidant activities of the treatments;

ii) use different inflammation inducers to stimulate the cells and measure additional pro-inflammatory cytokines/chemokines, i.e., IL-4, IL-13, IL-17A, IFN-γ, TNF-α, etc.;

iii) study cellular biological pathways involved in inflammation of the lacrimal functional unit; and

iv) test different pure olive pomace phenolic compounds present in the extracts, alone or in combination with hydroxytyrosol and oleuropein.

Subsequently, the immunosuppressive effect of the phenolic treatments was studied *in vitro* on human CD4+ T cells isolated from peripheral blood mononuclear cells of healthy donors, analyzing their proliferation and viability. However, samples were extracted only from three male subjects. Moreover, no effect was studied regarding cellular mechanisms of action of the antiproliferative effect of the tested phenolic solutions or over the cytokine/chemokine production by isolated stimulated CD4+T cells. Therefore, future studies should include:

i) blood samples obtained from a larger population, including both men and women, and different age groups; and

ii) studies of the mechanism of action of the phenolic solutions on these cells and their effect on cytokine/chemokine secretion.

The anti-inflammatory effect of hydroxytyrosol and a selected hydroxytyrosol-enriched olive pomace extract (namely, OPT3) was studied in a mouse dry eye model of desiccating stress conditions with scopolamine administration. The treatments were administered in parallel with the desiccating stress and thus, the development of dry eye disease. Both treatments were found to be effective in inhibiting the impairment of the disease. However, most patients have already established symptoms when they visit the ophthalmologists. Also, immunohistochemistry was performed to detect CD4+ and CD8+ infiltration in lacrimal functional unit tissues. However, the duration of desiccating stress conditions (14 days) may be considered short to produce enough lymphocyte agglomerates and hence, the data were not considered reliable to be presented. In addition, the phenolic concentrations selected were 100 μM (0.015 mg/mL) for hydroxytyrosol and 0.20 mg/mL for OPT3, demonstrating a strong anti-inflammatory effect. However, no other concentrations were tested for the selected treatments. Also, at the selected concentrations, OPT3 comprised 10 times less hydroxytyrosol compared to the pure compound tested. Therefore, a strong synergistic effect between hydroxytyrosol and other phenolic compounds present in OPT3 was demonstrated, as already confirmed by the *in vitro* studies on human ocular surface cells. Furthermore, it has been proved that oxidative stress plays a very important role in the impairment and development of inflammation in dry eye. However, in our *in vivo* study, no effect of the phenolic solutions was examined on the oxidative stress biomarkers of the lacrimal functional unit. Finally, it is well-known that each animal model represents only some of the aspects of an induced human disease and not all its characteristics. In our case, the *in vivo* model selected is based on the evaporative dry eye and does not represent the pathophysiological mechanisms of the aqueous deficient form of the disease. Thus, further studies may include:

i) application of the treatments after the establishment of the disease (i.e., after 14 days of desiccating stress/scopolamine administration), to examine the treating capacity of the phenolic solutions on the developed dry eye;

ii) a longer duration of desiccating stress/scopolamine administration, which would result in more visible lymphocyte agglomerates in the lacrimal functional unit tissues, to detect the effect of the treatments on their infiltration;

iii) different concentrations of hydroxytyrosol and OPT3 to increase their effectiveness and detect any safety issues;

iv) different pure phenolic compounds present in OPT3, alone or in combination with hydroxytyrosol;

v) examination of the antioxidant effect of the phenolic treatments *in vivo*, based on their proven *in vitro* findings;

vi) measurement of the levels of cytokines/chemokines in the tear fluid of the dry eye mice;

vii) screening of the anti-inflammatory effect of the treatments on an adoptive transfer dry eye mouse model using athymic mice; and

viii) different established animal models of dry eye, such as models representing the aqueous deficient dry eye, to study the effect of the treatments on different aspects of the disease.

Olive pomace extracts and compounds were also evaluated as future ophthalmic products. Different storage and pretreatment conditions were applied to olive pomace, to optimize the factors that could affect the stability and accessibility of polyphenols from the raw material. However, as mentioned previously, several factors can affect the richness of the material in the compounds of interest before the harvest, such as differences in environmental and cultivation conditions. For a plant material to be converted into a pharmaceutical drug, it is necessary to determine the range of concentration of the active ingredients in the raw material and establish that no significant variances occur among the differently produced olive pomace. Furthermore, although all analytical methods were tested and set according to the bibliography, validation of the methods was not completed. In addition, an evaluation of the stability profile and degradation by-products of aqueous solutions of phenolic compounds was established for the first time. However, for hydroxytyrosol, HPLC-DAD-MS/MS method was not adequate to identify the degradation by-products. Also, none of the solutions fulfilled the stability requirements for long-term storage, as all concentrations were below 95% of the initial quantity after 6 months. Finally, for the approval as a pharmaceutical product for human use, all active ingredients have to fulfill the genotoxicity requirements described by the ICH S2 (R1) guideline followed by EMA. In our study, we performed the comet test (DNA strand brake assay), which is one of the most common *in vitro* genotoxicity assays. However, there are additional *in vitro* (such as AMES test, mouse lymphoma Tk gene mutation assay, etc.) and *in vivo* (such as chromosomal damage on rodent hematopoietic cells) tests proposed by the same guideline, whereas eye irritation studies (described by the OECD Test Guideline 492) are also mandatory for the approval of the product. Therefore, future studies could:

i) validate all analytical methods to fulfill the industrial guidelines;

ii) apply different analytical methods to identify the degradation by-products of aqueous solutions of hydroxytyrosol after long-term storage;

iii) perform formulation studies for the aqueous phenolic solutions to increase their stability during long-term storage; and

iv) perform additional *in vitro* or *in vivo* genotoxicity assays, as well as eye irritation tests, as described by the industrial guidelines, to confirm that the phenolic solutions and their formulations are safe for human use.

The final objective of the research is to bring discoveries from the bench to the bedside. This was the purpose of the ITN IT-DED^3 (Integrated Training in Dry Eye Disease Drug Development) consortium; To deliver innovative research ideas, converting them into future therapeutic products for dry eye disease. This doctoral thesis was part of IT-DED^3, also representing the scope of IOBA: "*investigar para curar mejor*" (research to heal better). Hence, the most important future study would be to study the effect of topical administration of aqueous solutions of olive pomace extracts, as well as pure hydroxytyrosol, in patients with dry eye disease. To achieve this objective, the controlled experimental chamber (CER-lab) of IOBA can be used, as temperature, pressure, and humidity can remain stable and controlled. The performance of this study could prove clinically that olive pomace phenolic compounds could contribute to the treatment of dry eye disease.

Resumen

La enfermedad del ojo seco (EOS) es una enfermedad multifactorial de la superficie ocular, que se caracteriza por una pérdida de la homeostasis de la película lagrimal y que va acompañada de síntomas oculares, en la que la inestabilidad e hiperosmolaridad de la superficie ocular, la inflamación y daño de la superficie ocular, y las anomalías neurosensoriales desempeñan papeles etiológicos. Sus principales mecanismos fisiopatológicos incluyen inflamación y estrés oxidativo de la unidad funcional lagrimal, provocando una composición anormal de la película lagrimal y, por tanto, una capa de la película pre-corneal inestable. Además, también se ha observado una infiltración elevada de células inflamatorias (por ejemplo, linfocitos T CD4+) en la conjuntiva y la glándula lagrimal de pacientes con EOS, algo significativamente relacionado con un aumento en los niveles de citoquinas/quimioquinas lagrimales. Actualmente, la prevalencia de la EOS es alta, oscilando entre el 5 y el 50% según la población y afectando considerablemente la calidad de vida de muchos pacientes. La EOS también ocasiona una importante carga económica, no solo por los costes del tratamiento directo, sino también por los costes indirectos (pérdida de la productividad laboral). El uso de estudios preclínicos (modelos *in vitro* e *in vivo*) y clínicos ha contribuido a la comprensión de la enfermedad.

Los enfoques de tratamiento para la EOS se basan en medicamentos antinflamatorios como corticosteroides tópicos, administración tópica u oral de antibióticos, gotas de suero autólogo o inmunosupresores, como ciclosporina A. Los corticosteroides tópicos, como metilprednisolona, fluorometolona y dexametasona, se utilizan ampliamente para el tratamiento de muchas enfermedades inflamatorias, incluida la EOS. Sin embargo, su administración a largo plazo puede tener varias complicaciones, como hipertensión de la presión intraocular, infecciones y cataratas. La ciclosporina A es un inhibidor de la calcineurina, modificando la respuesta de las linfocitos T al inhibir la activación de la interleuquina (IL)-2, y es una de las pocas moléculas aprobadas con la indicación de tratamiento para la EOS. Sin embargo, en muchos países la ciclosporina A no está disponible comercialmente o solo se puede administrar en los casos de EOS más graves. Además, tiene muy baja solubilidad en agua y, por lo tanto, requiere formulaciones a base de aceite o surfactantes, que en su aplicación tópica ocular suelen causar irritaciones y problemas visuales, entre otros problemas. Lifitegrast es otro tratamiento disponible de tipo antiinflamatorio, que actúa inhibiendo la unión de la integrina LFA-1 (que se encuentra expresada en los linfocitos) a la molécula de adhesión intercelular – 1 (ICAM-1), que se expresa en los linfocitos T y el epitelio ocular inflamado. Sin embargo, aunque se comprobó que era eficaz para mejorar la sequedad y el malestar ocular, no se demostró ningún efecto en la tinción corneal después de casi 3 meses de tratamiento.

Por tanto, todavía hay una escasez de terapias eficaces para la EOS y se continúa buscando compuestos alternativos para una terapia eficaz con actividad antinflamatoria, y también antioxidante para reducir el estrés oxidativo. En este sentido, se ha demostrado que los compuestos fenólicos de origen natural pueden una opción terapéutica eficaz para la EOS. Entre ellos, los compuestos fenólicos presentes en el olivo han demostrado una amplia gama de actividades biológicas, incluida la prevención de la oxidación de lipoproteínas de baja densidad, efectos antioxidante y antiinflamatorio y la protección frente a agentes externos. El principal producto del olivo es el aceite de oliva virgen; existen varios procedimientos para producir el aceite de oliva del fruto de oliva (aceituna). De entre todos ellos, hoy en día el sistema de "separación por centrifugación bifásica" es el más utilizado, en el que se generan subproductos de desecho, como el alperujo,

las aguas residuales del molino, las hojas y los huesos. Se ha demostrado que solo el 1-2% de los compuestos fenólicos de la aceituna terminan en el aceite de oliva, ya que el 45% de ellos se quedan en el alperujo. El alperujo es el subproducto semisólido y constituye una mezcla de pulpa de aceituna, aguas de vegetación y de proceso, y huesos de aceituna. En peso, es el principal subproducto producido por el sistema bifásico. En concreto, se producen entre 7 y 30 millones de m^3 al año solo en la cuenca mediterránea, quedándose almacenados en pilas al aire libre. Tiene una alta carga orgánica, salinidad y humedad (aprox. 55-70%), y debido a su alto contenido en compuestos fenólicos posee propiedades antimicrobianas, pero también altamente fitotóxicas. Por lo tanto, constituye un factor de contaminación importante para la atmósfera, el agua y el suelo, y la mayoría de los países productores no permiten su vertido a los ríos o al suelo. Además, el alperujo producido contiene una cantidad de aceite residual significativa por lo que se procesa para recuperarlo. La recuperación del aceite residual produce el llamado "aceite de orujo", que se encuentra disponible en el mercado, y se realiza mediante el secado del alperujo y una extracción con disolventes orgánicos tóxicos (hexano). Por todo ello existe una creciente conciencia global para la valorización de este subproducto, incluyendo tratamientos fisicoquímicos, transformaciones biotecnológicas, extracción de compuestos útiles para la industria cosmética, depuración de aguas residuales y compostaje de residuos sólidos. Todas estas estrategias se pueden realizar en el alperujo desgrasado (después de la eliminación del aceite de orujo).

Una de las estrategias de valorización más importantes para el alperujo es la recuperación de sus compuestos fenólicos, ya que éstos pueden tener numerosas aplicaciones de alto valor añadido para la salud humana. Entre ellos, las clases químicas más citadas de los mismos son los fenoles simples, (por ejemplo, hidroxitirosol (HT) y tirosol (TY)) y los secoiridoides, (por ejemplo, oleuropeína (OL) y oleaceína (OLC)). Otros polifenoles tales como ácidos hidroxibenzoicos, ácidos hidroxifenilacéticos, ácidos hidroxicinámicos, ésteres de ácidos grasos fenólicos, metoxifenoles, glucósidos, flavonoides, iridoides, cumarinas, lignanos e hidroxi-isocromanos también se encuentran presentes. Muchos de estos compuestos contribuyen a las propiedades antimicrobianas y fitotóxicas del alperujo. Por otro lado, numerosas actividades biológicas, tales como antioxidante, antiinflamatoria, cardioprotectora y antiaterogénica, se han demostrado para el hidroxitirosol, el tirosol, la oleuropeína y la oleaceína. Para el hidroxitirosol también se han demostrado actividades antimicrobianas y quimioprotectoras, mientras que para oleuropeína también se han descrito actividades antihipertensivas, endocrinas, citostáticas, molusquicidas, antimicrobianas y antivirales, junto con modulación enzimática. Para la oleaceína, también se ha demostrado una mejora de la sensibilidad a la insulina, junto con la actividades antitumoral/anticancerígena. Además, extractos hidroalcohólicos crudos de oliva han demostrado actividades antirradicales y antiinflamatorias en células de la superficie ocular.

Por lo tanto, el alperujo rico en polifenoles, podría ser una fuente valiosa de agentes terapéuticos para la EOS, contribuyendo también a la valorización de un subproducto agroindustrial peligroso para el medio ambiente y al crecimiento sostenible de la industria oleícola.

1. Hipótesis

Es posible utilizar compuestos y extractos fenólicos presentes en los subproductos de la industria del aceite de oliva, como el alperujo, con probada actividad antioxidante y antinflamatoria, como agentes terapéuticos para enfermedades inflamatorias de base inmune de la superficie ocular, tales como la enfermedad del ojo seco o alergias.

2. Objetivos

Los objetivos generales y específicos para comprobar la hipótesis de esta tesis son:

2.1 Objetivo General:

Obtención óptima, evaluación del uso tópico ocular y evaluación de la estabilidad y de la seguridad de los compuestos fenólicos del alperujo para su futuro uso como tratamiento de la enfermedad del ojo seco.

2.2 Objetivos Específicos:

1. Establecer el método de pretratamiento/almacenamiento más eficiente para el alperujo que conduce a la mayor estabilidad de sus principales compuestos fenólicos (OL e HT) en el extracto obtenido.
2. Establecer las condiciones de extracción más eficientes para cada uno de los compuestos fenólicos seleccionados del alperujo, dando lugar a un extracto con la mayor concentración de los principios activos de interés.
3. Establecer *in vitro* la citotoxicidad de los compuestos puros y los extractos seleccionados en dos líneas celulares procedentes del epitelio conjuntival y corneal humano.
4. Determinar *in vitro* el efecto antinflamatorio de extractos derivados del alperujo (eligiendo el/los extracto/s de interés), y de sus compuestos fenólicos principales puros (OL, HT y su combinación), en dos líneas celulares procedentes de los epitelios conjuntival y corneal humano estimuladas con factor de necrosis tumoral (TNF)-α.
5. Determinar *in vitro* el efecto antioxidante de extractos derivados del alperujo (eligiendo el/los extracto/s de interés), y de sus compuestos fenólicos principales puros (OL, HT y su combinación), en dos líneas celulares procedentes de los epitelios conjuntival y corneal humano estimuladas con luz UV-B.
6. Determinar la actividad inmunorreguladora de los extractos elegidos y de sus compuestos fenólicos principales puros (OL e HT), en linfocitos T CD4+ procedentes de sangre periférica humana.
7. Determinar *in vivo* el efecto terapéutico de los extractos y compuestos seleccionados en un modelo murino de ojo seco.
8. Establecer *in vitro* la seguridad (genotoxicidad celular) de los compuestos puros y los extractos seleccionados en dos líneas celulares procedentes del epitelio conjuntival y corneal humano, ensayos necesarios para su aprobación como futuros productos farmacéuticos.
9. Determinar la estabilidad a largo plazo de soluciones acuosas de los compuestos puros y los extractos seleccionados, como requisito para su aprobación como futuros productos oftálmicos.

3. Metodología

3.1 Valorización del Alperujo

3.1.1 Reactivos

Los reactivos usados en esta parte experimental han sido agrupados y presentados por fabricante:

- *Carburos Metálicos* (Barcelona, España): CO_2 (99.95%).
- *Dávila Villalobos S.L.* (Valladolid, España): EtOH no desnaturalizado (99.9%).
- *Extrasynthese* (Genay, Francia): HT (≥98%), TY (≥99%), OL (≥98%).
- *Linde Gas* (Puc◎ol, España): N_2 (99.996%).
- *Linde Gas* (Puc◎ol, España): N_2 (99.996%).
- *Panreac Quimica SLU* (Barcelona, España): Reactivo de Folin-Ciocalteu, Na_2CO_3, $AlCl_3$, NaOH, metanol (MeOH, 99,9% LC-MS), dimetilsulfóxido (DMSO), ácido fosfórico, *n*-hexano.
- *Sigma-Aldrich* (St Louis, MO, EE. UU.): Trolox (ácido 6-hidroxi-2,5,7,8-tetrametilcromano-2-carboxílico), AAPH (2,2′-azobis-(2-metilpropionamidina) diclorhidrato), ácido gálico, catequina.
- *Thermo Fisher Scientific* (Rockford, IL, EE. UU.): $NaNO_2$.
- *Vetec Quimica* (Xerem Duque De Caxias, Rio de Janeiro, Brasil): Sal de fluoresceína sódica.

3.1.2 Materia Prima: Alperujo

El alperujo de variedad Arbequina (cosecha 2018) fue entregado por Oliduero (Medina del Campo, España). La caracterización detallada de la materia prima inicial se presenta en la Tabla 1. La humedad se determinó por gravimetría secando el material a 105 °C. La grasa y los extractos se definieron mediante 3 extracciones consecutivas en Soxhlet utilizando 3 disolventes diferentes: *n*-hexano durante 6 horas (grasa) y etanol (EtOH) y agua durante aprox. 18 horas cada uno (extractivos). El contenido de proteínas se determinó mediante el método Kjeldahl (con un factor de conversión de 6.25). El contenido de cenizas correspondió al carbón formado a 550 °C.

Tabla 1. Caracterización del alperujo

Humedad	Grasa	Ceniza	Proteína	Extractivos
$g_{H2O}/g_{ALPERUJO\ SECO}$	$mg/g_{ALPERUJO\ SECO}$	$mg/g_{ALPERUJO\ SECO}$	$mg/g_{ALPERUJO\ SECO}$	$mg/g_{ALPERUJO\ SECO}$
1.48 ± 0.01	200 ± 6	25.2 ± 1.7	143 ± 4	479 ± 7

3.1.3 Estudio del Efecto del Método de Almacenamiento/Pretratamiento en la Estabilidad de los Compuestos Fenólicos.

Para determinar en qué condiciones de almacenamiento/pretratamiento del alperujo era mayor la estabilidad de los principales compuestos fenólicos se analizó la misma en 4 condiciones diferentes de pretratamiento: fresco, descongelado, liofilizado (FD-OP) y secado. El material se sometió posteriormente a extracción convencional sólido – líquido (ver sección 3.1.4.1).

Condiciones de Pretratamiento/Almacenamiento

Se han probado cuatro condiciones de pretratamiento/almacenamiento diferentes para la materia prima, previo al proceso de extracción: fresco, congelación/descongelación (descongelado), liofilización (FD-OP) y congelación/descongelación+desecación (secado). Brevemente, una parte del alperujo fresco se sometió al llegar a extracción hidroalcólica (sin ningún pretratamiento previo). El resto del material se empaquetó en bolsas de plástico de aproximadamente 1 kg de peso bajo atmósfera de N_2 y se almacenó congelado a –20 °C durante 4 meses. El alperujo descongelado se sometió a extracción (apartado 3.1.4.1) directamente después de la descongelación. Otra parte de alperujo descongelado se liofilizó al vacío (18 kPa) (FD-OP) y se protegió de la luz durante 72 horas. El alperujo seco se produjo colocando el alperujo congelado en una cámara a 40 °C durante 24 horas para un secado lento. La humedad del alperujo fresco y descongelado fue 59.7 ± 0.2% (1.48 ± 0.01 $g_{H2O}/g_{ALPERUJO\ SECO}$) (Tabla 1). El material secado y FD-OP tenían aprox. 45% y 3% de humedad, respectivamente, y también se sometieron a extracción directamente después del pretratamiento.

Pretratamiento Desgrasante del Alperujo

El alperujo está compuesto por una fracción de aceite residual y componentes lipofílicos. La superficie ocular se compone principalmente de tejidos similares al agua, como la película lagrimal y el humor acuoso. Por lo tanto, para lograr una buena difusión del fármaco en los tejidos oculares, deben diseñarse formulaciones a base de agua. Por lo tanto, un paso de desgrasado previo a la extracción de fenoles se consideró necesario para eliminar cualquier aceite residual de la materia prima considerando su futura aplicación oftálmica, así como para recuperar el aceite de orujo.

Para el proceso de desgrasado se eligió FD-OP y se realizaron dos métodos diferentes: uno, un proceso convencional con *n*-hexano, y otro, con extracción de dióxido de carbono supercrítico ($scCO_2$). El efecto de ambos métodos se examinó sobre la estabilidad de los compuestos fenólicos en el extracto hidroalcohólico obtenido, así como su capacidad de extraer el aceite residual.

Proceso de Desgrasado con *n*-Hexano

En un matraz de fondo redondo, se mezclaron 4.33 g de FD-OP con 20 mL de *n*-hexano. El matraz se puso en un baño termostático a 30 °C durante 15 minutos con agitación magnética (750 rpm). Posteriormente, la solución se recogió, se centrifugó y se secó usando un rotavapor (Buchi Rotavapor R-200, Flawil, Suiza) a 30°C y aprox. 20 kPa. A continuación, se pesó el extracto seco para calcular el porcentaje del rendimiento del aceite eliminado.

