UNIVERSIDAD DE SEVILLA
FACULTAD DE FARMACIA
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ESTUDIO DE LÍPIDOS AISLADOS DE MICROALGAS Y POLIFENOLES EN MODELOS INFLAMATORIOS DE PIEL

TESIS DOCTORAL, SEVILLA 2017

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Que la presente Tesis Doctoral titulada "ESTUDIO DE LIPÍDOS AISLADOS DE MICROALGAS Y POLIFENOLES EN MODELOS INFLAMATORIOS DE PIEL" realizada por **AZAHARA MARÍA RODRÍGUEZ LUNA** para aspirar al grado de Doctora en Farmacia con Mención Internacional, ha sido llevada a cabo bajo su dirección.

En Sevilla, a 26 de Septiembre de 2016

V°B° de las Directoras

Fdo: Elena Talero Barrientos

Fdo: Virginia Motilva Sánchez

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Fdo: Fco. Javier Ávila Román

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Que la presente Tesis Doctoral titulada "ESTUDIO DE LIPÍDOS AISLADOS DE MICROALGAS Y POLIFENOLES EN MODELOS INFLAMATORIOS DE PIEL" realizada por **AZAHARA MARÍA RODRÍGUEZ LUNA** ha sido dirigida por la **Dra. Elena Talero Barrientos,** la **Dra. Virginia Motilva Sánchez** y el **Dr. Fco. Javier Ávila Román,** para aspirar al grado de Doctora en Farmacia con Mención Internacional, cumpliendo los requisitos para este tipo de trabajo.

Y para que así conste, firmo la presente.

En Sevilla, a 26 de Septiembre de 2016

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2. DATOS ACADÉMICOS

- Máster Oficial en Especialización Profesional en Farmacia. Especialidad en Gestión y Calidad asistencial en la Oficina de Farmacia. Facultad de Farmacia (US) 2013-2014. Nota media: 9,13. Mención a mejor expediente curso 2013-2014.

- Licenciado en Farmacia, (US) 2008-2013. Nota media: 7,1

- Protección y Experimentación Animal. Función A, B y C en Roedores, Lagomorfos, Peces y Anfibios 2017.

3. EXPERIENCIA PROFESIONAL E INVESTIGACIÓN

- Contrato como personal investigador para la ejecución del proyecto "Acción II.3 (B) V Plan Propio de Investigación de la US" (INV-2016-I-008), asociado al proyecto "Interés de los Polifenoles en Inflamación Crónica y Cáncer: Papel de los Sistemas Celulares de Regulación Mitocondrial y del Inflamasoma" Polfanat-II (P12-AGR-430, 2013/1302). Proyecto de Excelencia, Junta de Andalucía (Octubre 2016-Agosto 2017).

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- Contrato como personal investigador. Ayudas a la gestión de la investigación (PPI2015-I.5). V Plan Propio de Investigación, Universidad de Sevilla (Julio-Septiembre 2015).

- Asistente Honorario del Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla, durante el cursos 2015/2016.

- Contrato como personal investigador con cargo al proyecto "Desarrollo de una cadena de producción de microalgas marinas e hipersalinas y productos derivados orientada al mercado de la alimentación" ALGALIMENTO IPT-2011-1370-060000 (Proyecto INNPACTO) Ministerios de Economía y competitividad (Octubre 2013-Diciembre 2014).

- Contrato en Oficina de Farmacia (Junio-Agosto 2013).

- Alumna Interna en el Departamento de Farmacología de la Facultad de Farmacia de la Universidad de Sevilla, durante los cursos 2012/2013 y 2011/1012. Bajo la dirección de la Dra. Elena Talero Barrientos.

4. TAREAS DOCENTES

- Impartición de la asignatura de prácticas Farmacología y Farmacoterapia I, 4º Curso del Grado en Farmacia. 3 créditos totales impartidos durante el curso 2016/2017

5. PARTICIPACIÓN EN PROYECTOS.

- Colaboración en proyecto Internacional. Búsqueda de principios activos antiinflamatorios y anticancerígenos a partir de especies vegetales del Estado de Hidalgo. Área de química, Universidad Autónoma del Estado de Hidalgo, México. Responsable: J. Jesús Martín Torres Valencia (Desde 2015- Actualidad).

- Investigadora del proyecto Polfanat-II (P12-AGR-430 MO). Interés de los Polifenoles en Inflamación Crónica y Cáncer: Papel de los Sistemas Celulares de Regulación Mitocondrial y del Inflamasoma. Junta de Andalucía. Responsable: Dra. Virginia Motilva Sánchez. (Desde Septiembre 2015- Actualidad).

- Investigadora del proyecto ALGALIMENTO IPT-2011-1370-060000 (Proyecto INNPACTO). Desarrollo de una cadena de producción de microalgas marinas e hipersalinas y productos derivados orientada al mercado de la alimentación. (2013-2014). Ministerio de Economía y competitividad. Responsable: Dra. Virginia Motilva Sánchez (Desde Octubre 2013-Diciembre 2014).

6. GRUPO DE INVESTIGACIÓN

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7. ESTANCIAS Y PRÁCTICAS EXTERNAS.

- Estancia en el Departamento de Biología de la Universidad de Aveiro, Portugal. (Febrero-Mayo 2017). En colaboración con la Dra. Helena Oliveira. holiveira@ua.pt

- Prácticas en el Área de Dermatología del Hospital Universitario Virgen del Rocío. Área de Dermatología. Conocimiento, detección y tratamiento de patologías relacionadas con la piel. 160 horas. Sevilla (Mayo 2014).