Proceso de Desgrasado con Dióxido de Carbono Supercrítico ($scCO_2$)

En un extractor de acero inoxidable se cargaron 406.4 g de FD-OP con aproximadamente 3% de humedad, junto con cerámica Raschig, no solo para llenar el extractor por completo sino también para evitar la obstrucción y la formación de canales preferenciales. Se elevó la temperatura del sistema (60 °C) y se introdujo CO_2 en el extractor hasta la presión de extracción indicada (30 MPa). El caudal se fijó en 10.5

kg CO_2/h durante 3 horas. En el separador, la temperatura se fijó en 20 °C y la presión en 6 MPa. Una vez completado el experimento, se pesó el FD-OP desgrasado (FD-OP-DO) para calcular el porcentaje del rendimiento del aceite eliminado.

3.1.4 Extracción de Compuestos Fenólicos del Alperujo

Como referencia, se usó una extracción convencional sólido-liquido. A continuación, para obtener extractos de alperujo ricos en sus principales compuestos fenólicos bioactivos, se estableció un proceso de extracción secuencial optimizado, respetuoso para el medio ambiente y apropiado industrialmente. Se seleccionó la extracción de $scCO_2$ al FD-OP, seguida de una optimización de la extracción de líquido presurizado (*Pressurized liquid extraction*, PLE) a través del diseño de experimentos. Para el diseño de experimentos, las metodologías de superficie de respuesta son las que se utilizan principalmente, debido a su capacidad de determinar la interacción entre las variables del proceso. Entre ellas, se seleccionó el diseño compuesto central (*Central Composite Design*) por su mejor capacidad predictiva en comparación con otros diseños.

Extracción Convencional Sólido-Líquido

Para estudiar el efecto de las condiciones de almacenamiento/pretratamiento sobre la materia prima, todos los materiales (frescos, descongelados, FD-OP o secos) fueron sometidos a la misma extracción sólido-líquido convencional. También, el mismo método de extracción se usó para estudiar el efecto de los procesos de desgrasado (con n-hexano y con $scCO_2$). Brevemente, la materia prima se mezcló con el solvente (50.0% v/v de EtOH en agua) en un matraz de fondo redondo con una relación sólido/líquido de 0.5 $g_{ALPERUJO}$/ $mL_{SOLVENTE}$ a 70.0 °C. El matraz se colocó en un baño termostático a la temperatura deseada durante 1 hora. La velocidad de agitación se ajustó a 750 rpm. Cuando se completó la extracción, los extractos se centrifugaron a 6100*g* (Sigma 2-16P; Sigma Laborzentrifugen GmbH, Osterode am Harz, Alemania) durante 15 minutos a temperatura ambiente. El mismo proceso y condiciones de extracción se aplicaron a todas las materias primas pretratadas de manera diferente.

Extracción de Líquido Presurizado (PLE): Diseño de Optimización

Para la optimización de la PLE a través del diseño de experimentos, se seleccionó un diseño compuesto central, utilizando el software Statgraphics Centurion 18.0 (Statgraphics Technologies, Inc., Virginia, EE. UU.). Los factores establecidos fueron 3: el porcentaje de EtOH en agua, la temperatura y la relación sólido/líquido. Cada factor (numérico) se estimó en cinco niveles; ± 1 (puntos factoriales), ± a (puntos axiales) y un punto central replicado 10 veces, lo que resultó en un total de 24 experimentos (Tabla 2). El porcentaje de EtOH en agua se ajustó entre 8.0 y 92.0% v/v para cubrir un amplio intervalo de polaridad. La temperatura varió entre 65.0 y 185.0 °C para estudiar la posible descomposición de los compuestos. La relación sólido/líquido varió de 0.2 a 0.8 $g_{ALPERUJO}$/ $mL_{SOLVENTE}$ para describir el efecto de la transferencia de masa interna.

Tabla 2. Diseño compuesto central para la optimización de la extracción de líquido presurizado (PLE) - 24 experimentos.

Orden	Temperatura (°C)	Porcentaje de EtOH en agua	Relación Sólido/Liquido (g/mL)
1	160.7	75.0	0.7
2	185.0	50.0	0.5
3	89.3	25.0	0.3
4	**125.0**	**50.0**	**0.5**
5	125.0	50.0	0.8
6	**125.0**	**50.0**	**0.5**
7	125.0	8.0	0.5
8	**125.0**	**50.0**	**0.5**
9	160.7	75.0	0.3
10	**125.0**	**50.0**	**0.5**
11	**125.0**	**50.0**	**0.5**
12	89.3	75.0	0.7
13	160.7	25.0	0.7
14	125.0	50.0	0.2
15	160.7	25.0	0.3
16	**125.0**	**50.0**	**0.5**
17	**125.0**	**50.0**	**0.5**
18	89.3	25.0	0.7
19	**125.0**	**50.0**	**0.5**
20	**125.0**	**50.0**	**0.5**
21	125.0	92.0	0.5
22	**125.0**	**50.0**	**0.5**
23	65.0	50.0	0.5
24	89.3	75.0	0.3

Los 10 puntos centrales se presentan en negrita.

Dentro de las distintas opciones del material usado para el estudio PLE, se seleccionó el alperujo liofilizado desgrasado con $scCO_2$ (FD-OP-DO). En cada experimento de extracción PLE, se mezcló una cantidad adecuada (g) de FD-OP-DO (de 1.4 a 7.1 g, dependiendo de la relación sólido/líquido) con 20 mL de solvente. La velocidad de agitación se fijó en 750 rpm. El sistema se calentó y la extracción comenzó cuando se alcanzó la temperatura deseada (el tiempo varió entre 5 y 8 minutos). Antes de iniciar el proceso, se ajustó la presión a 10 MPa mediante aplicación de N_2. El procedimiento fue estático y duró 20 minutos (según la bibliografía). Cuando se completó la extracción, el reactor se enfrió en un baño de hielo hasta llegar a temperatura ambiente (el tiempo varió entre 3 y 5 minutos). El extracto obtenido se centrifugó a 6100*g* (Sigma 2-16P; Sigma Laborzentrifugen GmbH, Osterode am Harz, Alemania) durante 15 minutos a temperatura ambiente y se almacenó a –20 °C y en oscuridad hasta su análisis.

3.1.5 Extractos Elegidos

En base a los resultados obtenidos un total de 5 extractos fueron elegidos para los estudios posteriores: dos extractos producidos con extracción convencional sólido-líquido, y tres extractos producidos por la optimización PLE. Las condiciones y el método de extracción, el pretratamiento de la materia prima, así como los códigos de cada extracto están incluidos en la Tabla 3.

Tabla 3. Extractos seleccionados para estudios posteriores

Extracto	Material usado	Condiciones		
		Temperatura (°C)	% de EtOH en agua	Relación Sólido/Liquido ($g_{ALPERUJO}$/ $mL_{SOLVENTE}$)
CONV	Liofilizado	70.0	50.0	0.5
CONV-2	Liofilizado desgrasado con dióxido de carbono supercrítico	70.0	50.0	0.5
OPT1		66.0	10.0	0.8
OPT2		66.0	92.0	0.8
OPT3		184.0	90.0	0.8

El extracto CONV-2 se usó como referencia para el estudio de optimización del PLE (apartado 3.1.4), comparando la riqueza del extracto en los polifenoles de interés (apartado 3.1.7) con los extractos OPT1, OPT2 y OPT3. CONV se usó como referencia para comparar la seguridad y la actividad antioxidante, antinflamatoria e inmunosupresora *in vitro* de los extractos OPT1, OPT2 y OPT3 (apartados 3.2 y 3.3).

3.1.6 Identificación Putativa por HPLC-DAD-MS/MS de Compuestos Fenólicos de Interés en el Extracto Derivado de Alperujo Liofilizado y Desgrasado con Extracción de Dióxido de Carbono Supercrítico (Extracto CONV-2)

La identificación putativa de los compuestos fenólicos presentes en el extracto CONV-2 se realizó mediante un sistema HPLC-DAD-MS/MS en colaboración con la facultad de Farmacia de Lisboa. Los análisis de HPLC se realizaron en un Waters Alliance 2695 (Waters, Irlanda, Reino Unido) equipado con una bomba cuaternaria, un desgasificador de disolvente, un inyector automático (volumen de inyección: 10 μL) y un horno de columna. El sistema está acoplado a un detector de matriz de fotodiodos Waters 996 PDA (Waters, Irlanda, Reino Unido) con una absorción de longitud de onda de barrido de 210 a 600 nm. La columna utilizada fue una LiChrospher 100 RP-18 5 μm (250 × 4.0 mm) (Sigma-Aldrich, San Luis, Misuri, EE. UU.) a 35 °C. Se aplicó un método de gradiente con dos eluyentes: A (agua MiliQ con 0,5% de HCOOH), y B (metanol). Se estableció un caudal de 0.3 mL/min y se aplicó el siguiente programa de elución: 0-15 min isocrático al 70%; Gradiente lineal de 15-45 min al 60% A; 45-60 min isocrático 60% A; Gradiente lineal de 60-75 min al 55% A; 75-105 min isocrático a 55% A, y finalmente regresando a las condiciones iniciales durante 20 minutos. La detección por espectrometría de masas en tándem (MS/MS) se realizó en un triple cuadrupolo Micromass Quattro Micro (Waters, Irlanda, Reino Unido) utilizando una fuente de ionización por electro-pulverización en el modo de iones negativos (ESI-). La temperatura de la fuente era de 120 °C, y los voltajes del capilar y de la fuente fueron de 2.5 kV y 20 V, respectivamente. Los compuestos separados por HPLC se ionizaron y los espectros de masas se registraron en el modo de barrido completo con *m*/*z* de 60 a 1100. Las energías de colisión se optimizaron para cada compuesto (10, 20 y 30 eV). Se utilizó N_2 de alta pureza tanto como gas de secado como de nebulización. Se usó argón (Ar) de ultra alta pureza como gas de colisión. Para la adquisición y procesamiento de datos, se utilizó el software MassLynx versión 4.1 (Waters, Irlanda, Reino Unido).

3.1.7 Caracterización de los Extractos Fenólicos Obtenidos del Alperujo

Se evaluó la riqueza de todos los extractos de alperujo obtenidos de manera diferente en sus principales compuestos fenólicos bioactivos (OL, HT y TY). También, se estudió la riqueza de los extractos en OLC, ya que se encontró que era el polifenol más abundante en el extracto CONV-2 (referencia) mediante el análisis HPLC-DAD-MS/MS. La eficiencia de extracción se definió por el rendimiento de extracción (EY) y los extractos también se caracterizaron en términos de contenido fenólico total (TPC), contenido total de flavonoides (TFC) y actividad antioxidante química (CAA).

Rendimiento de Extracción (EY)

Los extractos se secaron de acuerdo con el siguiente procedimiento: primero, se evaporó el EtOH usando un rotavapor (Buchi Rotavapor R-200, Flawil, Suiza) a 60 °C y aprox. 20 kPa. A continuación, el extracto conteniendo principalmente agua se liofilizó al vacío (18 kPa) y protegido de la luz durante 48 horas (Lyoquest-55, Telstar, Terrassa, España). Después, el extracto seco se pesó y se calculó el EY.

Determinación del Contenido Fenólico Total (TPC)

El TPC de los extractos se determinó mediante el método Folin-Ciocalteu. Brevemente, 40 μL de muestra se mezclaron con 3 mL de agua destilada y 200 μL de reactivo Folin-Ciocalteu. Después de 5 minutos, se añadieron 600 μL de Na_2CO_3 (20% w/v) y la mezcla se incubó a 40 °C durante 30 minutos. La absorbancia se midió a 765 nm (UV 2550, espectrofotómetro UV/Vis, Shimadzu GmbH, Kioto, Japón). Los resultados de TPC se calcularon utilizando una curva de calibración de ácido gálico (GA).

Determinación del Contenido Ttotal de Flavonoides (TFC)

1 mL de extracto se diluyó a 10 mL con agua destilada, se agregaron 300 μL de $NaNO_2$ (5% w/w) y la mezcla se dejó reaccionar durante 5 minutos. Posteriormente, 500 μL de $AlCl_3$ (2% w/w) y 500 μL de NaOH (1 M) se añadieron en la solución y la mezcla se incubó a temperatura ambiente durante 6 minutos. La absorbancia se midió a 510 nm (UV 2550, espectrofotómetro UV/Vis, Shimadzu GmbH, Kioto, Japón) y los resultados de TFC se calcularon usando una curva de calibración de catequina.

Determinación de la Actividad Antioxidante Química (CAA)

El método utilizado para la evaluación de la CAA de los extractos fue el ensayo de capacidad de absorbancia de radicales de oxígeno (ORAC). Los valores de ORAC se calcularon usando una ecuación de regresión entre la concentración de trolox y el área bajo el decaimiento de la curva de sal de fluoresceína sódica (AUC) para cada muestra de acuerdo con la curva de calibración para trolox. Para cada muestra, se probaron cuatro concentraciones diferentes (diluyendo el extracto de 4000 a 11000 veces) creando una curva de calibración para cada extracto. La fluorescencia se midió a 530 nm_{em} y 485 nm_{ex}, utilizando un lector de fluorescencia de microplacas FL800 (Bio-Tek Instruments, Winooski, Vermont, EE. UU.).

Determinación del Contenido en OL, HT, TY y OLC: Análisis HPLC-DAD

La determinación del contenido en OL, HT, TY y OLC se realizó mediante análisis por HPLC-DAD. El sistema HPLC usado fue un Waters e2695 *Separation module* con un muestreador automático (volumen de inyección: 20 µL) y una bomba cuaternaria junto con un detector de matriz de fotodiodos Waters 2998 ajustado a 280 nm (Waters, Irlanda, Reino Unido). La columna utilizada fue una C18 Mediterranean Sea (250 × 4,6 mm, 5 µm) a 35 °C (Teknokroma Analıtica S.A., Barcelona, España). También se utilizó una pre-columna OptiGuard de 1 mm (Sigma-Aldrich, San Luis, Misuri, EE. UU.). Se aplicó un método de gradiente usando eluyente A (agua acidificada a pH = 3 con ácido fosfórico) y eluyente B (metanol). Se estableció un flujo de 1 mL/min y se aplicó el siguiente programa de elución: gradiente lineal de 0 a 10 min de 90 a 80% A, 10-16 min 80% A isocrático; Gradiente lineal de 16 a 20 min a 70% A; 20-25 min 70% A isocrático; Gradiente lineal de 25 a 35 min a 60% A; 35-40 min 60% A isocrático; Gradiente lineal de 40 a 45 min a 55% A; 45-55 min 55% A isocrático; Gradiente lineal de 55 a 60 min a 40% A; Gradiente lineal de 60 a 65 min a 30% A; y gradiente lineal de 65 a 70 min hasta 0% A. Las soluciones de todos los compuestos estándares se prepararon en DMSO y se inyectaron usando el mismo método analítico y las mismas condiciones con las muestras. OLC se calculó como equivalentes de OL (OLE). Para la adquisición y procesamiento de datos, se utilizó el software Empower 3 (Waters, Irlanda, Reino Unido).

3.2 Estudios *In vitro* en Células Epiteliales de la Superficie Ocular Humana

3.2.1 Reactivos

Los reactivos usados en esta parte de la tesis han sido agrupados y presentados por fabricante:

- *BioNova scientific* (Fremont, CA, EE.UU.): TNF-α.
- *Extrasynthese* (Genay, Francia): HT (≥98%), OL (≥98%).
- *bioNova scientific* (Fremont, CA, EE. UU.): OxiSelect 96-Well Comet Assay Kit.
- *Panreac* (Barcelona, España): EtOH.
- *Sigma-Aldrich* (St Louis, MO, EE. UU.): diacetato de 2',7'-diclorodihidrofluoresceína (H_2DCF-DA), insulina bobina, DMSO, fluoruro de fenilmetilsulfonilo (PMSF), aprotinina, ortovanadato sódico (Na_3VO_4), 2,3-bis-(2-metoxi-4-nitro-5-sulfofenil)-2H-tetrazolio-5-carboxanilida (XTT), metilsulfato de 5-metilfenazinio (PMS), Kit magnético Milliplex Human Cytokine / Chemokine HCYTOMAG-60K-5 plex (IL-1beta, IL-6, IL-8 / CXCL8, IL-17A e IP-10).
- *Thermo Fisher Scientific* (Rockford, IL, EE. UU.): matraces, placas, puntas, pipetas de cultivo de plástico, Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 (DMEM/F12) + GlutaMax, DMEM (medio de cultivo sin $NaHCO_3$, piruvato sódico y rojo fenol), RIPA buffer de lisis RIPA, Dulbecco's tampón fosfato salino (DPBS), suero fetal bobino (FBS), factor de crecimiento epitelial humano (EGF), insulina humana, penicilina, estreptomicina, ácido bicinconínico (BCA), solución tampón Tris-EDTA.

3.2.2 Cultivos Celulares

Para los estudios *in vitro* de esta parte de la tesis, se seleccionaron dos líneas celulares epiteliales humanas de la superficie ocular: HCE e IM-ConjEpi.

La línea celular HCE es una línea celular epitelial corneal humana inmortalizada con el antígeno T grande del virus simio 40 (SV40) y que nos fue cedida por el Profesor Arto Urti (Universidad de Helsinki, Finlandia). Las células HCE se cultivaron en medio DMEM/F-12 + GlutaMax, suplementado con 10 ng/mL de EGF, FBS al 10%, 5 μg/mL de insulina humana, 100 U/mL de penicilina y 0.1 mg/mL de estreptomicina.

La línea celular IM-ConjEpi es una línea celular epitelial conjuntival humana inmortalizada con el antígeno T grande del SV40, adquirida de Innoprot (Derio, España). Las células IM-ConjEpi se cultivaron en medio DMEM/F-12 + GlutaMax suplementado con 10 ng/mL de EGF, FBS al 10%, 1 μg/mL de insulina bovina, 5000 U/mL de penicilina y 5000 μg/mL de estreptomicina.

3.2.3 Preparación de las Soluciones Fenólicas

El día del experimento, HT y OL se disolvieron en medio de cultivo (DMEM/F-12 + GlutaMax puro sin ningún suplemento), mientras que los extractos CONV, OPT1, OPT2 y OPT3 fueron disueltos en medio de cultivo suplementado con EtOH al 0.4%. A continuación, se realizaron diluciones seriadas para alcanzar las concentraciones deseadas (1-150 μM para HT, 5/325 μM para OL, 0.05-0.80 mg/mL para CONV, 0.05-0.40 mg/mL para OPT1, 0.05-0.40 mg/mL para OPT2 y 0.005-0.600 mg/mL para OPT3) con medio de cultivo. Las mezclas de OL con HT (5 μM de OL con 10, 25, o 50 μM de HT y una mezcla de 10 μM de OL con 50 μM de HT) se prepararon mezclando la doble concentración de cada compuesto en volúmenes iguales.

3.2.4 Seguridad Ocular para los Compuestos Fenólicos y Extractos

Antes de probar el efecto antioxidante y antinflamatorio *in vitro* sobre las células epiteliales de la superficie ocular humana, es necesario establecer las concentraciones máximas permisibles (no tóxicas) que no disminuyan la viabilidad celular por debajo del 90%. También, las pruebas obligatorias para la aprobación y liberación de una sustancia o un producto farmacéutico en el mercado están descritas por la directriz ICH S2 (R1) de la EMA, que determina los diferentes ensayos aceptables para el cribado de posibles daños genéticos, a fin de predecir y evitar posibles riesgos humanos. Entre ellos, el ensayo Comet se propone como una técnica confiable y común para detectar la genotoxicidad *in vitro* de un compuesto, detectando varios tipos de daño del ADN en células individuales.

Ensayo de Viabilidad Celular (XTT)

Las células HCE e IM-ConjEpi se plantaron en placas de 96 pocillos y se dejaron crecer en medio

suplementado durante 3 días, hasta alcanzar una confluencia de 90% de la superficie del pocillo. Después, el medio de cultivo se reemplazó por DMEM/F-12 + GlutaMax sin ningún suplemento. A las 24 horas, se descartó el medio y las células se trataron con las diferentes concentraciones de OL, HT, OL + HT, CONV, OPT1, OPT2 o OPT3 y se incubaron durante 24 horas a 37 °C. Células tratadas solo con vehículo se usaron como control negativo, mientras que células tratadas con 0.001% de cloruro de benzalconio se usaron como control positivo. Pasadas las 24 horas, los sobrenadantes se eliminaron y DMEM/F-12 sin rojo fenol se añadió, junto con 10 μL de 3 mg de PMS/mL de DPBS mezclado con 4 mL de 1 mg de XTT/mL de DMEM/F-12 sin rojo fenol. Después de 4 horas de incubación a 37 °C, se midió la absorbancia (450 nm y 660 nm) en un espectrofotómetro UV/vis (SpectraMax M5; Molecular Devices, Sunnyvale, CA, EE. UU.).

Ensayo de Genotoxicidad (Comet)

De acuerdo con las instrucciones del fabricante, células HCE o IM-ConjEpi se mezclaron con agarosa líquida, se colocaron en un portaobjetos de 96 pocillos recubierto con agarosa y se dejaron a 4 °C durante 15 minutos hasta solidificación. Después, el portaobjetos se sumergió en tampón de lisis (2.5 μM de NaCl, 100 mM de Na_2EDTA, 10 mM de Tris-HCl, N-laurosil-sarcosina al 1%, Triton X-100 al 1%, DMSO al 10%, pH = 10) a 4 °C durante 60 minutos. Posteriormente, se transfirió a un recipiente lleno de solución alcalina pre-enfriada (300 mM de NaOH, 1 mM de Na_2EDTA) y se dejó sumergido a 4 °C durante 30 minutos para permitir que el ADN se desenrollara. Pasados los 30 minutos, utilizando la misma solución alcalina, se realizó electroforesis alcalina durante 30 minutos a 35 voltios y 300 mA. Finalmente, el portaobjetos se lavó 3 veces con H_2O destilada pre-enfriada, se secó con etanol al 70% y se tiñó con tinte de ADN Vista Green durante 15 minutos en la oscuridad. Las imágenes se adquirieron con un microscopio de epi-fluorescencia invertido (DMI 6000 B, Leica, Wetzlar, Alemania) utilizando un filtro de isotiocianato de fluoresceína, y se analizaron utilizando el software de análisis de imágenes CASP 1.2.3 (CASPlab, Wroclaw, Poland).

3.2.5 Efectos en Inflamación y Estrés Oxidativo Celular

El efecto antiinflamatorio y antioxidante de extractos crudos derivados del alperujo, y de sus compuestos fenólicos principales puros (OL, HT y su combinación) fue estudiado *in vitro* en las dos líneas celulares, una derivada del epitelio corneal humano (HCE) y otra derivada del epitelio conjuntival humano (IM-ConjEpi), utilizando para ello dos estímulos: 1) TNF-α para inducir inflamación y 2) luz UV-B para inducir estrés oxidativo.

Previamente a estos ensayos, se llevó a cabo un ensayo de viabilidad celular con dichos tratamientos (descrito en el apartado *3.2.4.1)*, a fin de determinar sus concentraciones máximas permitidas (no tóxicas) para ambas líneas celulares. Posteriormente, el efecto antinflamatorio y antioxidante de los extractos del alperujo, y de OL, HT y su combinación, fue estudiado midiendo la secreción de citoquinas/quimioquinas y ROS en células estimuladas con TNF-α y luz UV-B, respectivamente.

Estimulación con TNF-α y Tratamiento con las Soluciones Fenólicas

Células HCE e IM-ConjEpi se plantaron en placas de 24 pocillos y se dejaron crecer durante 3 días, a fin de alcanzar una confluencia de 90% de la superficie del pocillo. Después, el medio de cultivo se reemplazó por DMEM/F-12 + GlutaMax sin ningún suplemento. A las 24 horas, se descartó el medio y las células se trataron con las diferentes concentraciones de OL, HT, OL + HT, CONV, OPT1, OPT2 y OPT3 durante 2 horas a 37 °C. Posteriormente, los pretratamientos se descartaron y las células se estimularon con 25 ng/mL de TNF-α en presencia de OL, HT, OL + HT, CONV, OPT1, OPT2 y OPT3 durante 24 horas. Células sin TNF-α o tratamientos se utilizaron como controles. Pasadas las 24 horas, los medios de cada muestra se recogieron y se centrifugaron a 18.800 x *g* durante 10 minutos. Las placas con células adheridas y los sobrenadantes se almacenaron a –80 °C antes de ser analizados.

Medida de la Secreción de Citoquinas/Quimioquinas

La concentración en los sobrenadantes de las células de las citoquinas IL-1β, IL-6, IL-8, IL-17A y Proteína inducible por interferón-γ (IP)-10 se midieron simultáneamente mediante tecnología X-MAP® (Luminex Corp), utilizando Kits de análisis comerciales. Brevemente, 25 µL de cada sobrenadante se incubaron en presencia de una mezcla de microesferas tapizadas con los anticuerpos respectivos a 4 °C durante toda la noche en agitación. Después, se añadió una solución de anticuerpos de detección (biotinilados) y se volvieron a incubar durante 1 hora a temperatura ambiente, seguida de otra incubación con una solución de estreptavidina-ficoeritrina durante 30 minutos a temperatura ambiente con agitación. Finalmente, la placa se leyó en un equipo Magpix (Luminex Corporation, Austin, TX, EE. UU.), realizando curvas de calibración de cada estándar de citoquina/quimioquina y utilizando el software BeadView (Upstate, Reino Unido).

Estimulación con Luz UV-B y Medida de Especies Reactivas de Oxígeno

Células HCE e IM-ConjEpi se plantaron en placas de 24 pocillos y se dejaron crecer durante 3 días, a fin de alcanzar una confluencia de 90% de la superficie del pocillo. Después, el medio de cultivo se reemplazó por DMEM/F-12 + GlutaMax sin ningún suplemento. A las 24 horas, se descartó el medio y se trataron las células con las diferentes concentraciones de OL, HT, OL + HT, CONV, OPT1, OPT2 y OPT3 durante 1 hora a 37 °C. Posteriormente, los pretratamientos se descartaron y las células se incubaron durante 30 minutos en presencia de 10 µM de H_2DCF-DA. A continuación, la solución de H2DCF-DA se descartó, y las células se volvieron a tratar con los tratamientos, se irradiaron durante 15 segundos con una lámpara UV-B con intensidad de 107.25 mJ/cm^2 (Bio-Rad, Inc., Hercules, CA, EE. UU.) y se incubaron durante 1 hora. Células sin tratamientos y células no irradiadas se utilizaron como controles. La intensidad de fluorescencia intracelular (488 nm_{ex} y 522 nm_{em}) se midió en un espectrofotómetro UV/vis (SpectraMax M5; Molecular Devices, Sunnyvale, CA, EE. UU.). Las placas con células adheridas se almacenaron a –80 °C para su posterior análisis de contenido en proteínas total.

Determinación de la Proteína Total

La concentración en proteína total en cada pocillo fue determinada en las células adheridas en las placas. Brevemente, las células en cada pocillo fueron lisadas añadiendo buffer de lisis RIPA suplementado con 0.1 mg/mL de PMSF, 60 μg/mL de aprotinina y 0.1 μM de Na_3VO_4 y se incubaron a 4 °C durante 30 minutos. Finalmente, se centrifugaron y se guardaron a –80 °C antes de ser analizadas.

La determinación de la cantidad de la proteína total fue determinada con el ensayo BCA. Brevemente, 25 μL de muestra, junto con estándares, se trataron con el reactivo de trabajo BCA (BCA en 0.1 mM de hidróxido sódico y sulfato de cobre (II) al 4%) y se incubaron durante 30 minutos a 37 °C. A continuación, se midió la absorbancia a 562 nm de las muestras en un espectrofotómetro (SpectraMax M5; Molecular Devices, Sunnyvale, CA, EE. UU.) y los datos se analizaron utilizando el software SoftMax® Pro (Molecular Devices, Sunnyvale, CA, EE. UU.).

3.3 Efecto *In vitro* sobre la Proliferación de Linfocitos T Humanos (CD4+)

El efecto inmunosupresor del extracto OPT3 del alperujo, junto con HT, fue estudiado *in vitro* en la proliferación de linfocitos T CD4+ procedentes de sangre periférica humana (PBMC).

3.3.1 Reactivos

Los reactivos usados en esta parte de la tesis han sido agrupados y presentados por fabricante:

- *Extrasynthese* (Genay, Francia): HT (≥98%).
- *GE Healthcare* (Chicago, IL, USA): Ficoll-Paque PLUS.
- *Gibco* (Grand Island, NY, EE. UU.): medio de cultivos Roswell Park Memorial Institute (RPMI) 1640 + L-glutamine, fitohemaglutinina - forma M (PHA-M).
- *Invitrogen* (Waltham, MA, EE. UU.): reactivo de viabilidad celular alamarBlue HS.
- *Miltenyi Biotech* (Bergisch Gladbach, North Rhine-Westphalia, Alemania): kit de aislamiento de linfocitos T CD4+ humanos, columnas LS, separador QuadroMACS.
- *Thermo Fisher Scientific* (Rockford, IL, EE. UU.): matraces, placas, puntas, pipetas de cultivo de plástico, Dulbecco's tampón fosfato salino (DPBS), penicilina, estreptomicina.

3.3.2 Donantes

La sangre periférica se obtuvo de tres pacientes varones sanos con una edad promedio de 28 ± 3 años, después de haber firmado un consentimiento informado. Este estudio siguió los Principios de

la Declaración de Helsinki y el protocolo fue aprobado por el Comité de Ética de la Universidad de Valladolid.

3.3.3 Aislamiento de las Células Mononucleares de Sangre Periférica

Se obtuvieron hasta 9 mL de sangre periférica de cada uno de los 3 donantes. Las células mononucleares se aislaron mediante gradiente de densidad con Ficoll-Paque PLUS (GE Healthcare, Chicago, IL, EE. UU.) a 400*g* durante 30 minutos, a temperatura ambiente. Las células se recogieron, se lavaron con solución DPBS y se utilizaron posteriormente para aislar linfocitos T CD4+ humanos.

3.3.4 Aislamiento y Cultivo de los Linfocitos CD4+

Los linfocitos T CD4+ humanas se aislaron de las células mononucleares mediante separación magnética usando un separador QuadroMACS. Los linfocitos T CD4+ intactas se seleccionaron negativamente utilizando el kit de aislamiento de linfocitos T CD4+ humanas y columnas LS, siguiendo el protocolo del fabricante. Los linfocitos T CD4+ aisladas se cultivaron en medio de cultivo RPMI 1640 + L-glutamina suplementado con FBS al 10% y penicilina/estreptomicina.

3.3.5 Preparación de las Soluciones Fenólicas

HT y OPT3 se disolvieron en DPBS a concentraciones de 400 μM, 1200 μM, 3.2 mg/mL y 1.6 mg/mL respectivamente, se dividieron en alícuotas y se almacenaron a –20 °C. El día del experimento, se dejó una alícuota de HT, OL, CONV u OPT3 a temperatura ambiente hasta su descongelación, y se diluyó con medio de cultivo celular para alcanzar las concentraciones deseadas. Al final de cada día, la cantidad restante se descartaba, para asegurar que no ocurriera ninguna degradación en los compuestos.

3.3.6. Ensayo de Proliferación

Linfocitos T CD4+ humanos se sembraron en placas de 96 pocillos y se activaron con 1% de PHA-M, excepto el control inactivado. A continuación, las células se trataron durante 48 horas con los tratamientos (soluciones fenólicas o medio de cultivos-control). Pasadas ese tiempo, se añadió reactivo de viabilidad celular alamarBlue HS al 10% a cada pocillo y se incubó durante 3 horas, antes de leer la fluorescencia (560 nm_{ex} y 590 nm_{em}) en un espectrofotómetro Spectra Max M5 (Molecular Devices Corporation, Sunnyvale, CA).

3.4 Modelo Murino de Ojo Seco

El efecto antiinflamatorio e inmunomodulador del extracto OPT3, elegido por su mejor actividad

in vitro, junto con HT, fue estudiado en un modelo murino de EOS (modelo de estrés por desecación durante 14 días). Este estudio se realizó en las instalaciones para animales de la Facultad de Medicina de la Universidad de Colonia (Colonia, Alemania). Se evaluó el efecto de dichos compuestos en el volumen lagrimal, la integridad del epitelio corneal (mediante tinción corneal con fluoresceína (CFS)), el contaje de linfocitos T CD4+ y CD8+ en los ganglios linfáticos y la expresión de los genes IP-10 y TNF-α en la córnea, la conjuntiva y las glándulas lagrimales.

3.4.1 Reactivos

Los reactivos, los cebadores seleccionados (Tabla 4) y los anticuerpos (Tabla 5) usados en esta parte de la tesis han sido agrupados y presentados por fabricante:

- *Alzet* (Cupertino, CA, EE. UU.): bombas osmóticas.
- *Biomol* (Hamburg, Alemenia): HT (≥98%).
- *Bio-rad* (Bonn, Germany): SsoFast EvaGreen supermix.
- *Drummond Scientific* (Broomall, PA, EE. UU.): tubos capilares de vidrio.
- *eBioscience* (San Diego, CA, EE. UU.): Fc block anti-mouse CD16/32, fijador estabilizador, tinción con tinción de viabilidad eFluor450fixable.
- *Gibco* (Grand Island, NY, EE. UU.): medio de cultivo RPMI 1640 + L-glutamina.
- *Invitrogen* (Waltham, MA, EE. UU.): agua destilada ultrapura sin DNasa/RNasa.
- *Oasis Medical* (San Dimas, CA, EE. UU.): hebras de rojo fenol (Zone Quick Thread).
- *Qiagen* (Hilden, Alemania): Mini kit RNeasy Plus, tampón RLT.
- *Sigma-Aldrich* (St Louis, MO, EE. UU.): bromhidrato de escopolamina, sal sódica de fluoresceína, β-mercaptoetanol, tampón de borato y solución salina normal.
- *Thermo Fisher Scientific* (Rockford, IL, EE. UU.): Kit de síntesis de ADNc de primera hebra de Revertaid, FBS, penicilina, estreptomicina, DPBS, N, O-bis (trimetilsilil) acetamida (BSA), ácido etilendiaminotetraacético (EDTA).

Tabla 4. Cebadores seleccionados para qRT-PCR

ARNm	Secuencia	Temperatura de desnaturalización (*melting*)	Fabricante
HPRT	F: 5′-TTGGATACAGGCCAGACTTTGTTG-3′ R: 5′-GATTCAACTTGCGCTCATCTTAGGC-3′	60 °C	Thermo Fisher Scientific (Rockford, IL, EE. UU.)
IP-10	F: 5′-ATATACGCGTTGACATTGATTATTGACTAG-3′ R: 5′-ATTGCTAG-CAGCTGGTTCTTTCCGCCTC-3′	60 °C	
TNF-α	F: 5′- AGGACTCAAATGGGCTTTCC-3′ R: 5′-CAGAGGCAACCTGACCACTC-3′	63 °C	

F = cebador directo, R = cebador inverso, IP-10 = proteína inducida por interferón γ – 10, TNF-α = Factor de necrosis tumoral -α, HPRT = hipoxiantina-guanina fosforribosiltransferasa.

Tabla 5. Panel de anticuerpos utilizado para el análisis de citometría de flujo (FACS)

Anticuerpos (clon)	Objetivo	Conjugación	Fabricante
Anti-ratón CD3 (17A2)	Linfocitos T	aloficocianina-cianina 7 (APC-Cy7)	Biolegend (San Diego, CA, EE. UU.)
Anti-ratón CD4 (GK1.5)	Linfocitos T	isotiocianato de fluoresceína (FITC)	
Anti-ratón CD8a (53-6.7)	Linfocitos T	APC	

3.4.2 Animales e Inducción del Estrés por Desecación

Se utilizaron ratones C57BL/6 (hembras) de 10 a 12 semanas de edad obtenidos de Charles River Laboratories (Sulzfeld, Alemania). Los ratones fueron expuestos a estrés por desecación durante 14 días, colocando las jaulas en una cámara de ambiente controlado (condiciones: humedad 30 ± 5%, flujo de aire forzado 10 horas/día y temperatura 25 ± 1 °C). Se administró bromohidrato de escopolamina de forma continua (0.1 mg/día) mediante bombas osmóticas implantadas subcutáneamente. Los experimentos terminaron el día 14 y los ganglios linfáticos regionales, los ojos completos con conjuntiva, así como las glándulas lagrimales, se diseccionaron y se procesaron como se describe más adelante.

Todos los experimentos con animales siguieron las regulaciones de la Universidad de Colonia, la ley de protección animal alemana (LANUV) y la declaración de la Asociación para la Investigación en Visión y Oftalmología (ARVO) para el uso de animales en la investigación oftálmica y de la visión. También, fueron aprobados por la instalación descentralizada de animales (EURL 2010/63) de la Facultad de Medicina de la Universidad de Colonia.

3.4.3 Aplicación Tópica de los Tratamientos

HT (100 μM) y OPT3 (0.2 mg/mL) se disolvieron en tampón de borato (vehículo), se dividieron en alícuotas y se almacenaron a –20 °C. Cada día de tratamiento, se usó una alícuota de HT u OPT3 y luego se descartó, para asegurar que no ocurriera ninguna degradación en los compuestos. Los ratones se distribuyeron aleatoriamente en tres grupos (n = 5 ratones/grupo): (1) HT, (2) OPT3 y (3) vehículo (control). Todos los grupos estuvieron expuestos a las mismas condiciones de estrés por desecación. Los tratamientos se aplicaron el mismo día con la configuración del estrés por desecación (día 1) y se administraron por vía tópica tres veces al día en ambos ojos (5 μL/ojo) durante 14 días.

3.4.4 Integridad del Epitelio Corneal: Tinción Corneal con Fluoresceína

Para evaluar el daño corneal (los días 0 y 11), se utilizó la tinción corneal con fluoresceína, aplicando 5 μL/ojo de sal sódica de fluoresceína al 5% en DPBS. La solución se limpió cuidadosamente después de 1 minuto y se realizó la clasificación bajo luz azul en un estereomicroscopio. Se utilizó un esquema de calificación de Oxford modificado con severidades que variaban del grado 0 al 5.

3.4.5 Volumen Lagrimal

Para la medida del volumen lagrimal (los días 0 y 11), una hebra de rojo fenol se colocó en el canto lateral de cada ojo durante 10 segundos y el cambio de color (de amarillo a rojo) en la parte húmeda (absorción de lágrimas) se midió en milímetros.

3.4.6 Análisis de Citometría de Flujo (FACS): Porcentaje de Contaje de Linfocitos CD3+, CD4+ y CD8+ en los Ganglios Linfáticos

Los ganglios linfáticos de todos los ratones en cada uno de los 3 grupos (tratados con vehículo, HT u OPT3) se trituraron a través de un filtro de células y las suspensiones de células individuales se transfirieron a tampón FACS (BSA al 0,5%, FBS al 1% y EDTA al 1:50 en DPBS). Después de bloquear con el Fc block anti-ratón CD16/32 durante 15 minutos, las muestras se tiñeron durante 30 minutos a 4 °C y protegidas de la luz con anticuerpos anti-ratón CD3, CD4 y CD8a a 0.10 mg/mL, 0.125 mg/mL y 0.05 mg/mL, respectivamente. Posteriormente, las células se lavaron y re-suspendieron en DPBS, y la tinción se realizó con tinción de viabilidad eFluor450fixable. Después de volver a lavar las células, se añadió un fijador estabilizador y las muestras teñidas se analizaron en un FACS Canto (BD, Alemania), extrayendo los resultados con el software FlowJo (FlowJo LLC, Tree Star Inbc., Ashland, OR, EE. UU.).

3.4.7 Expresión de Genes de Citoquinas/Quimioquinas

La expresión de los genes de *IP-10* y *TNF-α* en la córnea, la conjuntiva y las glándulas lagrimales se estudió mediante qRT-PCR. Una vez diseccionadas, las córneas y conjuntivas de dos ojos por ratón se agruparon en el mismo tubo, mientras que para las glándulas lagrimales se utilizó una muestra (la glándula del ojo izquierdo) por ratón. Todas las muestras se colocaron en tampón RLT con 10 μL/mL de β-mercaptoetanol y el aislamiento del ARN se realizó utilizando un RNeasy Plus Mini Kit, de acuerdo con las instrucciones del fabricante. La transcripción inversa se realizó utilizando un kit de síntesis de ADNc First-Stand de Revert Aid, siguiendo las instrucciones del fabricante. Para cuantificar las transcripciones de *IP-10* y *TNF-α*, se realizó una qRT-PCR basada en SYBR Green en las muestras de ADNc. Para las reacciones qRT-PCR, se utilizó un volumen de 20 μL con 20 ng de ADNc y 0.75 μM de cada cebador directo e inverso, junto con SsoFast EvaGreen Supermix. La incubación incluyó 2 minutos a 95 °C seguidos de 45 ciclos de 5 segundos a 95 °C y 30 segundos a 60 °C, utilizando un sistema de detección CFX96 Touch Real-Time PCR (Bio-Rad, Bonn, Alemania). Los resultados de *IP-10* y *TNF-α* se calcularon mediante el método de umbral comparativo utilizando un gen de mantenimiento, el *HPRT*.

3.5 Estudios de Estabilidad a Largo Plazo de Soluciones Acuosas de Extractos de Alperujo Seleccionados y de los Compuestos Puros

Las pruebas obligatorias para la aprobación y liberación de una sustancia o un producto farmacéutico en el mercado incluyen la estabilidad de almacenamiento a largo plazo. La guía de la EMA CPMP/ICH/2736/99 determina las diferentes condiciones de temperatura y humedad en las que un producto farmacéutico debe permanecer estable, cubriendo el almacenamiento, envío y uso posterior del producto. Dependiendo de las condiciones de almacenamiento previstas del producto futuro, se describen las condiciones intermedias, aceleradas y a largo plazo. Por lo general, el almacenamiento a largo plazo debe cubrir un mínimo de 12 meses de duración, mientras que, para los estudios intermedios o acelerados, se propone un período de 6 meses. De acuerdo con la guía CPMP/ICH/2736/99, soluciones acuosas de HT, OL, OPT3 y CONV se almacenaron en cuatro condiciones diferentes de temperatura y humedad.

3.5.1 Reactivos

Los reactivos usados en esta parte de la tesis han sido agrupados y presentados por fabricante:

- *Panreac Quimica SLU* (Barcelona, España): MeOH (99.9% LC-MS), DMSO, ácido fosfórico.
- *Extrasynthese* (Genay, Francia): HT (≥98%), OL (≥98%).
- *Sigma-Aldrich* (St Louis, MO, EE. UU.): $MgSO_4$ anhidro, KI, NaBr, NaCl.

3.5.2 Condiciones de Almacenamiento

Todos los compuestos y extractos se disolvieron en agua Milli-Q y se filtraron a través de un filtro de fluoruro de polivinilideno de 0.20 μm. Se añadió un total de 3 mL de cada solución acuosa fenólica a viales de vidrio ámbar, se desgasificaron con N_2, se cerraron herméticamente con un tapón de goma y se sellaron con cápsulas de aluminio. Las 4 temperaturas de estudio fueron elegidas según la pautas científicas ICH: 5, 25, 30 y 40 °C. Para fijar la humedad deseada para cada temperatura, se utilizaron sales adecuadas; para 5 °C, se seleccionó el $MgSO_4$ anhidro (para lograr que no haya humedad), para 25 °C: NaBr (para alcanzar una humedad de 57.57% ± 0,40), para 30 °C: KI (estableciendo una humedad de 67.89% ± 0,3) y para 40 °C: NaCl (estableciendo una humedad de 74.68% ± 0,13). Los viales se colocaron en grandes recipientes de plástico, junto con una cantidad suficiente de cada sal, se cerraron herméticamente y se dejaron en diferentes salas de temperatura controlada y protegidas de la luz durante 6 meses. Se tomaron muestras inmediatamente después de la preparación (considerada t_0) y posteriormente en los días 1 (24 h), 2, 4, 6, 8, 10, 12, 14 y 30 (1 mes). Después del primer mes, se tomó una muestra cada 30 días, hasta una duración total de 6 meses. Posteriormente se analizó el contenido en HT y OL en cada muestra obtenida a cada tiempo y condición. Para CONV, se midió el contenido de HT y OL, mientras que para OPT3 se calculó solo el contenido de HT debido a la falta de OL en este extracto.

3.5.3 Determinación del Contenido en OL e HT en las Soluciones Acuosas: Análisis HPLC-DAD

Para cuantificar los compuestos HT y OL tanto en soluciones puras como en los extractos CONV y OPT3, se utilizó el método HPLC-DAD. El sistema HPLC-DAD, las condiciones, el método de gradiente y los eluyentes usados se describen en la sección 2.4.6 de este capítulo.

3.5.4 Identificación Putativa por HPLC-DAD-MS/MS en las Soluciones Acuosas

Se realizó un análisis HPLC-DAD-MS/MS para soluciones acuosas de extractos seleccionados (CONV y OPT3), para comparar la composición de los dos extractos. También, se realizó el mismo análisis a todas las muestras (ambos extractos, OL e HT) expuestas a 40 °C, para identificar posibles subproductos de degradación durante el almacenamiento a largo plazo. El método de gradiente de HPLC, los eluyentes y el programa de elución utilizados, junto con las condiciones aplicadas, se describen en la sección 2.3.4.

4. Resultados Más Relevantes

4.1 Valorización del Alperujo

4.1.1 Efecto del Método de Almacenamiento/Pretratamiento

La OLC se identificó mediante HPLC-DAD-MS/MS como el polifenol más abundante en el extracto de CONV-2 (referencia – material liofilizado y desgrasado con $scCO_2$). También, se observó un comportamiento opuesto al observado en OL. Por lo tanto, se incluyó en el estudio del efecto del almacenamiento/pretratamiento de la materia prima en la estabilidad de los principales compuestos fenólicos. Entre las diferentes condiciones aplicadas, la liofilización (Tabla 6) y la extracción posterior del aceite residual con $scCO_2$ (Tabla 7) resultaron ser las más adecuadas en cuanto al efecto sobre la riqueza en los principales compuestos fenólicos de los extractos hidroalcohólicos producidos. Además, el rendimiento del aceite obtenido por $scCO_2$ y por el método convencional (*n*-hexano) fue similar en ambos casos (Tabla 7).

Tabla 6. Resultados obtenidos para extractos generados por condiciones de extracción convencional, utilizando alperujo (OP) pretratado en diferentes condiciones.

Material/ condición	CAA ($mmol_{TE}/g_{DE}$)	TPC (mg_{GAE}/g_{DE})	TFC (mg_{CATE}/g_{DE})	OL (mg/g_{DE})	OLC (mg_{OLE}/g_{DE})	HT (mg/g_{DE})	TY (mg/g_{DE})	EY ($mg_{DE}/g_{OP\ seco}$)
Fresco	3.64 ± 0.15^{bc}	117 ± 11^{a}	8.0 ± 0.7^{b}	6.0 ± 0.8^{b}	3.7 ± 0.7^{b}	1.0 ± 0.3^{bc}	1.5 ± 0.3^{ab}	134 ± 15^{b}
Descongelado	3.8 ± 0.3^{b}	130 ± 1^{a}	10.9 ± 0.4^{a}	10.1 ± 1.3^{c}	5.2 ± 0.3^{b}	1.4 ± 0.5^{ac}	1.3 ± 0.2^{b}	116 ± 4^{ab}
Liofilizado	4.36 ± 0.08^{a}	131 ± 27^{a}	11.2 ± 1.3^{a}	3.4 ± 0.5^{a}	12.0 ± 3.3^{a}	1.9 ± 0.3^{a}	1.9 ± 0.2^{a}	94 ± 6^{a}
Seco	3.2 ± 0.12^{c}	105.0 ± 0.5^{a}	5.0 ± 0.2^{c}	2.53 ± 0.01^{a}	2.4 ± 0.3^{b}	0.17 ± 0.07^{b}	0.28 ± 0.01^{c}	112 ± 6^{ab}

Los valores con diferentes letras minúsculas en la misma columna son significativamente diferentes ($p < 0.05$). Los resultados se presentan como promedio ± desviación estándar. Respuestas medidas: actividad antioxidante química (CAA, expresada como mmol de equivalentes de trolox (TE)/g de extracto seco (DE)); contenido fenólico total (TPC, expresado como mg de equivalentes de ácido gálico (GAE)/g DE; contenido total de flavonoides (TFC, expresado como mg de equivalentes de catequina (CATE)/g DE), riqueza de extracto en oleuropeína (OL), oleaceína (OLC), hidroxitirosol (HT) y tirosol (TY) (expresado como mg de compuesto/g DE, OLC se calculó como equivalentes de OL: OLE) y rendimiento de extracción (EY, expresado como mg de DE/g de OP seco).

4.1.2 Extracción de Compuesto Fenólicos

Combinando dos técnicas de extracción sostenibles ($scCO_2$ seguido de PLE), se produjeron extractos fenólicos derivados del alperujo altamente concentrados en un tiempo de extracción 3 veces menor y con un consumo de solvente 1.6 veces menor en comparación con el método sólido-líquido convencional, utilizando exclusivamente mezclas de etanol-agua. Para cada respuesta, se encontraron diferentes condiciones óptimas (Tabla 8). En particular, en comparación con el extracto de referencia (CONV-2, obtenido de forma convencional a partir de alperujo liofilizado y desgrasado con $scCO_2$ – Tabla 3), a temperatura, porcentaje de EtOH en agua y relación sólido/líquido altos, se produce un extracto con 5 y 3 veces más HT y TY, respectivamente. En las mismas condiciones, el TPC se puede

casi duplicar. Por el contrario, se puede obtener un extracto con contenido 6 veces mayor en OL a temperatura baja, y relación sólido/líquido y porcentaje de EtOH en agua altos. OLC demostró un aumento de casi 5 veces a temperatura y porcentaje de EtOH en agua bajos, y relación sólido/líquido alta. En cuanto a CAA, se puede casi duplicar a temperatura y porcentaje de EtOH en agua altos, y relación sólido/líquido baja.

En base a estos resultados, para los ensayos posteriores se seleccionaron 3 extractos optimizados (OPT). OPT1, con la actividad química antioxidante (CAA) más alta (8.8 mmol de equivalentes de trolox/g de extracto seco), OPT2, con la concentración más alta en OL (11.4 mg/g de extracto seco), y OPT3, enriquecido en HT (7.7 mg/g de extracto seco), TY (4.1 mg/g de extracto seco) y TPC (336 mg de equivalentes de ácido gálico/g de extracto seco).

4.2 Resultados *In vitro*

4.2.1 Seguridad Ocular en Células Epiteliales de la Superficie Ocular Humana

Se determinaron las concentraciones máximas permitidas en células del epitelio corneal y conjuntival humano por ensayo de viabilidad celular para el HT, la OL y su combinación, junto con los 3 extractos de PLE óptimos elegidos (OPT1, OPT2 y OPT3) y el extracto CONV (producido utilizando alperujo liofilizado no desgrasado y con extracción sólido-líquido convencional – Tabla 3). Para CONV y OPT1, la concentración máxima se determinó a 0.8 mg/mL, para OPT2 y OPT3 a 0.4 mg/mL, para HT a 150 μM, para OL a 300 μM y para OL+HT a 5+50 μM. Según el modelo *in vitro* de genotoxicidad en las mismas líneas celulares, no se detectó ningún efecto genotóxico ni para OL, HT y su combinación, ni para los extractos (CONV, OPT1, OPT2 y OPT3) (Figura 1) en las concentración máxima de cada tratamiento determinada por la citotoxicidad.

4.2.2 Actividad Antioxidante y Antinflamatoria en Células Epiteliales de la Superficie Ocular Humana

Entre los 4 extractos (CONV, OPT1, OPT2 y OPT3) testados, CONV y OPT3 demostraron el mejor efecto antinflamatorio (Figura 2.A-C) y antioxidante (Figura 2.D) en células HCE, por lo que dichos 2 extractos se seleccionaron para el posterior estudio dosis-dependiente.

Según estudios dosis-dependientes en el modelo *in vitro* de inflamación de la superficie ocular, CONV y OPT3 demostraron un efecto antinflamatorio sobre las células del epitelio corneal y conjuntival humano mediante la inhibición de la secreción de citoquinas/quimioquinas (Figuras 3 y 4) (en HCE; 0.40 mg/mL de CONV para IL-6 e IL-1β, y 0.60 mg/mL de CONV para IL-8, mientras que 0.20 mg/mL de OPT3 para IL-6, IL-8 e IP-10. En IM-ConjEpi; 0.20 mg/mL de CONV y 0.05 mg/mL de OPT3 para IP-10). La actividad antinflamatoria de OPT3 se demostró en concentraciones de 2 a 4 veces más bajas en comparación con CONV.

Principalmente HT, pero también OL y la combinación de los dos compuestos en concentraciones bajas, tienen un efecto antinflamatorio sobre las células del epitelio corneal y conjuntival humano mediante la inhibición de la secreción de citoquinas/quimioquinas en estudios dosis-dependientes

Tabla 7. Efecto de los dos métodos de desgrasado seleccionados sobre las respuestas medidas para los extractos fenólicos convencionales producidos utilizando alperujo (OP) liofilizado comparados con extractos de OP liofilizado no desgrasado.

Material/tratamiento desgrasante	CAA ($mmol_{TE}/g_{DE}$)	TPC (mg_{GAE}/g_{DE})	TFC (mg_{CATE}/g_{DE})	OL (mg/g_{DE})	OLC (mg_{OLE}/g_{DE})	HT (mg/g_{DE})	TY (mg/g_{DE})	EY ($mg_{DE}/g_{OP\ SECO}$)	Rendimiento de aceite (%)
OP liofilizado/ no desgrasado (referencia)	*4.36 ± 0.08[a]*	*131 ± 27[a]*	*11.2 ± 1.3[a]*	*3.4 ± 0.5[a]*	*12.0 ± 3.3[a]*	*1.9 ± 0.3[a]*	*1.9 ± 0.2[a]*	*94 ± 6[a]*	-
OP liofilizado / *n*-hexano	4.8 ± 0.5[a]	152 ± 15[a]	9 ± 3[a]	2.6 ± 0.2[a]	12.1 ± 1.3[a]	1.9 ± 0.2[a]	1.8 ± 0.2[a]	93 ± 11[a]	2.4 ± 0.5
OP liofilizado /scCO$_2$	4.66 ± 0.14[a]	180 ± 11[b]	11.2 ± 1.3[a]	3.3 ± 0.8[a]	11.8 ± 1.6[a]	1.80 ± 0.1[a]	1.78 ± 0.10[a]	121 ± 25[a]	2.40 ± 0.15

Los valores con diferentes letras minúsculas en la misma columna son significativamente diferentes ($p < 0.05$). Los resultados se presentan como promedio ± desviación estándar. Respuestas medidas: actividad antioxidante química (CAA, expresada como mmol de equivalentes de trolox (TE)/g de extracto seco (DE)); contenido fenólico total (TPC, expresado como mg de equivalentes de ácido gálico (GAE)/g DE; contenido total de flavonoides (TFC, expresado como mg de equivalentes de catequina (CATE)/g DE), riqueza de extracto en oleuropeína (OL), oleaceína (OLC), hidroxitirosol (HT) y tirosol (TY) (expresado como mg de compuesto/g DE, OLC se calculó como equivalentes de OL: OLE) y rendimiento de extracción (EY, expresado como mg de DE/g de OP seco). scCO2: extracción de CO_2 de carbono supercrítico.

Tabla 8. Comparación entre la extracción convencional y las condiciones optimizadas de extracción de líquido presurizado (PLE) para cada respuesta

Respuestas	Extracción convencional *(extracto CONV-2)*				Extracción optimizada con PLE				Incremento (%)
	Condiciones			Valor	Condiciones			Valor	
	T (°C)	EtOH%	S/L ($g_{OP}/mL_{SOLVENTE}$)		T (°C)	EtOH%	S/L ($g_{OP}/mL_{SOLVENTE}$)		
EY ($mg_{DE}/g_{ALPERUJO\ SECO}$)				121	184.0	8.0	0.2	396	**227**
CAA ($mmol_{TE}/g_{DE}$)				4.66	66.1	8.0	0.8	8.9	**89**
					183.9	92.0	0.2	8.8	**87**
TPC (mg_{GAE}/g_{DE})				180	183.9	84.7	0.8	340	**89**
					183.9	91.9	0.2	332	**84**
TFC (mg_{CATE}/g_{DE})	70.0	50.0	0.5	9	66.4	8.0	0.8	22	**158**
Riqueza en OL (mg_{OL}/g_{DE})				2.4	66.4	92.0	0.8	13.8	**475**
Riqueza en OLC (mg_{OLE}/g_{DE})				11	66.1	19.3	0.8	52	**373**
Riqueza en HT (mg_{HT}/g_{DE})				1.79	183.9	90.0	0.8	9.5	**428**
Riqueza en TY (mg_{TY}/g_{DE})				1.78	183.9	92.0	0.8	5.3	**194**
					66.1	8.0	0.2	4.9	**172**

Respuestas medidas para los extractos de alperujo (OP): rendimiento de extracción (EY, expresado como mg de DE/g de OP seco), actividad antioxidante química (CAA, expresada como mmol de equivalentes de trolox (TE)/g de extracto seco (DE)); contenido fenólico total (TPC, expresado como mg de equivalentes de ácido gálico (GAE)/g DE; contenido total de flavonoides (TFC, expresado como mg de equivalentes de catequina (CATE)/g DE), y riqueza de extracto en oleuropeína (OL), oleaceína (OLC), hidroxitirosol (HT) y tirosol (TY) (expresado como mg de compuesto/g DE, OLC se calculó como equivalentes de OL: OLE). Factores: temperatura (T), porcentaje de etanol (EtOH) en agua (EtOH%) y relación sólido/líquido (S/L).

en el modelo *in vitro* de inflamación de la superficie ocular (Figuras 5, 6 y 7) (en HCE; 50, 100 y 150 μM de hidroxitirosol para IL-6, IL-8 e IP-10, e IL-1β, respectivamente, mientras que 5+50 μM de oleuropeína+hidroxitirosol para IL-6 e IP-10. En IM-ConjEpi; 25 μM de hidroxitirosol, 200 μM de oleuropeína y 5+50 μM de oleuropeína+hidroxitirosol para IP-10).

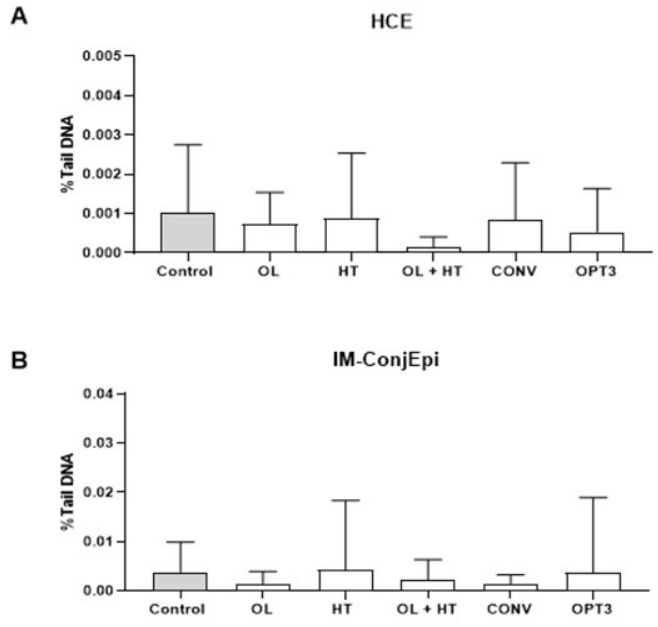

Figura 1. Efecto genotóxico (ensayo comet alcalino) de soluciones acuosas de extractos de alperujo (0.8 mg/mL del convencional — CONV y 0.4 mg/mL del optimizado — OPT3), 300 μM de oleuropeína (OL), 100 μM de hidroxitirosol (HT) y su mezcla (5 μM+50 μM OL+HT) en células epiteliales corneales (HCE) (**A**) y conjuntivales (IM-ConjEpi) (**B**) humanas.

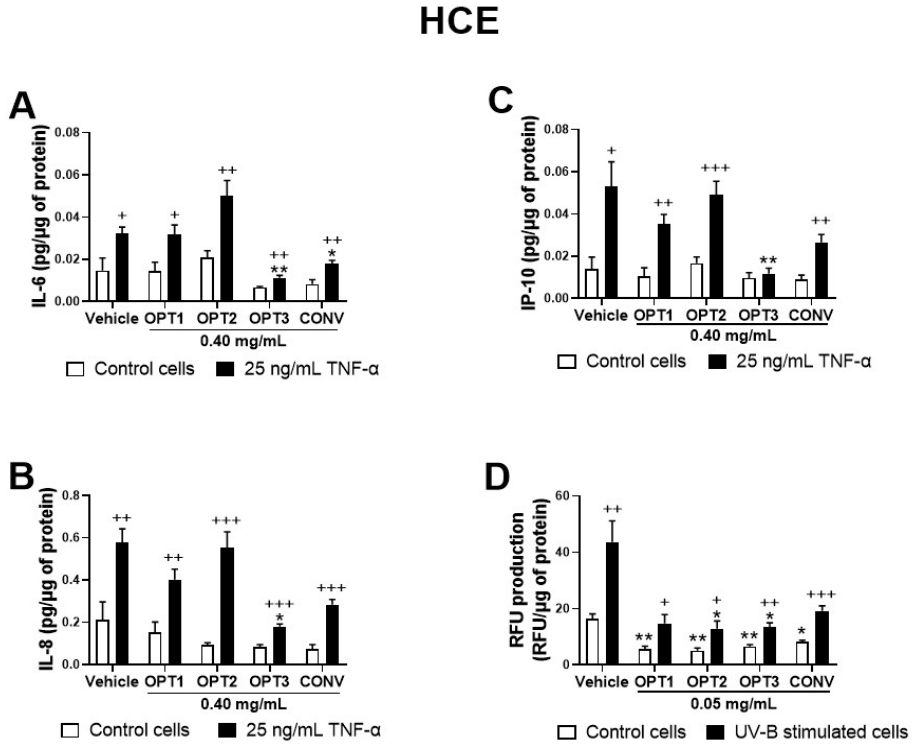

Figura 2. Cribado de la actividad antiinflamatoria y antioxidante del extracto convencional (CONV) y los 3 extractos optimizados (OPT1, OPT2 y OPT3) derivados del alperujo sobre la liberación de citoquinas/quimioquinas (IL-6, IL-8 e IP-10) inducida por TNF-α (24 horas) (**A**-**C**), y la producción de especies reactivas de oxígeno (ROS) inducida por luz UV-B (**D**) en células epiteliales corneales humanas (HCE).

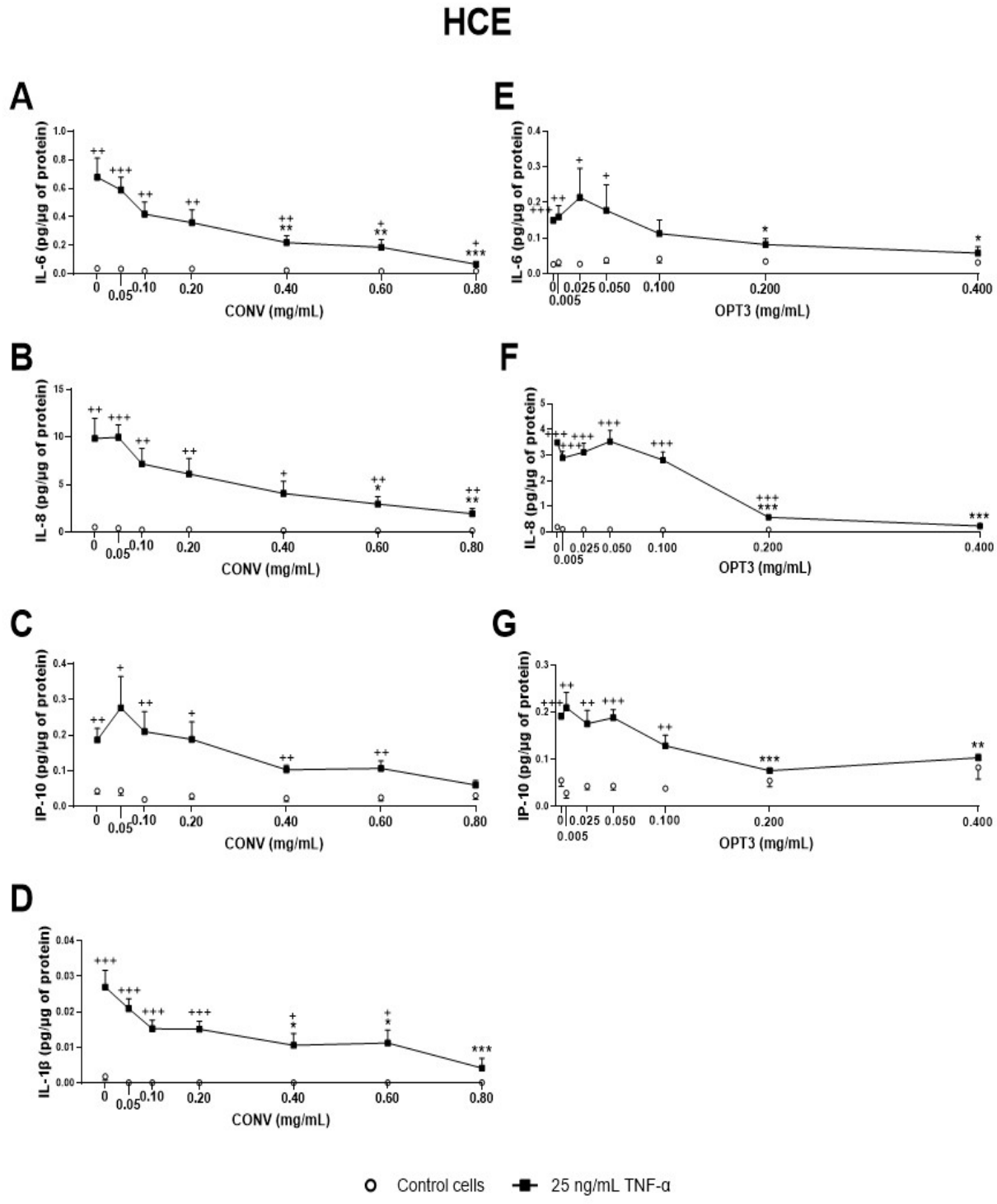

Figura 3. Efecto de los extractos de alperujo convencional (CONV) (**A**-**D**) y optimizado seleccionado (OPT3) (**E**-**G**) sobre la liberación de citoquinas/quimioquinas (IL-6, IL-8, IP-10 e IL-1β) inducida por TNF-α (24 horas) en células del epitelio corneal humano (HCE).

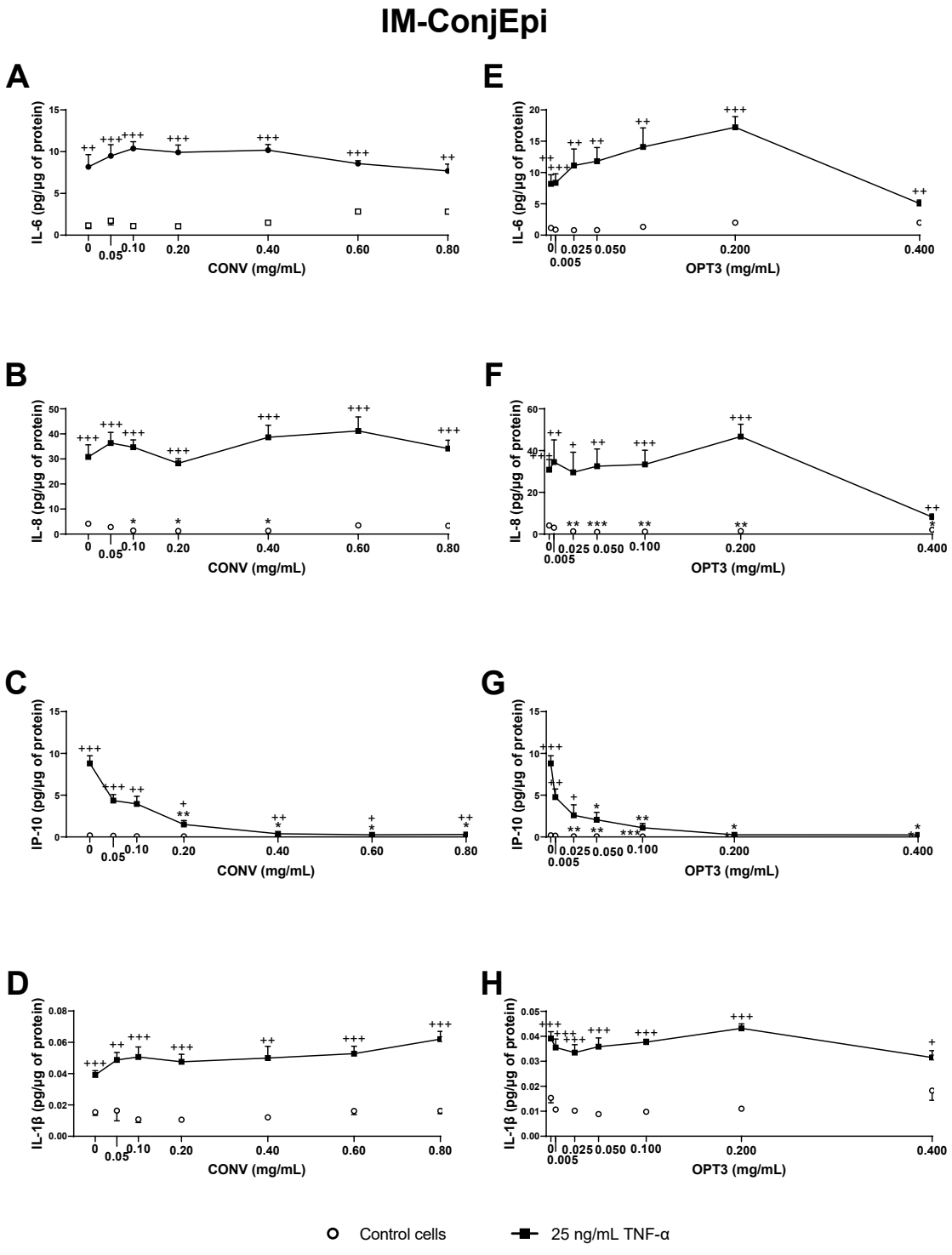

Figura 4. Efecto de los extractos de alperujo convencional (CONV) (**A**-**D**) y optimizado seleccionado (OPT3) (**E**-**H**) sobre la liberación de citoquinas/quimioquinas (IL-6, IL-8, IP-10 e IL-1β) inducida por TNF-α (24 horas) en células del epitelio conjuntival humano (IM-ConjEpi).

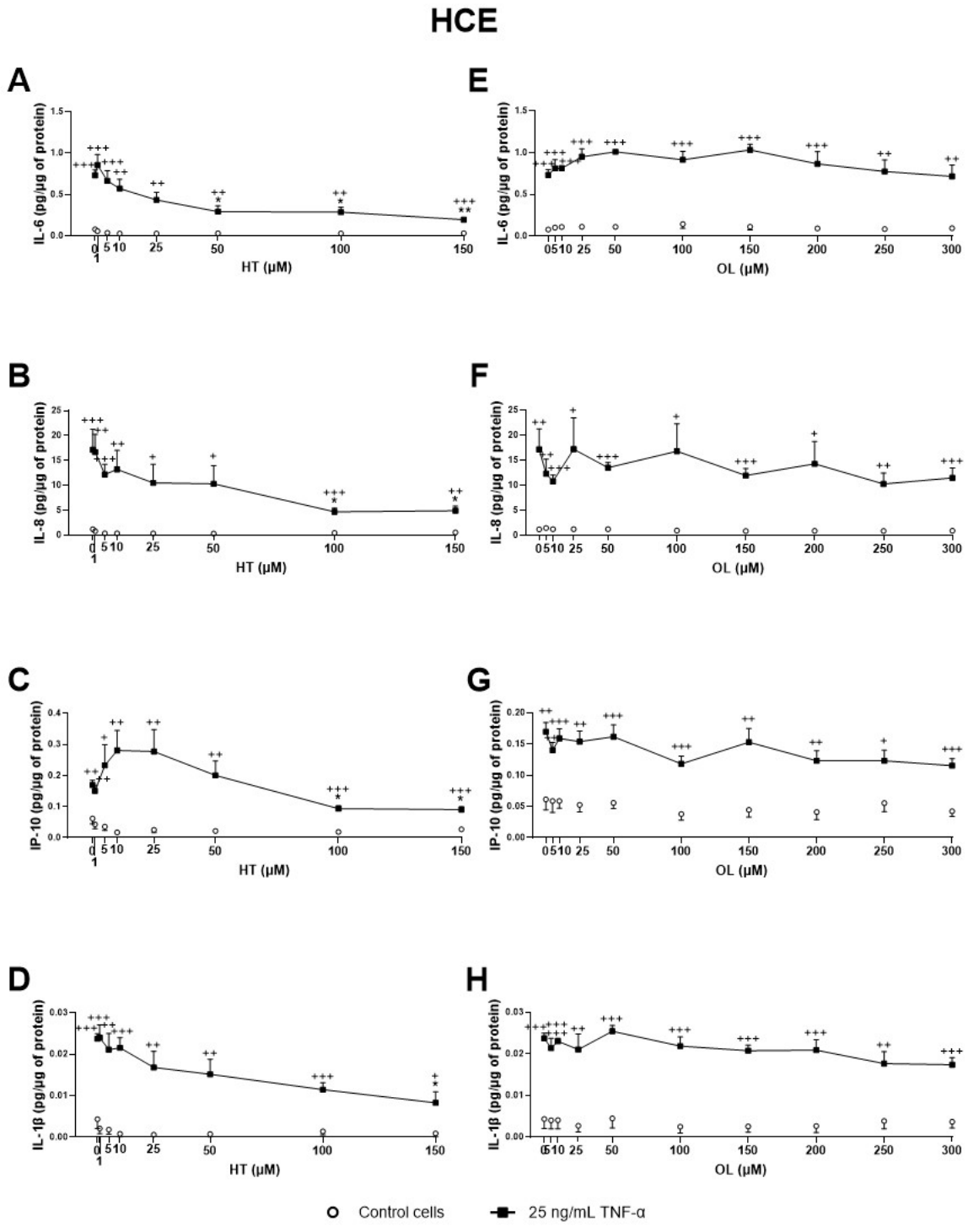

Figura 5. Efecto de hidroxitirosol (HT) (**A**-**D**) y oleuropeína (OL) (**E**-**H**) sobre la liberación de citoquinas/quimioquinas (IL-6, IL-8, IP-10 e IL-1β) inducida por TNF-α (24 horas) en células del epitelio corneal humano (HCE).

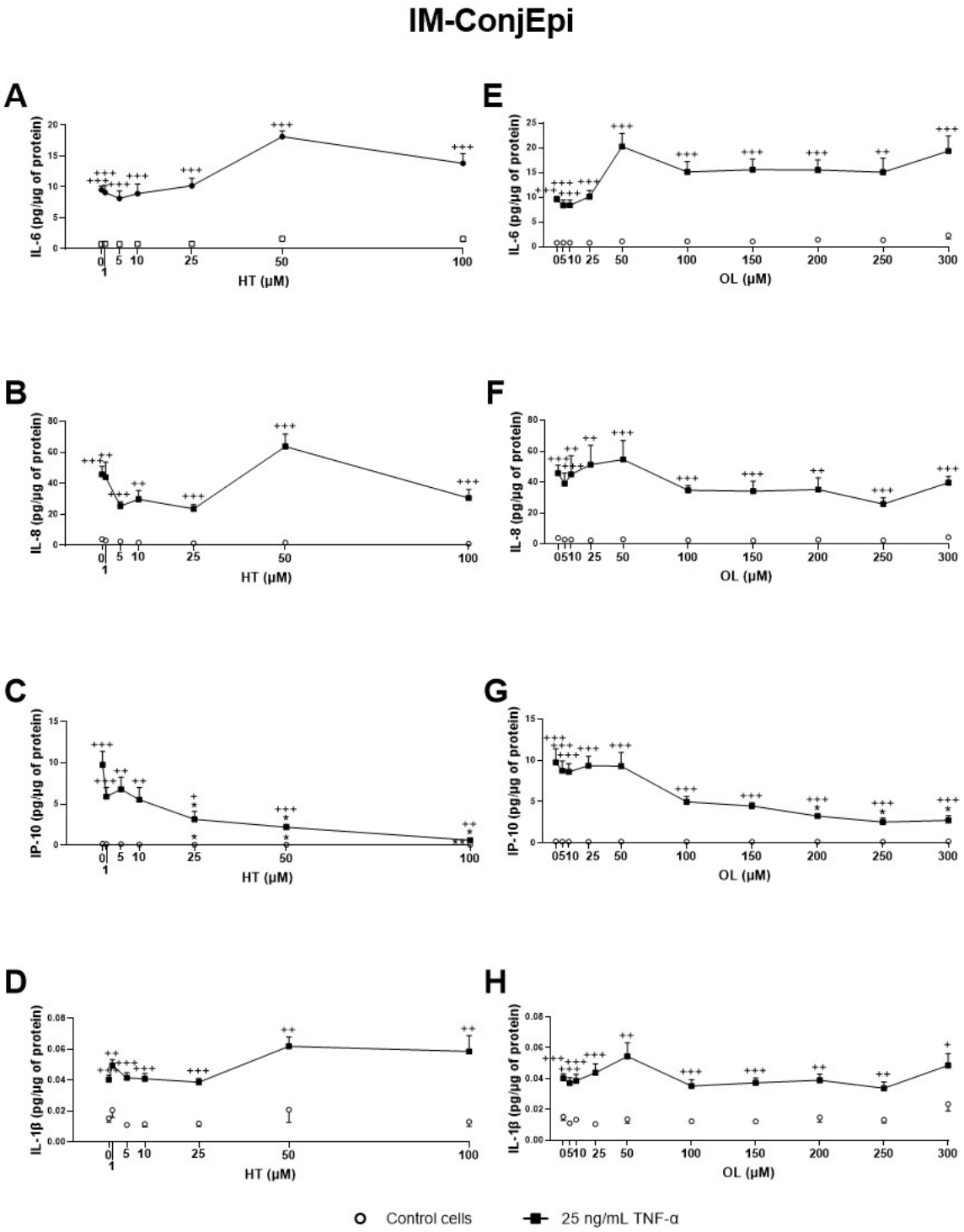

Figura 6. Efecto de hidroxitirosol (HT) (**A**-**D**) y oleuropeína (OL) (**E**-**H**) sobre la liberación de citoquinas/quimioquinas (IL-6, IL-8, IP-10 e IL-1β) inducida por TNF-α (24 horas) en células del epitelio conjuntival humano (IM-ConjEpi).

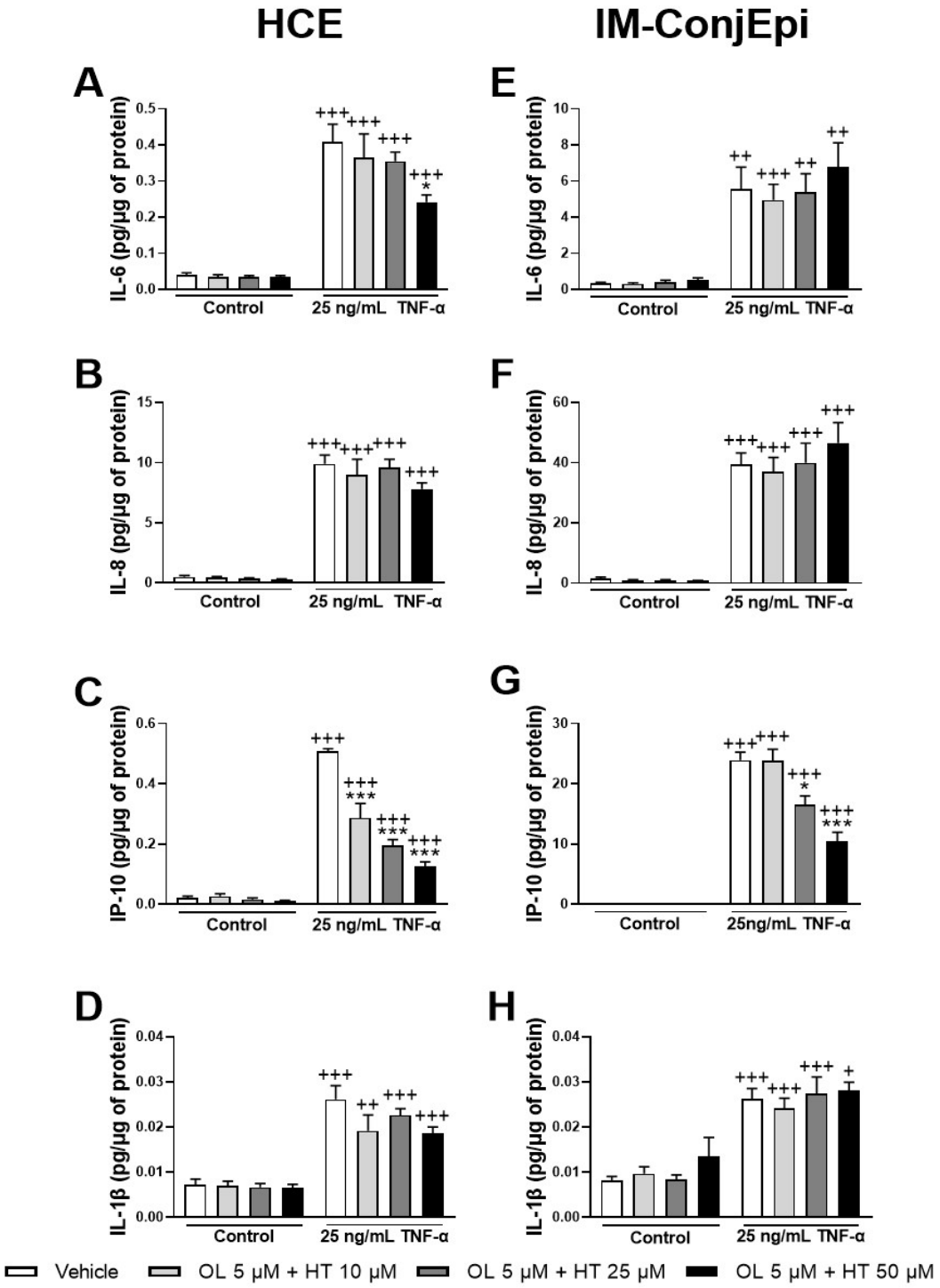

Figura 7. Efecto de la mezcla de hidroxitirosol (HT) y oleuropeína (OL) sobre la liberación de citoquinas/quimioquinas (IL-6, IL-8, IP-10 e IL-1β) inducida por TNF-α (24 horas) en células del epitelio corneal (HCE) (**A–D**) y conjuntival (IM-ConjEpi) (**E–H**) humano.

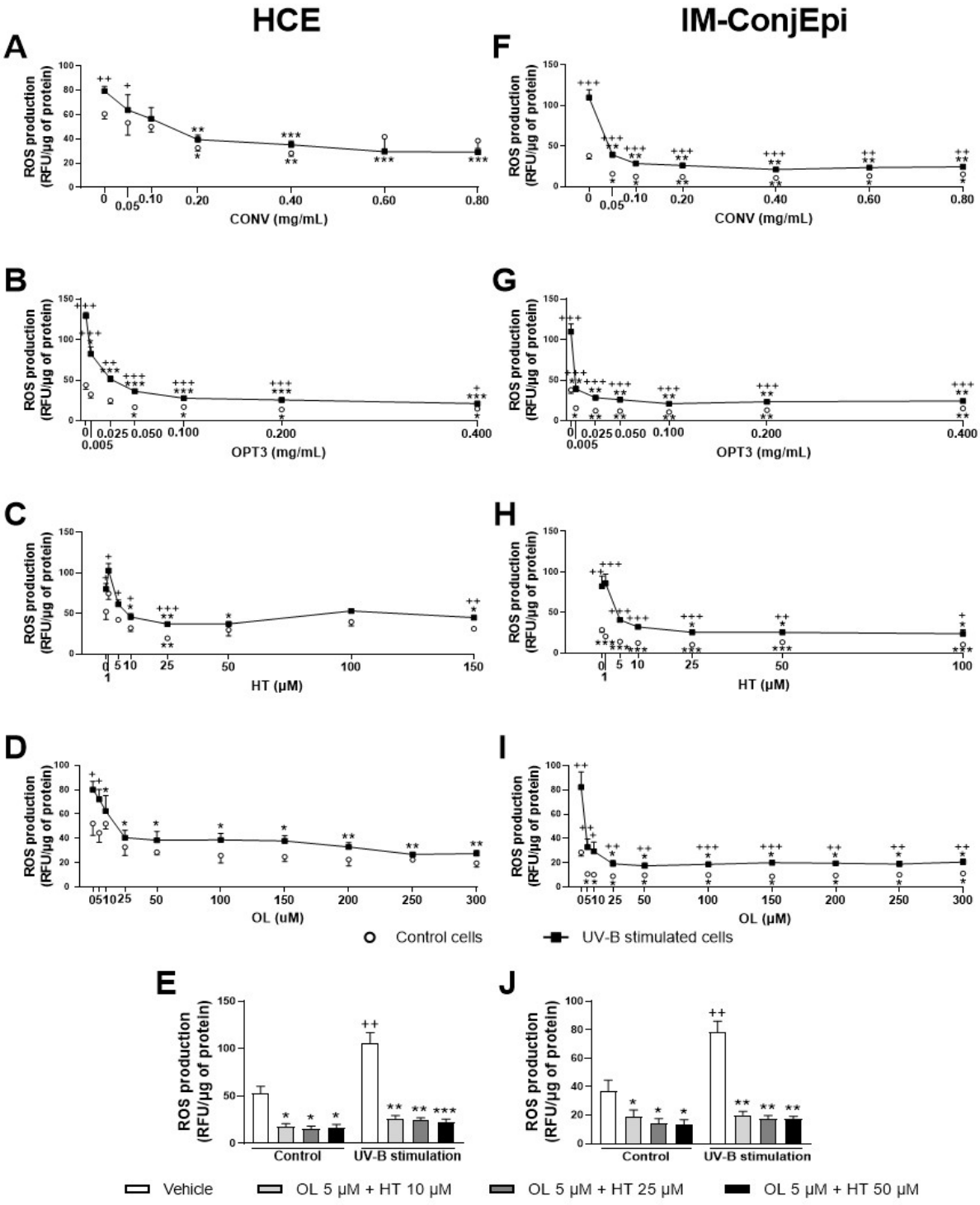

Figura 8. Efecto de oleuropeína (OL), hidroxitirosol (HT) y su combinación, junto con los extractos de alperujo convencional (CONV) y optimizado seleccionado (OPT3) sobre la producción de especies de oxígeno reactivas (ROS) inducida por UV-B en células del epitelio corneal (HCE) (**A**–**E**) y conjuntival (IM-ConjEpi) (**F**–**J**) humano.

Según estudios dosis-dependientes en el modelo *in vitro* de estrés oxidativo de la superficie ocular, CONV y OPT3 demostraron un efecto antioxidante significativo sobre las células del epitelio corneal y conjuntival humano mediante la inhibición de la producción de especies reactivas de oxígeno (Figura 8.A, B, F G) (En HCE; 0.20 mg/mL de CONV y 0.005 mg/mL de OPT3. En IM-ConjEpi; 0.05 mg/mL de CONV y 0.005 mg/mL de OPT3). La actividad antioxidante de OPT3 se demostró en concentraciones de 10 a 40 veces más bajas en comparación con CONV. OL, HT y su combinación en concentraciones bajas (10 μM de hidroxitirosol, 10 μM de oleuropeína y 5+10 μM de oleuropeína+hidroxitirosol en HCE; 25 μM de hidroxitirosol, 25 μM de oleuropeína y 5+10 μM de oleuropeína+hidroxitirosol en IM-ConjEpi) tienen un efecto antioxidante sobre las células del epitelio corneal y conjuntival humano mediante la inhibición de la producción de especies reactivas de oxígeno en estudios dosis-dependientes en el modelo *in vitro* de estrés oxidativo de la superficie ocular (Figura 8.C, D, E, H, I, J).

4.2.3 Efecto sobre la Proliferación de Linfocitos T Humanos (CD4+)

OPT3 e HT redujeron significativamente *in vitro* la proliferación activada por PHA-M de los linfocitos T humanos (CD4+) a las 48 horas (Figura 9).

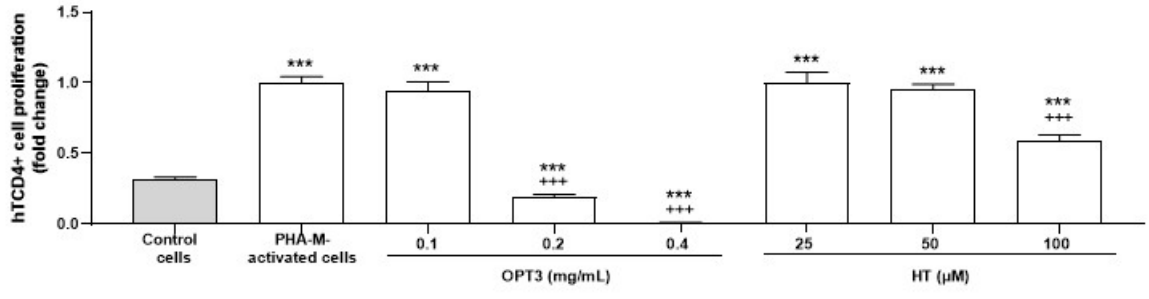

Figura 9. Efecto del extracto de alperujo seleccionado (OPT3) e HT, sobre la proliferación de linfocitos T humanos CD4+ (hTCD4+) activados por fitohemaglutinina - forma M (PHA-M).

4.3 Resultados del Efecto en un Modelo de EOS *In vivo*

Con respecto al modelo de EOS de ratón, OPT3 e HT redujeron significativamente la tinción corneal con fluoresceína (Figura 10.A), el contaje de linfocitos T en los ganglios linfáticos cervicales (Figura 10.B) y la expresión de genes de citoquinas en la glándula lagrimal, la córnea y la conjuntiva (Figura 11) de ratones expuestos a estrés por desecación.

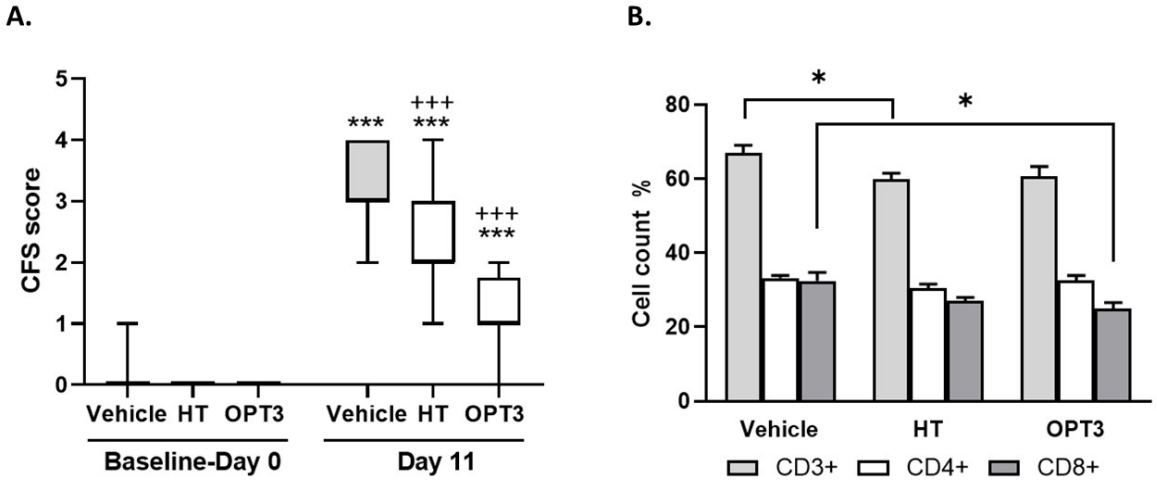

Figura 10. Efecto de la aplicación tópica de HT (100 μM) y el extracto de alperujo seleccionado (OPT3) (0.2 mg/mL) sobre la tinción corneal con fluoresceína (CFS) (**A**) y el contaje de linfocitos T CD3+, CD4+ y CD8+ (análisis FACS) en los ganglios linfáticos cervicales (**B**) de ratones expuestos a estrés por desecación.

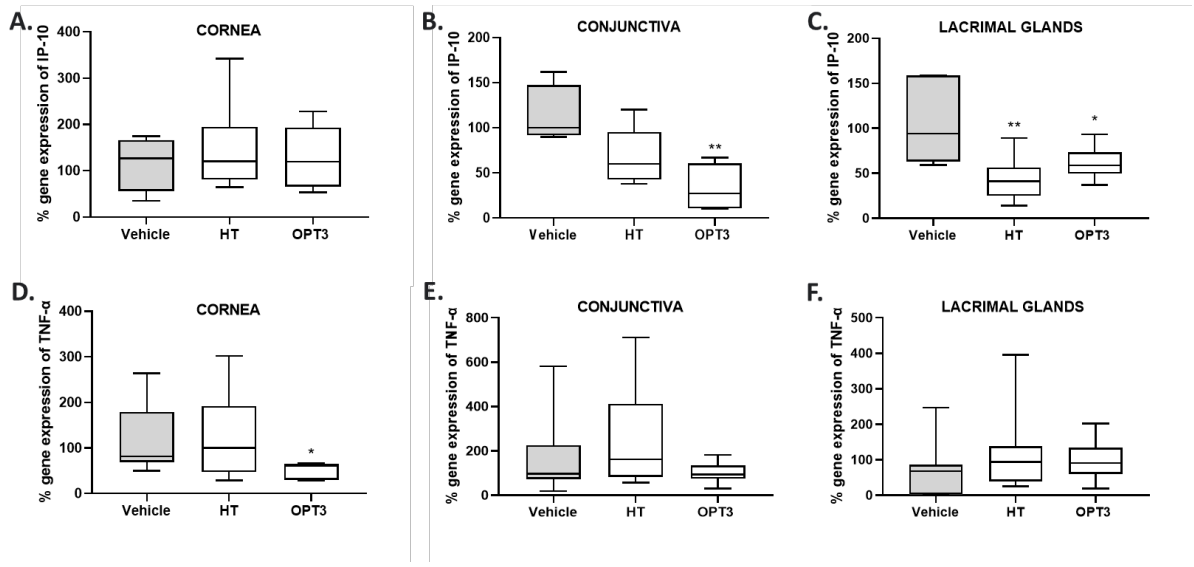

Figura 11. Efecto de la aplicación tópica de HT (100 μM) y el extracto de alperujo seleccionado (OPT3) (0.2 mg/mL) sobre la expresión de genes de IP-10 (**A**-**C**) y TNF-α (**D**-**F**) en la córnea (**A**, **D**), conjuntiva (**B**, **E**) y glándulas lagrimales (**C**, **F**) de ratones expuestos a estrés por desecación.

4.4 Estabilidad a Largo Plazo de Soluciones Acuosas de Extractos de Alperujo y Compuestos Puros

Con respecto a la estabilidad de almacenamiento a largo plazo, OL y HT demostraron un perfil de estabilidad notablemente diferente como soluciones puras o extractos en las cuatro diferentes condiciones de temperatura/humedad probadas (Figura 12). Así, OL en CONV estaba altamente degradado (aprox. 50%) después del día 2 en todas las condiciones, mientras que por el contrario como solución estándar era estable hasta 1 mes a 25 °C o menos. HT como solución pura demostró una buena estabilidad solo a 5 °C. Sin embargo, en el extracto, HT permaneció estable o incluso aumentó con el tiempo proporcionalmente con la reducción de secoiridoides en todas las condiciones. Un análisis HPLC-DAD-MS/MS de las soluciones degradadas nos permitió identificar los diferentes perfiles de degradación de OL pura o en el extracto.

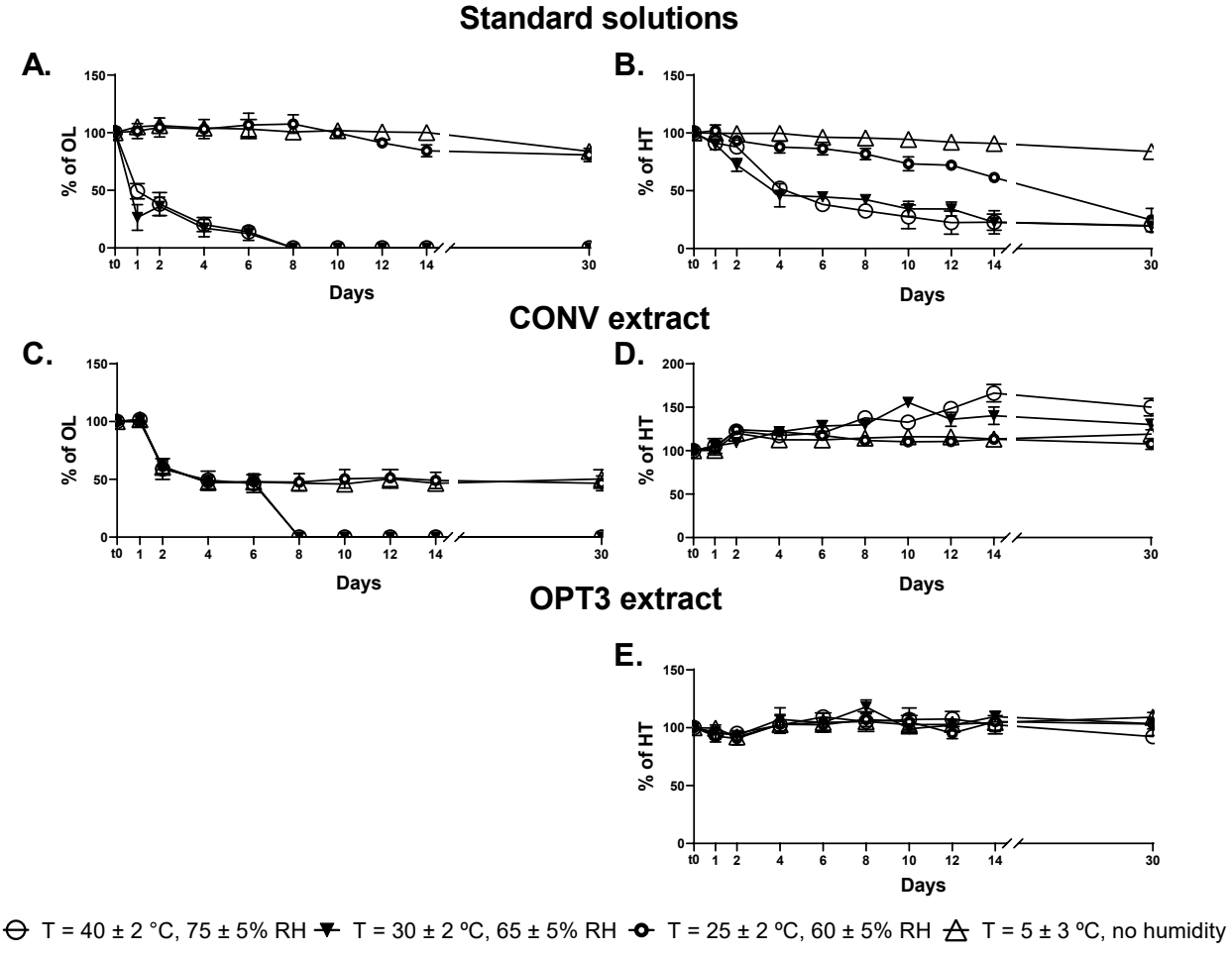

Figura 12. Estudios de estabilidad hasta 30 días de soluciones acuosas de oleuropeína (OL) estándar (**A**), hidroxitirosol (HT) estándar (**B**), OL (**C**) y HT (**D**) en el extracto convencional (CONV) e HT (**E**) en el extracto optimizado (OPT3) en cuatro condiciones diferentes de temperatura (T) y humedad relativa (RH). Los resultados se presentan como promedio de porcentaje de cada compuesto (HT u OL) con respecto a la cantidad inicial de t_0 ± desviación estándar.

5. Conclusiones

Esta tesis es una contribución en la búsqueda de nuevas terapias en el tratamiento de la enfermedad del ojo seco a partir de compuestos bioactivos de origen natural. En concreto, se propone el uso de extractos fenólicos del alperujo de oliva por su demostrada actividad antiinflamatoria y antioxidante. Esta aplicación de alto valor añadido para este un subproducto de la industria oleícola, potencialmente peligroso para el medio ambiente, es de gran importancia para el desarrollo sostenible de esta industria. También, los resultados de este estudio ilustran cómo las técnicas de extracción sostenibles e intensificadas demuestran ser competitivas frente a las convencionales, estableciendo también una alta selectividad hacia compuestos con alta actividad biológica para el tratamiento de la enfermedad del ojo seco. Las principales conclusiones que responden a los objetivos específicos planteados se muestran a continuación.

1. La liofilización seguida de un proceso de desgrasado con dióxido de carbono supercrítico es la condición de procesamiento más adecuada del alperujo, obteniéndose un extracto hidroalcohólico con una mayor concentración en comparación con el material fresco en sus principales compuestos fenólicos (incrementos entre el 90-224%, según el compuesto), así como una mayor actividad antioxidante química (incremento del 22%).
2. El uso de la extracción líquida presurizada permite establecer condiciones óptimas de extracción diferentes para maximizar la riqueza del extracto seco en los distintos compuestos de interés (oleuropeína, hidroxitirosol y oleaceína) así como en la actividad antioxidante química reduciendo el consumo de solvente (mezclas hidroalcohólicas) en 1.6 veces y el tiempo de extracción en 1/3, y un incremento entre 89-475% (según el compuesto) en la riqueza respecto al proceso convencional.
3. Los extractos de alperujo, así como los compuestos fenólicos puros (oleuropeína, hidroxitirosol y su combinación) no presentan problemas de citotoxicidad en células epiteliales corneales y conjuntivales humanas a concentraciones por debajo de 0.80 mg/mL para el extracto convencional (CONV) y el extracto enriquecido en oleaceína y con la mayor actividad antioxidante química (OPT1), 0.40 mg/mL para el extracto enriquecido en oleuropeína (OPT2) y el extracto enriquecido en hidroxitirosol (OPT3), 100 μM para hidroxitirosol, 300 μM para oleuropeína y 5+50 μM para oleuropeína+hidroxitirosol, por lo que pueden ser usados en esas dosis con seguridad.
4. El uso de hidroxitirosol y de los extractos CONV y OPT3 podrían ser de utilidad para paliar la respuesta inflamatoria de tipo innato del epitelio corneal y/o conjuntival en patologías de la superficie ocular humana.
5. El uso de hidroxitirosol, oleuropeina, solos o en combinación a bajas dosis, y de los extractos CONV y OPT3 podrían ser de utilidad como terapias antioxidantes para paliar la respuesta de estrés oxidativo de tipo innato del epitelio corneal y/o conjuntival en patologías de la superficie ocular humana.
6. La capacidad de actividad inmunosupresora sobre linfocitos T CD4+ de hidroxitirosol así como del extracto OPT3 sugiere que ambos tratamientos podrían ser de utilidad como terapias en patologías inflamatorias con base inmune de la superficie ocular humana.

7. Los tratamientos OPT3 e hidroxitirosol se perfilan como prometedores agentes terapéuticos para el tratamiento de la enfermedad del ojo seco, basado en las propiedades inmunorreguladoras y antinflamatorias mostradas *in vivo* en los ganglios linfáticos cervicales, la glándula lagrimal, la córnea y la conjuntiva de ratones expuestos a estrés por desecación (modelo animal de ojo seco).
8. Los extractos de alperujo CONV y OPT3, así como los compuestos fenólicos puros (oleuropeína, hidroxitirosol y su combinación) se pueden utilizar de forma segura como productos oftálmicos tópicos en la superficie ocular humana en base a su no genotoxicidad a concentraciones de 0.80 mg/mL para CONV, 0.40 mg/mL para OPT3, 100 μM para hidroxitirosol, 300 μM para oleuropeína y 5+50 μM para oleuropeína+hidroxitirosol), algo necesario para su aprobación como futuros productos farmacéuticos.
9. El establecimiento por primera vez de las interacciones entre oleuropeína e hidroxitirosol, y el perfil de degradación de las soluciones acuosas de oleuropeína en los estudios de estabilidad, establece una línea de base de evaluación necesaria para la futura aprobación de extractos de alperujo y sus compuestos principales como productos oftálmicos.

Appendix I

Patent Submission

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica utilizando la conexión segura de la O.E.P.M. De acuerdo con lo dispuesto en el art. 16.1 del Reglamento de ejecución de la Ley 24/2015 de Patentes, se han asignado a su solicitud un número de expediente y una fecha de recepción de forma automática. La fecha de presentación de la solicitud a la que se refiere el art. 24 de la Ley le será comunicada posteriormente.

Número de solicitud:	P202031306	
Fecha de recepción:	28 diciembre 2020, 14:21 (CET)	
Oficina receptora:	OEPM Madrid	
Su referencia:	ES2080.29	
Solicitante:	Universidad de Valladolid	
Número de solicitantes:	1	
País:	ES	
Título:	COMPUESTOS PARA SU USO EN LA PREVENCIÓN Y/O TRATAMIENTO DE LA INFLAMACIÓN Y EL ESTRÉS OXIDATIVO DEL SEGMENTO ANTERIOR DEL OJO	
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Fecha y hora de recepción:	28 diciembre 2020, 14:21 (CET)	

Codificación del envío:	54:B9:72:65:E2:DB:5C:EF:53:C4:0B:00:CC:62:6A:D3:AE:ED:D3:CD

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(2) FORMULARIO 5101. TIPO DE SOLICITUD: PRIMERA PRESENTACIÓN SOLICITUD DIVISIONAL CAMBIO DE MODALIDAD TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA PCT: ENTRADA FASE NACIONAL	 [✓] [ ] [ ] [ ] [ ]
(3) EXP. PRINCIPAL O DE ORIGEN: MODALIDAD: N.ºSOLICITUD: FECHA SOLICITUD:	
4) LUGAR DE PRESENTACIÓN:	OEPM, Presentación Electrónica
(5-1) SOLICITANTE 1: DENOMINACIÓN SOCIAL: UNIVERSIDAD PÚBLICA NACIONALIDAD: CÓDIGO PAÍS: NIF/NIE/PASAPORTE: CNAE: PYME: DOMICILIO: LOCALIDAD: PROVINCIA: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: EMPRENDEDOR: PERSONA DE CONTACTO: MODO DE OBTENCIÓN DEL DERECHO: INVENCIÓN LABORAL: CONTRATO: SUCESIÓN: OTROS: PORCENTAJE DE TITULARIDAD:	 Universidad de Valladolid [✓] España ES Q4718001C Plaza del de Santa Cruz, 5 bajo Valladolid 47 Valladolid 47002 España ES [ ] [✓] [ ] [ ] [ ] 100,00 %
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(7) TITULO DE LA INVENCION:	COMPUESTOS PARA SU USO EN LA PREVENCIÓN Y/O TRATAMIENTO DE LA INFLAMACIÓN Y EL ESTRÉS OXIDATIVO DEL SEGMENTO ANTERIOR DEL OJO
(8) NUMERO DE INFORME TECNOLÓGICO DE PATENTES (ITP):	
(9) SOLICITA LA INCLUSIÓN EN EL PROCEDIMIENTO ACELERADO DE CONCESION SI NO	 [ ] [✓]
(10) EFECTUADO DEPÓSITO DE MATERIA BIOLÓGICA: SI NO	 [ ] [✓]
(11) DEPÓSITO: REFERENCIA DE IDENTIFICACION: INSTITUCIÓN DE DEPÓSITO: NÚMERO DE DEPÓSITO: ORIGEN BIOLÓGICO:	
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(16) REMISION A UNA SOLICITUD ANTERIOR: PAIS DE ORIGEN: CODIGO PAIS: NUMERO: FECHA:	
(17) AGENTE DE PROPIEDAD INDUSTRIAL: APELLIDOS: NOMBRE: CODIGO DE AGENTE: NUMERO DE PODER:	 Pons Ariño Angel 0499/5

(18) DIRECCIÓN A EFECTOS DE COMUNICACIONES: DIRECCIÓN ASOCIADA AL PRIMER SOLICITANTE DOMICILIO: LOCALIDAD: CÓDIGO POSTAL: PAÍS RESIDENCIA: CÓDIGO PAÍS: TELÉFONO: FAX: CORREO ELECTRÓNICO: MEDIO PREFERENTE DE COMUNICACIÓN	
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(20) EL SOLICITANTE SE ACOGE A LA REDUCCIÓN DE TASAS PARA EMPRENDEDORES PREVISTA EN EL ART. 186 DE LA LEY 24/2015 DE PATENTES Y, A TAL EFECTO, APORTA LA SIGUIENTE DOCUMENTACIÓN ADJUNTA:	[ ]
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(22) FIRMA: FIRMA DEL SOLICITANTE O REPRESENTANTE: LUGAR DE FIRMA: FECHA DE FIRMA:	 50534279J ANGEL PONS (R: B84921709) MADRID 28 Diciembre 2020

Appendix II

Additional work: “Purification of the conventional olive pomace extract”

This piece of additional work was conducted at the Instituto de Biologia Experimental e Tecnológica (iBET) (Oeiras, Portugal) during a 3-month industrial secondment. It consisted of a purification study performed to increase the concentration of the conventional olive pomace extract (CONV) in the bioactive compounds hydroxytyrosol (HT), oleuropein (OL), and oleacein (OLC). Since the objective was the establishment of a purification protocol for each phenolic compound, the CONV extract was selected over the OPT3, as it comprised all aforementioned substances and it had also shown remarkable *in vitro* activity. In the OPT3 extract, there was a lack of OL content, as well as very low richness in OLC.

Introduction

One of the most important limiting factors for the use of an extract as a potential drug product is its heterogeneity, which together with the complexity of the isolation, fractionation, and structural identification processes, can lead to a lack of detection of the bioactive compounds present in it.[1-3] Apart from the compounds of interest (in our case, phenolic compounds), an obtained extract may comprise several unwanted components, such as mono- or polysaccharides,[4] cellulose,[5] proteins,[6] or mechanically recovered pigments, terpenes, fats, and waxes.[7]

Several methods have been described to purify the compounds of interest from the rest of the components present in an extract. For phenolic compounds, membrane processing (microfiltration, ultrafiltration, and nanofiltration), reverse osmosis and osmotic distillation, as well as adsorption technology have widely been studied.[8] For the adsorption of phenolic compounds from the extract solution, adsorbent (such as the different types of Amberite® XADs and Diaion® HP20) and ion exchange (such as Amberlite™ IRA) resins are used.[9,10] Ion exchange resins exert differences principally on their functional groups.[11]

According to the bibliography, among the adsorbent resins, XAD4, XAD7, and XAD16 have extensively been used to recover the phenolic compounds from olive mill wastewaters.[12,13] Also, the good adsorption capacities of HP20 resin have been reported,[14] whereas, among the ion exchange resins, the weak base IRA resins (IRA67 and IRA96) have been applied to olive mill wastewaters, demonstrating a high recovery of the present polyphenols.[15,16] Hence, for the purification study, one ion exchange (IRA96) and 4 different adsorbents (XAD4, XAD7, XAD16, and HP20) resins were selected and compared. A first screening was performed, using pure aqueous solutions of HT and OL, to establish the experimental parameters. Subsequently, the 3 most effective resins were tested in the aqueous solution of CONV extract.

Materials and Methods

Plant Material

Olive pomace from 2018 crop (Arbequina variety) was kindly offered by Oliduero (Medina del Campo, Valladolid, Spain). It was stored at -20 °C and then, freeze-dried under vacuum (18 kPa) and in darkness for 72 h (Lyoquest-55, Telstar, Terrassa, Spain).

Materials

Milli-Q water was obtained from a Millipore unit, and non-denaturalized EtOH (99.9%) from Dávila

Villalobos S.L. (Valladolid, Spain). Methanol (MeOH, 99.9% LC–MS), dimethyl sulfoxide (DMSO), and phosphoric acid were purchased from Panreac Quimica SLU (Barcelona, Spain), whereas HT (≥98%), TY (≥99%), and OL (≥98%) from Extrasynthese (Genay, France). Amberite® XAD4, Amberite® XAD7, Amberite® XAD16, Diaion® HP20, and AmberliteTM IRA96 resins, as well as HCl 37%, were obtained from Sigma-Aldrich (Madrid, Spain).

CONV Extract Preparation

The CONV extract was produced using freeze-dried olive pomace. The extraction conditions, as well as the procedure followed, are described in detail in Chapter 1 (*Experimental section: Extraction of phenolic compounds. Conventional solid-liquid extraction*).

Purification Protocol Establishment: Standard Aqueous Solutions of OL and HT

Aqueous solutions of HT (120 ppm) and OL (240 ppm) were prepared using Milli-Q water. Subsequently, 0.1 g of dry resin was added to 50 mL of solution and the mixture was left under stirring for 18 h at 22 °C. For IRA96 and HT, the temperatures of 37 and 60 °C were also tested. Samples were taken at 20, 40, 60 (1h), 120 (2h) and 1080 (18h) min. After 18h, the solution was filtered under vacuum (büchner funnel) and the resin was recovered and placed into a beaker with 50 mL of solvent to perform desorption at 22 °C under stirring. For OL, EtOH was used for all resins, while for HT 5 different solvent mixtures (EtOH, EtOH acidified with 0.5%, w/w HCl 37%, 50% v/v EtOH acidified-water, 50% v/v EtOH-water, and water) were tested using IRA96. For desorption, samples were taken at 10, 20, 30, 40, 50, 60 (1h) and 120 (2h) min. All samples were measured using a UV Spectrophotometer (Genesys 10S UV-Vis, MA, USA) at 281nm, creating a calibration curve for each compound (range for OL: 5-240 ppm, for HT: 2.5-120 ppm). Results are expressed as mg of compound adsorbed/g of resin or mg of compounds desorbed.

Purification of CONV Extract

To perform the purification study at the CONV extract, the EtOH was firstly removed from the freshly produced CONV hydroalcoholic extract. To perform that, the liquid extract was put in a round bottom flask and then, into a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland) at 60°C and ca. 20 kPa. Subsequently, the extract containing only water was filtered under vacuum to remove any residual non-dissolved quantity. Then, 0.1g of dry resin was added to 12.5 mL of solution and the mixture was left under stirring for 24 h at 22 °C. Samples were taken at 10, 20, 40, 60 (1 h), 120 (2 h), 1080 (18 h), and 1440 (24 h) min. After 24h, the solution was filtered under vacuum (büchner funnel) and the resin was recovered and placed into a beaker with 12.5 mL of solvent (EtOH for XAD4 and XAD16, and 50% v/v EtOH acidified-water for IRA96). For desorption, samples were taken at 10, 20, 30, 40, 50, 60 (1 h) and 120 (2 h) min. All samples were analyzed by HPLC-DAD.

HPLC-DAD Analysis

The content in OL, OLC, TY, and HT of the purified solutions was determined by an HPLC-DAD system. The apparatus, the analytical methods, and the conditions are all described in Chapter 1 (*Experimental section: Extract characterization. HPLC-DAD analysis*). OLC was expressed as OL equivalents (OLE).

Results

Purification Protocol Establishment

For OL adsorption (Fig. 1a), all tested resins were able to retain a high percentage of the compound present in the solution (66% for XAD7, 68% for IRA96, 81% for HP20, 91% for XAD4, and 96% for XAD16) after 18 h. Apart from IRA96 that desorbed only 15% of the adsorbed OL (Fig. 1c), the rest of the resins were able to release almost all OL retained (92% for XAD16, 97% for XAD4 and XAD7, and 100% for HP20) in a short time (10 min). Regarding HT adsorption, from Fig. 1b it can be seen that only IRA96 was able to adsorb 56% of the initial quantity. Taking into account that the maximum allowable temperature for this resin is 60 °C, the effect of the temperature during the adsorption process has been studied (Fig. 2). However, the increase in temperature did not enhance HT adsorption remarkably. Since EtOH was able to release only 7% of the HT adsorbed (Fig. 1d), 4 more solvents mixtures have been applied with 50% v/v EtOH acidified-water being the most efficient (76% efficacy of desorption). Also, the desorption of HT was found to be time-dependent (Fig. 1d), with the maximum values measured at 2 h.

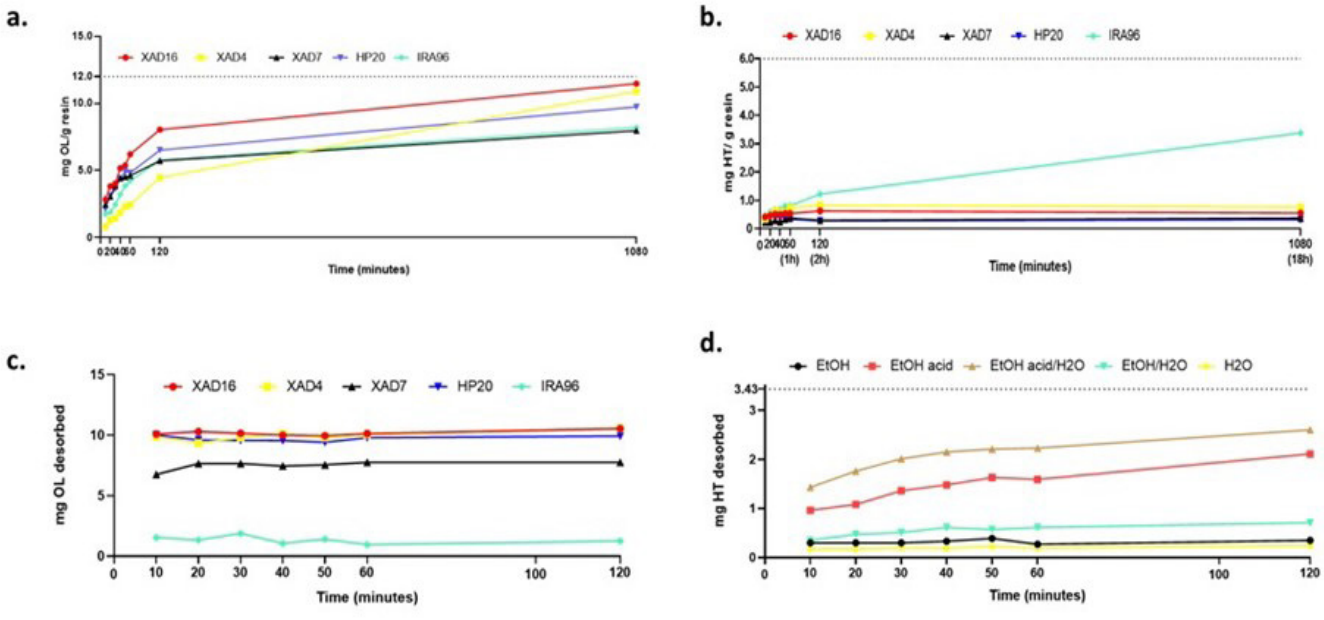

Figure 1. Adsorption (as mg of compound/g of resin – **a** and **b**) and desorption (as mg of compound obtained – **c** and **d**) at 22 °C of oleuropein (OL) (**a**, **c**) and hydroxytyrosol (HT) (**b**, **d**) as standard solutions.

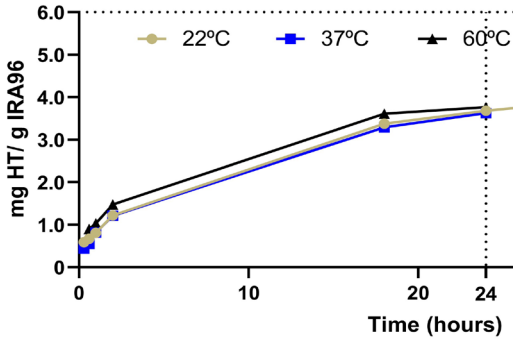

Figure 2. Adsorption of hydroxytyrosol (HT) (as mg of HT/g of resin) at 22, 37, and 60 °C.

Purification of CONV Extract

According to previous results, XAD4, XAD16, and IRA96 have been selected to be further tested with the extract. The desorption of XAD4 and XAD16 was performed using EtOH, while for IRA96 50% v/v EtOH acidified-water was used. All adsorption and desorption procedures were performed at 22 °C.

Figure 3 presents the mg of OL (Fig. 3a), OLC (Fig. 3c), HT (Fig. 3e), or TY (Fig. 3g) adsorbed from the CONV extract per g of resin tested, together with the mg of each compound desorbed (Fig. 3b for OL, Fig. 3d for OLC, Fig. 1f for HT and Fig. 1h for TY). All tested resins were able to retain equally OL. However, only IRA96 released all OL adsorbed (99% of quantity retained), while XAD4 and XAD16 a 70% of it, which was contrary to the results obtained from the purification protocol establishment study. For OLC, XAD4 was more effective in adsorbing, returning also a high percentage (83%). As already expected from the purification test with pure compounds, HT was only adsorbed by IRA96, which was able to desorb 100% of the retained quantity. Regarding TY, all tested resins were equally effective during the adsorption process. However, only IRA96 was able to release 58% of the retained quantity, as the XAD4 and XAD16 desorbed only 3% and 1% of it respectively.

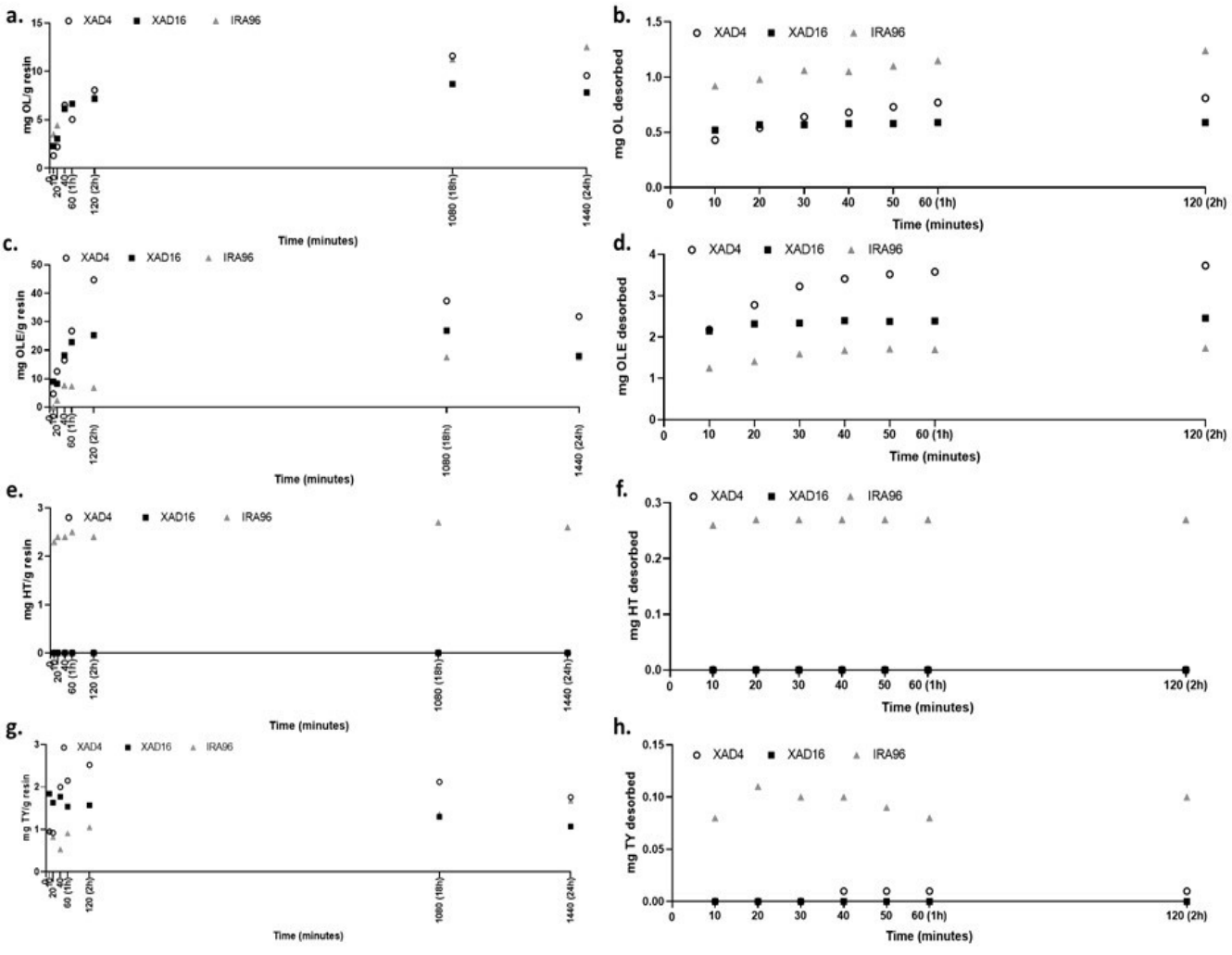

Figure 3. Adsorption (as mg of compound/g of resin – **a**, **c**, **e**, **g**) and desorption (as mg of compound obtained – **b**, **d**, **f**, **h**) at 22 °C of oleuropein (OL) (**a**, **b**), oleacein (OLC) (**c**, **d**), hydroxytyrosol (HT) (**e**, **f**) and tyrosol (TY) (**g**, **h**) applied in the conventional (CONV) OP extract. OLC is expressed as OL Equivalents (OLE).

Conclusions

1. The ion exchange IRA96 resin was the only one able to retain and desorb all phenolic compounds of interest in high percentage. It is important also to highlight that data on pure compounds cannot always be extrapolated to compounds as part of the extract.

2. The established adsorption and desorption purification protocol will be useful for the separation of unwanted compounds (such as polysaccharides and sugars) from the compounds of interest (phenolic compounds) in any olive pomace extract selected in the future.

3. Further studies of column fractionation should be performed to complete the purification study.

Appendix II

References

1. Kuphcan, S.M. Drugs from Natural Products—Plant Sources. In *Drug Discovery Science and Development in a Changing Society*; American Chemical Society: Washington, DC, 1971; Vol. 16, pp. 1–13.
2. Newman, D.J.; Cragg, G.M.; Kingston, D.G.I. Natural Products as Pharmaceuticals and Sources for Lead Structures. *Pract. Med. Chem. Fourth Ed.* **2015**, 101–139, doi:10.1016/B978-0-12-417205-0.00005-5.
3. Poulev, A.; O'Neal, J.M.; Logendra, S.; Pouleva, R.B.; Timeva, V.; Garvey, A.S.; Gleba, D.; Jenkins, I.S.; Halpern, B.T.; Kneer, R.; et al. Elicitation, a New Window into Plant Chemodiversity and Phytochemical Drug Discovery. *J. Med. Chem.* **2003**, *46*, 2542–2547, doi:10.1021/JM020359T.
4. Noggle, G.R.; Zill, L.P. The quantitative analysis of sugars in plant extracts by ion-exchange chromatography. *Arch. Biochem. Biophys.* **1952**, *41*, 21–28, doi:10.1016/0003-9861(52)90500-6.
5. Ranganagowda, R.P.G.; Kamath, S.S.; Bennehalli, B. Extraction and Characterization of Cellulose from Natural Areca Fiber. *Mater. Sci. Res. India* **2019**, *16*, 86–93, doi:10.13005/MSRI/160112.
6. Kumar, M.; Tomar, M.; Potkule, J.; Verma, R.; Punia, S.; Mahapatra, A.; Belwal, T.; Dahuja, A.; Joshi, S.; Berwal, M.K.; et al. Advances in the plant protein extraction: Mechanism and recommendations. *Food Hydrocoll.* **2021**, *115*, 106595, doi:10.1016/J.FOODHYD.2021.106595.
7. Alara, O.R.; Abdurahman, N.H.; Ukaegbu, C.I. Extraction of phenolic compounds: A review. *Curr. Res. Food Sci.* **2021**, *4*, 200–214, doi:10.1016/J.CRFS.2021.03.011.
8. Kelly, N.P.; Kelly, A.L.; O'Mahony, J.A. Strategies for enrichment and purification of polyphenols from fruit-based materials. *Trends Food Sci. Technol.* **2019**, *83*, 248–258, doi:10.1016/J.TIFS.2018.11.010.
9. Kammerer, D.R.; Kammerer, J. Enrichment and Fractionation of phenolic Compounds using Adsorbent- and Ion-Exchange-Resins. *Dtsch. Leb. Zeitschrift für Leb. und Leb.* **2013**, *109*, 25–32.
10. Kammerer, D.R.; Kammerer, J.; Carle, R. Resin Adsorption and Ion Exchange to Recover and Fractionate Polyphenols. In *Polyphenols in Plants: Isolation, Purification and Extract Preparation*; 2014; pp. 219–230 ISBN 9780123979346.
11. Vagliasindi, F.G.A.; Belgiorno, V.; Napoli, R.M.A. Water treatment in remote and rural areas: A conceptual screening protocol for appropriate POU/POE technologies. *Environ. Eng. Renew. Energy* **1998**, 329–336, doi:10.1016/B978-0-08-043006-5.50049-5.
12. Zagklis, D.P.; Vavouraki, A.I.; Kornaros, M.E.; Paraskeva, C.A. Purification of olive mill wastewater phenols through membrane filtration and resin adsorption/desorption. *J. Hazard. Mater.* **2015**, *285*, 69–76, doi:10.1016/j.jhazmat.2014.11.038.
13. Agalias, A.; Magiatis, P.; Skaltsounis, A.L.; Mikros, E.; Tsarbopoulos, A.; Gikas, E.; Spanos, I.; Manios, T. A new process for the management of olive oil mill waste water and recovery of natural antioxidants. *J. Agric. Food Chem.* **2007**, *55*, 2671–2676, doi:10.1021/jf063091d.
14. Fan, L.; Sun, G.; Qiu, J.; Ma, Q.; Hess, P.; Li, A. Effect of seawater salinity on pore-size distribution on a poly(styrene)-based HP20 resin and its adsorption of diarrhetic shellfish toxins. *J. Chromatogr. A* **2014**, *1373*, 1–8, doi:10.1016/j.chroma.2014.11.008.
15. Víctor-Ortega, M.D.; Ochando-Pulido, J.M.; Airado-Rodríguez, D.; Martínez-Férez, A. Experimental design for optimization of olive mill wastewater final purification with Dowex Marathon C and Amberlite IRA-67 ion exchange resins. *J. Ind. Eng. Chem.* **2016**, *34*, 224–232, doi:10.1016/j.jiec.2015.11.013.
16. Bertin, L.; Ferri, F.; Scoma, A.; Marchetti, L.; Fava, F. Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction. *Chem. Eng. J.* **2011**, *171*, 1287–1293, doi:10.1016/j.cej.2011.05.056.

Appendix III

Additional work: "*In vitro* immunosuppressive activity of oleuropein and the conventional olive pomace extract"

Introduction

The importance of activated T cells for the pathophysiology of dry eye disease (DED) has already been described in the Introduction (*section 2.4*). On human corneal (HCE) and conjunctival (IM-ConjEpi) epithelial cells, oleuropein (OL) and the olive pomace conventional extract (CONV) demonstrated strong antioxidant activity. For CONV, a remarkable anti-inflammatory activity was observed as well on both cell lines. Although OL had no anti-inflammatory effect on HCE cells, on IM-ConjEpi it significantly decreased interferon γ-induced protein (IP)-10 secretion. We also already described in chapter 3 the immune-suppressive effect of HT and OPT3 on human inflammatory CD4+ T cells.

To test whether CONV and OL could also affect human inflammatory cells, the immunosuppressive effect of both treatments was tested *in vitro* as well, on CD4+ T cells isolated from peripheral blood. This appendix presents briefly the results obtained from this study.

Materials and Methods

Cell Line Isolation and Culture Conditions

Human CD4+ T cells were used for this experiment, which were isolated from peripheral blood mononuclear cells (PBMCs) of 3 healthy volunteers. The isolation procedure, as well as the cell culture conditions, are described in detail in Chapter 3 (*Section 2.2.1*).

Preparation of Phenolic Solutions

OL (≥98% purity, Extrasynthese, Genay, France) and CONV were dissolved in Dulbecco's phosphate-buffered saline (DPBS – Thermo Fisher Scientific, Rockford, IL, USA) at 1200 µM and 3.2 mg/mL respectively, and stored at –20 °C in tubes. On the day of the experiment, a tube of OL or CONV was diluted with cell medium to reach the desired concentrations: 200-300 µM for OL and 0.4-0.8 mg/mL for CONV.

Cell Proliferation Inhibition Study

Cells were seeded in 96-well plates (10^5 cells/well) and activated with 1% phytohemagglutinin – M form (PHA-M – GIBCO, Grand Island, NY, USA). Then, they were treated for 48 h with 0.4, 0.6, and 0.8 mg/mL of CONV, and 200, 250, and 300 µM of OL. Cells non-activated and cells treated with culture medium were used as control. After the 48 h, the cells were incubated for 3 h in the presence of 10% alamarBlue HS Cell Viability reagent (Invitrogen, Waltham, MA, USA), and then the fluorescence was read at 560 nm_{ex} and 590 nm_{em} on a Spectra Max M5 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). Three independent experiments were performed in duplicates.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD). For data analysis, the SPSS software was used (SPSS 15.0, SPSS Inc., Chicago, IL, USA). Normality tests were performed using the Kolmogorov-Smirnov test and homogeneity of variances using the Levene's test. *T*-test or *t*-test with Welch's correction was performed between the groups and p-values lower than 0.05 were considered statistically significant.

Results

The proliferation of human CD4+ T cells was significantly activated by 1% PHA-M (ρ-value < 0.001) after 48 h (Figure 1). Both treatments, CONV and OL, significantly reduced PHA-M-activated human CD4+ T cell proliferation at 48 h at all concentrations tested (Figure 1). In particular, 0.4 mg/mL of CONV decreased it by 87 ± 4 % (ρ-value < 0.001), 0.6 mg/ml of CONV by 99 ± 4 % (ρ-value < 0.001), 0.8 mg/ml of CONV by 99.6 ± 0.3 % (ρ-value < 0.001), 200 µM of OL by 62 ± 12 % (ρ-value < 0.001), 250 µM of OL by 86 ± 15 % (ρ-value < 0.001), and 300 µM of OL by 96 ± 2 % (ρ-value < 0.001). For all treatments, the decrease in T cell proliferation was also significant compared to the basal levels (non-activated cells).

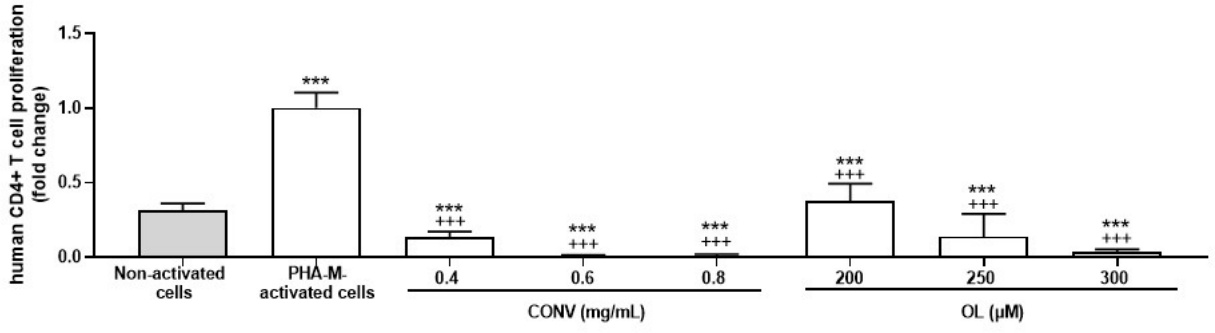

Figure 1. Effect of a conventional olive pomace extract (CONV) and oleuropein (OL), on the proliferation of phytohemagglutinin–M(PHA-M)–activated human CD4+ T cells. Cells were activated with 1% PHA-M and treated for 48h with CONV (0.4-0.8 mg/mL) or OL (200-300 µM). Cells non-activated and cells treated with culture medium were used as control. Cell proliferation was measured with alamarBlue HS assay (n = 3). *** ρ-value < 0.001, compared to non-stimulated cells; +++ ρ-value < 0.001, compared to vehicle-treated-PHA-M-activated cells.

Conclusions

The results obtained suggest that both CONV and OL could have a beneficial therapeutic effect in ocular immune-based inflammatory disease by exerting a strong immunosuppressive effect on human CD4+ T cells.

Appendix IV

Additional work: “Effect of the treatments on the osmolarity of the aqueous solution”

Introduction

The tear fluid is the outermost and most prominent barrier of the ocular surface, and is the first part of the lacrimal functional unit that comes in direct contact with the topical drug. Topical eye drops have to pass through the tear film to be retained and achieve a therapeutic effect on the ocular surface. Around 7-10 µL of fluid can be retained in the *cul-de-sac*, whereas an eye drop has a volume that varies from 20-50 µL. Hence, the physiology of the tear fluid can be highly affected through eye drop application. The average pH of the tear fluid is 7.4, its viscosity approximately 3 mPas and its surface tension about 44 mN/m, whereas its normal osmolarity ranges from 310 to 350 mOsm/L, being regulated by proteins and inorganic ions, such as Na+, K+, or Cl-, that are present in the tear fluid. Thus, drugs have to be isotonic, non-irritant, non-toxic, and of neutral pH to be effectively applied on the tear film.[1]

The osmolarity of the ophthalmic drug influences the ocular surface. In general, it should have an osmolarity value that ranges from 270 to 340 mOsm/L, to maintain the structural integrity of the eye.[2] For dry eye, reduction of tear hyperosmolarity is the first treatment line, using tear supplements that balance the osmolarity. Although no therapeutic effect was observed using moderately hypotonic (215 mOsm/L) or isotonic (305 mOsm/L) solutions, a clear therapeutic outcome was observed by applying hypotonic eye drops (150 mOsm/L).[3] Apart from the pharmaceutically active ingredient, the carbohydrate type and concentration have been found to affect solution osmolarity.[4]

Therefore, for this study, the osmolarity of aqueous solutions of OPT3 and CONV extracts at the different biologically effective concentrations was measured. Previously, the dry extract content in carbohydrates (free sugars and total polysaccharides) was calculated for conventional (CONV) and all optimized (OPT1, OPT2, and OPT3) olive pomace extracts.

Materials and Methods

Chemicals

Milli-Q water was obtained from a Millipore unit. EtOH non denaturalized (99.9%) was bought from Dávila Villalobos S.L. (Valladolid, Spain), whereas glucose (99%) and fructose (99%) from Sigma-Aldrich (Madrid, Spain).

Total Polysaccharides Determination

The total polysaccharides content was calculated according to the Laboratory Analytical Procedure of the National Renewable Energy Laboratory.[5] Briefly, 0.8 mL of sulfuric acid (72%) were added to 20 mL of extract aqueous solution at 5.0 mg/mL and autoclaved at 121 °C for 1 h. Subsequently, solutions were filtered (pore size 0.22 µm, Diameter 25 mm, Nylon; FILTER-LAB) and analyzed by HPLC-IR.

HPLC-IR Analysis

The determination of free sugar and total polysaccharides content in the dry extracts was performed by direct analysis of the extract dissolved in milli-Q water (5.0 mg/mL) and the same solution after acid

hydrolysis as previously described, respectively. The HPLC apparatus from Waters (Waters, Ireland, UK) was equipped with an isocratic pump (Waters 1515), an automatic injector (Waters 717), and an IR detector (Waters 2414). A Supelcogel Pb (Supelco) column was used at 85 °C and a flow rate of 0.5 mL/min, using milli-Q water as mobile phase.

Osmolarity Measurement

Aqueous solutions of CONV, and OPT3 were prepared at a concentration of 100 µM, 10.00 mg/mL, and 10.00 mg/mL, respectively, using Milli-Q water. Subsequently, serial dilutions were performed with Milli-Q water to reach the range of concentrations tested (and demonstrated activity) *in vitro* for each treatment; 0.20-0.80 mg/mL for CONV and 0.05-0.40 mg/mL mg/mL. For both extracts, the concentration of 10.00 mg/mL was also selected to be measured, the verify the effect of the extract on osmolarity at extrapolated concentrations.

The samples were shipped to AVIZOR S.A. (Madrid, Spain) directly after preparation, using ice to maintain the temperature at 4 °C and avoid any degradation of the products. The samples were analyzed in the laboratories of Quality Control of AVIZOR S.A. on an Osmometer (OsmoTECH® PRO; Advanced Instruments, Norwood, MA, USA) using 300 µL per sample.

Results

Free Sugar and Total Polysaccharides Content

Table 1 summarizes the extract richness in polysaccharides and oligomers for CONV, OPT1, OPT2, and OPT3 extracts. Among the sugar compounds, only fructose and/or glucose were found to be present in the solutions, in very low quantities. CONV, OPT1, and OPT2 were produced at low temperatures (66-70 °C), whereas OPT3 at a very high percentage of EtOH in water (90.0%). Hence, the low richness of all extracts in free sugars and total polysaccharides was expected.

Table 1. Free sugar and total polysaccharides content in conventional (CONV) and optimized (OPT1, OPT2, and OPT3) extracts

Extract	Total polysaccharides content		Free sugar content	
	Glucose (mg/g_{DE})	Fructose (mg/g_{DE})	Glucose (mg/g_{DE})	Fructose (mg/g_{DE})
CONV	0.034	0.025	0.029	0.011
OPT1	0.062	0.047	0.062	0.034
OPT2	0.018	0.012	0.016	0.009
OPT3	0.004	0.014	0.000	0.010

Osmolarity of Aqueous Solutions

Table 2 includes the values of osmolarity expressed as mOsm/L for all the concentrations of the different solutions of the treatments. As it can be observed, all solutions were found to be hypotonic.

Table 2. Osmolarity values for aqueous solutions of conventional (CONV) and selected optimized (OPT3) extract at different concentrations

Sample	Osmolarity (mOsm/L)
CONV 0.20 mg/mL	1
CONV 0.40 mg/mL	2
CONV 0.80 mg/mL	4
CONV 10.00 mg/mL	46
OPT3 0.05 mg/mL	1
OPT3 0.20 mg/mL	1
OPT3 0.40 mg/mL	2
OPT3 10.00 mg/mL	25

Conclusions

1. The lack of effect of the free sugars and total polysaccharides present in the olive pomace extract on the osmolarity of the final solution suggests the use of extract as crude, without further necessary purification studies.

2. None of the treatments affect the osmolarity of the final solution significantly, proving that they can be incorporated into isotonic or hypotonic solutions, without additional and extended osmolarity studies, and used topically without causing hyperosmolarity issues in the ocular surface.

References

1. Nayak, K.; Misra, M. A review on recent drug delivery systems for posterior segment of eye. *Biomed. Pharmacother.* **2018**, *107*, 1564–1582, doi:10.1016/J.BIOPHA.2018.08.138.
2. Dutescu, R.M.; Panfil, C.; Schrage, N. Osmolarity of Prevalent Eye Drops, Side Effects, and Therapeutic Approaches. *Cornea* **2015**, *34*, 560–566, doi:10.1097/ico.0000000000000368.
3. Agarwal, P.; Craig, J.P.; Rupenthal, I.D. Formulation Considerations for the Management of Dry Eye Disease. *Pharmaceutics* **2021**, *13*, 207, doi:10.3390/PHARMACEUTICS13020207.
4. Shi, X.; Summers, R.W.; Schedl, H.P.; Flanagan, S.W.; Chang, R.; Gisolfi, C. V. Effects of carbohydrate type and concentration and solution osmolality on water absorption. *Med. Sci. Sports Exerc.* **1995**, *27*, 1607–1615, doi:10.1249/00005768-199512000-00005.
5. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP)*; 2008.

Appendix V

Acceptance by the ethics committee for drug research - Patient Information Sheet

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COMITÉ DE ÉTICA DE LA INVESTIGACIÓN CON MEDICAMENTOS
ÁREA DE SALUD VALLADOLID

Valladolid a 17 de junio de 2021

En la reunión del CEIm ÁREA DE SALUD VALLADOLID ESTE del 17 de junio de 2021, se procedió a la evaluación de los aspectos éticos del siguiente proyecto de investigación.

PI 21-2340 NO HCUV	EFECTO DE COMPUESTOS Y EXTRACTOS POLIFENÓLICOS SOBRE CÉLULAS MONONUCLEATES PROCEDENTES DE SANGRE PERIFÉRICA HUMANA.	PROYECTO EUROPEO IT-DED3 I.P.: AMALIA ENRÍQUEZ DE SALAMANCA EQUIPO: LAURA GARCÍA POSADAS, SORAYA RODRÍGUEZ ROJO, NIKOLAOS KATSINAS, CARMEN GARCÍA-VAZQUEZ IOBA

A continuación, les señalo los acuerdos tomados por el CEIm ÁREA DE SALUD VALLADOLID ESTE en relación a dicho Proyecto de Investigación:

Considerando que el Proyecto contempla los Convenios y Normas establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética, se hace constar el **informe favorable** y la **aceptación** del Comité de Ética de la Investigación con Medicamentos Área de Salud Valladolid Este.

Un cordial saludo.

Dr. F. Javier Álvarez.
CEIm Área de Salud Valladolid Este
Hospital Clínico Universitario de Valladolid
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Junta de Castilla y León

HOJA DE INFORMACIÓN AL PACIENTE

Le ofrecemos participar en el estudio titulado "Efecto de compuestos y extractos polifenólicos sobre células mononucleares procedentes de sangre periférica humana"

Promotor del Estudio: IOBA

Duración del Estudio: 1 año

Responsables del Estudio: Amalia Enríquez de Salamanca Aladro

Centro: IOBA, Paseo de Belén 17, 47011 Valladolid

Teléfono: 983186369

Propósito del estudio

Nos dirigimos a usted para informarle sobre el desarrollo del estudio en el que se le propone participar. Su participación en el estudio consiste en donar una muestra de sangre para aislar células mononucleares (linfocitos T) y estudiar sobre ellas los efectos de ciertos compuestos polifenólicos de origen natural.

Nuestra intención es que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello, lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación.

Objetivos y justificación del estudio.

El síndrome de ojo seco (SOS) es una enfermedad inflamatoria de la superficie ocular que afecta aproximadamente al 15 % de la población mundial. Uno de los problemas de la terapia médica es la falta de tratamientos efectivos para el síndrome de ojo seco; siendo el uso de lágrimas artificiales para reducir la sintomatología el único tratamiento hasta el momento disponible en España.

El objetivo final del estudio es desarrollar un tratamiento efectivo basado en compuestos de origen natural para el SOS. Para ello, se pretende estudiar si los compuestos naturales objeto de estudio son capaces de disminuir la proliferación y el tipo de células mononucleares estimuladas en experimentos in vitro así como la producción de moléculas inflamatorias.

Participación voluntaria

Debe saber que su participación en este estudio es **voluntaria** y que puede decidir no participar o cambiar su decisión y retirar el consentimiento en cualquier momento, sin que por ello se altere la relación con su médico, ni se produzca perjuicio alguno en su tratamiento.

Tanto ahora como durante el transcurso del mismo, puede plantear cualquier duda o pregunta que considere sobre su participación en el estudio.

Aunque no percibirá ninguna compensación económica o de otro tipo por su participación en el estudio, la información generada en el mismo podría ser fuente de beneficios comerciales. En tal caso, están previstos mecanismos para que estos beneficios reviertan en la salud de la población, aunque no de forma individual en el participante.

Es posible que los estudios realizados aporten información relevante para su salud o la de sus familiares. Vd. tiene derecho a conocerla y trasmitirla a sus familiares si así lo desea.

Condiciones del estudio

Si se decide a participar, usted accede a que se le realice una extracción de sangre venosa de aproximadamente 15 mL (volumen máximo). La sangre se procesará para aislar las células mononucleares (PBMC) y éstas se utilizarán para determinar el efecto de los compuestos objeto de estudio, que tienen propiedades antiinflamatorias.

Usted deberá notificar al responsable del estudio si en el momento de la misma padece alguna enfermedad (muy especialmente si padece de algún tipo de alergia o proceso inflamatorio crónico) y/o está tomando algún tipo de medicación, bien sea bajo prescripción o no.

En el caso de requerirse una nueva extracción por imposibilidad de obtener los datos necesarios de la muestra ya extraída para la realización del estudio, se le requerirá para que realice una visita para realizar otra extracción de sangre.

Para este procedimiento no se le administrará tratamiento extraordinario alguno.

Consideraciones sobre las muestras biológicas obtenidas

Es importante que Vd., como potencial donante de muestras, conozca varios aspectos importantes:

A) La donación de muestras es totalmente voluntaria.

B) Puede plantear todas las dudas que considere sobre su participación en este estudio.

C) Se solicita su autorización para la toma y uso en investigación biomédica de las células mononucleares procedentes de sangre periférica (PBMC) obtenidas. En dichas muestras se analizará "in vitro" el efecto de compuestos polifenólicos de origen natural sobre la proliferación, estimulación y producción de moléculas inflamatorias en condiciones basales y/o estimuladas, utilizando los métodos que el investigador principal considere necesarios para avanzar en la línea de trabajo arriba expuesta.

D) Se le tomará un volumen relativamente pequeño (15 ml máximo) de sangre venosa mediante una punción en el brazo una única vez, si bien si por algún motivo fuera imposible obtener datos necesarios de dicha muestra obtenida se le requerirá para que realice otra visita para realizar una nueva extracción de sangre. La donación de sangre apenas tiene efectos secundarios; lo más frecuente es la aparición de pequeños hematomas en la zona de punción que desaparecen transcurridos 1 o 2 días.

E) No percibirá ninguna compensación económica o de otro tipo por las muestras donadas y éstas no tendrán valor comercial. No obstante, la información generada a partir de los estudios realizados sobre su muestra podría ser fuente de beneficios comerciales. En tal caso, están previstos mecanismos para que estos beneficios reviertan en la salud de la población, aunque no de forma individual en el donante.

F) Las muestras y los productos obtenidos de las mismas serán almacenados y custodiados en las neveras/congeladores de los laboratorios del Grupo de Superficie Ocular del IOBA, lugar designado para este fin por el Investigador Principal del Estudio. La persona responsable de la custodia es la Dra. Amalia Enríquez de Salamanca. La muestra quedará

allí depositada hasta su uso y si no se hubiese consumido en su totalidad al finalizar los análisis del estudio para el que fue recogida se procederá a su destrucción.

No obstante, siempre podrá solicitar que se destruyan sus muestras dirigiéndonos una solicitud con copia de su DNI a La Fundación General de la Universidad de Valladolid en Plaza de Santa Cruz, 5 bajo del 47002 de Valladolid o al mail **protecciondatos@funge.uva.es**.

Pruebas que se realizarán durante el estudio

El estudio consta de 1 visita. En ella, se le realizarán algunas preguntas para confirmar que es apto para el estudio y si así es, tras la firma del consentimiento informado se le realizará una extracción de sangre venosa de aproximadamente 15 mL (volumen máximo).

Riesgos que entraña el presente estudio

Usted será tratado siempre según los postulados para la investigación clínica en seres humanos recogidos en la Declaración de Helsinki. El estudio no tiene como objetivo evaluar ni comparar la eficacia de ningún fármaco o tratamiento. En ningún momento se le administrará ninguna medicación o se le realizará prueba alguna que no pertenezca a la rutina médica mejor para su caso. No se trata pues, de un ensayo clínico, sino de **un estudio de recogida de datos** de manera sistematizada, que permitirá conocer e intentar solucionar mejor sus posibles problemas oculares.
La extracción de sangre tiene como principal inconveniente la percepción de una molestia mínima derivada de la extracción de sangre. Muy infrecuentemente se pueden producir un hematoma leve, o una hemorragia leve local.

Dado a que no es esperable que aparezcan acontecimientos adversos en lo que concierne a los sujetos que participan en la investigación, no se han tomado especiales medidas orientadas a su tratamiento.

Confidencialidad

Sus datos van a formar parte de un fichero automatizado y manual denominado Pacientes cuyo responsable es el IOBA (Fundación General de la Universidad de Valladolid).

En todo momento se seguirán las normativas establecidas en la Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales, lo cual garantiza el que su identificación será siempre confidencial fuera del equipo oftalmológico que cuidará su salud. Sólo se emplearán los datos de su historia clínica para correlacionarlos con los obtenidos en los análisis de los datos del estudio, estando en todo momento desvinculado su nombre de los mismos.

Sus datos serán tratados para la finalidad de prestarle la asistencia sanitaria necesaria, para realizar la gestión administrativa y también, para fines de investigación y docencia médica.

Los datos registrados serán tratados estadísticamente de forma codificada y serán guardados de forma indefinida, lo que permitirá que puedan ser utilizados por el grupo del investigador principal en estudios futuros de investigación relacionados con la línea de trabajo arriba expuesta.

Dichos datos podrán ser cedidos a otros investigadores designados por el Investigador Principal Amalia Enríquez de Salamanca para trabajos relacionados con esta línea, siempre al servicio de proyectos que tengan alta calidad científica y respeto por los principios éticos. En estos dos últimos casos, se solicitará antes autorización al CEIm (Comité de Ética de la Investigación con Medicamentos) Área de Salud Valladolid Éste.

En todo momento el participante tendrá derecho de acceso, rectificación, cancelación y oposición dirigiéndonos una solicitud con copia de su DNI a la Fundación General de la Universidad de Valladolid en Plaza de Santa Cruz, 5 bajo del 47002 de Valladolid o al mail protecciondatos@funge.uva.es. Así mismo, tiene derecho a dirigirse a la Agencia de Protección de Datos si no queda satisfecho.
Los resultados de este estudio podrán ser divulgados en revistas científicas, congresos y otro tipo de reuniones médicas, pero siempre guardando la confidencialidad de sus datos personales.

Otra información relevante

A partir de los estudios que se realicen se podría obtener información de importancia para su salud y la de sus familiares. La información que se obtenga del análisis le será comunicada, exclusivamente a Vd., cuando sea relevante para su salud.

Si usted decide retirar el consentimiento para participar en este estudio, ningún dato nuevo será añadido a la base de datos. También debe saber que puede ser excluido del programa si los responsables del estudio lo consideran oportuno.

CONSENTIMIENTO INFORMADO

EFECTO DE COMPUESTOS POLIFENÓLICOS DE ORIGEN NATURAL SOBRE CÉLULAS MONONUCLEARES PROCEDENTES DE SANGRE PERIFÉRICA HUMANA

Promotor del Estudio: IOBA
Responsables del Estudio: Dra. Amalia Enríquez de Salamanca Aladro (IP), Dra. Laura García Posadas, D. Nikolaos Katsinas, D. Carmen García-Vázquez, D. Diego Oliveros Gútiez.
Tf. 983186369
Centro: IOBA

Yo, _____
Nombre y Apellidos de paciente o representante legal

He leído la Hoja de Información que se me ha entregado, he podido hacer preguntas sobre el estudio y he recibido suficiente información sobre el mismo.

He hablado con _____
Nombre y Apellidos del investigador

Comprendo que **mi participación es voluntaria** y que **puedo retirarme del estudio cuando quiera**, sin tener que dar explicaciones, y **sin que esto repercuta en mis cuidados médicos**.

	SI	NO
• Presto libremente mi conformidad para participar en el estudio y doy mi consentimiento para el acceso y utilización de mis datos en las condiciones detalladas en la hoja de información.	☐	☐
• Accedo a que los investigadores contacten conmigo en el futuro si fuera necesario obtener nuevos datos.	☐	☐
• Accedo a que los investigadores el estudio contacten conmigo en caso de que los estudios realizados aporten información relevante para mi salud o la de mis familiares.	☐	☐
• Autorizo a que la muestra sobrante caso de que la haya pueda ser incluida en la **Colección de muestras nº 1417** "Investigación en Ciencias de la Visión", dada de alta por el IOBA en el registro nacional de Biobancos y sea utilizada en estudios posteriores de naturaleza similar dentro de la línea de investigación en Inflamación de la Superficie Ocular.	☐	☐

Una vez firmado el presente documento se me entregará una copia del mismo.

FIRMA DEL PACIENTE / REPRESENTANTE LEGAL	NOMBRE Y APELLIDOS	FECHA

Yo he explicado por completo los detalles relevantes del estudio al paciente nombrado anteriormente y/o la persona autorizada a dar el consentimiento en su nombre.

FIRMA DEL INVESTIGADOR	NOMBRE Y APELLIDOS	FECHA

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CONSENTIMIENTO INFORMADO

APARTADO PARA LA REVOCACIÓN DEL CONSENTIMIENTO
(DEBERÁ CONTACTARSE CON EL INVESTIGADOR PRINCIPAL)

Yo, revoco el consentimiento de participación en el estudio arriba firmado con fecha:

FIRMA DEL PACIENTE / REPRESENTANTE LEGAL	NOMBRE Y APELLIDOS	FECHA

IOBA – Campus Miguel Delibes – Paseo de Belén 17 – 47011 – Valladolid
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