- Estancia en el Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia. (Octubre 2014). En colaboración con el Dr. Miguel Payá Peris. miguel.paya@uv.es

- Prácticas en Farmacia Hospitalaria en Hospital Universitario Virgen de Valme. 400 horas. Sevilla. 2013.

- Prácticas en Oficina de Farmacia "República Argentina 10, D. Pedro A Villalba Orellana". 400 horas. Sevilla 2013.

8. PUBLICACIONES

De los Reyes, C., Ortega, M.J., Rodríguez-luna, A., Talero, E., Motilva, V., Zubía, E. 2016. Molecular Characterization and Anti-inflammatory Activity of Galactosylglycerides and Galactosylceramides from the Microalga Isochrysis galbana. J. Agric. Food Chem. 1–39. doi:10.1021/acs.jafc.6b03931

Ávila-Román, J., Talero, E., Rodríguez-Luna, A., García-Mauriño, S. and Motilva, V. 2016. Anti-inflammatory effects of an oxylipin-containing lyophilised biomass from a microalga in a murine recurrent colitis model. Br. J. Nutr. 1–9. doi: 10.1017/S0007114516004189.

Talero, E., García-Mauriño, S., Ávila-Román, J., Rodríguez-Luna, A., Alcaide, A., Motilva, V., 2015. Bioactive Compounds Isolated from Microalgae in Chronic Inflammation and Cancer. Mar. Drugs 13, 6152–209. doi:10.3390/md13106152

9. COMUNICACIONES A CONGRESOS

1. Rodríguez-Luna, A., Ávila-Román, J., González-Rodríguez., M.L., Talero, E., Zubía, E., Motilva, V. Topical pre-treatment with a carotenoid from microalgae reduces cutaneous UVB-induced inflammation in hairless mice. Oral presentatation. 37th National Meeting of the Spanish Society of Pharmacology (SEF) (Barcelona, 2017).

2. Rodríguez-Luna, A., Oliveira, H., Ávila-Román, J., Talero E., Motilva, V. Efectos del carotenoide F-111 y del polifenol RA-303, solos o en combinación, sobre la producción de ROS y la apoptosis en queratinocitos humanos HaCaT irradiados con UVB. IX Reunión de jóvenes farmacólogos de Andalucía. Comunicación oral (Sevilla, 2017).

3. A. Rodríguez-Luna, E. Talero, FJ. Ávila-Román, V. Motilva Topical pre-treatment with a carotenoid from microalgae reduces

cutaneous UVB-induced inflammation in hairless mice. II Jornadas de Doctorado en Farmacia. Comunicación oral (Sevilla, 2017).

4. A. Rodríguez-Luna, E. Talero, FJ. Ávila-Román, V. Motilva ¿Son necesarias nuevas terapias en fotoprotección? Nuestra experiencia con productos de origen natural. Jornadas Internacionales de Investigación e Innovación en Ciencias de la Salud. Póster (Sevilla, 2016).

5. A. Rodríguez-Luna, FJ. Ávila-Román, I. Sosa-Rosado, ML. González-Rodríguez, MJ. Cózar, C. de los Reyes, E. Zubía, V. Motilva, E. Talero. Estudio de fórmulas farmacéuticas con compuestos de origen marino para el tratamiento de patologías inflamatorias de la piel. VIII Reunión de Jóvenes Farmacólogos de Andalucía. Comunicación oral (Málaga, 2016).

6. I. Sosa-Rosado, A. Rodríguez-Luna, FJ. Ávila-Román, V. Motilva, E. Talero. Estudio de productos naturales marinos como estrategia terapéutica en modelos de inflamación y cáncer de colon. VIII Reunión de Jóvenes Farmacólogos de Andalucía. Comunicación oral (Málaga, 2016).

7. FJ. Ávila-Román, A. Rodríguez-Luna, E. Talero, I. Sosa-Rosado, C. de los Reyes, E. Zubía, S. García-Mauriño, V. Motilva. Our experience in the use of microalgae products in inflammatory process: Importance of fatty acids and carotenoids compounds. European Roadmap for an Algae-based industry. European Conference Olhão. Póster (Portugal 2016).

8. E. Talero, A. Rodríguez-Luna, V. Cechinel Filho, R. Niero, LB. Mariano, V. Motilva. In vitro antioxidant, cytotoxic and anti-inflammatory activity of uncommon xanthones from branches of Garcinia achachairu Rusby. International Conference of polyphenols and health. Póster (Tours, Francia, 2015).

9. A. Rodríguez-Luna, E. Talero, C. de los Reyes, E. Zubía, MC. Terencio, V. Motilva. Compuestos aisaldos de la microalga Isochrysis galbana previenen la hiperplasia en un modelo de psoriasis en ratón. I Jornadas Internacionales de Ciencias de la Salud. Comunicación oral (Sevilla, 2015).

10. A. Rodríguez-Luna, E. Talero, FJ. Ávila-Román, A. Alcaide, MJ. Cózar, ML. González-Rodríguez, C. de los Reyes, E. Zubía, MC. Terencio, V. Motilva. Topical application of glycolipids and fucoxanthin from microalgae prevents inflammation in a murine model of TPA-induced hyperplasia. 36° Congreso de la Sociedad Española de Farmacología. Póster (Valencia, 2015).

11. FJ. Ávila-Román, E. Talero, A. Rodríguez-Luna, A. Alcaide, C. de los Reyes, E. Zubía, S. García-Mauriño , V. Motilva. Pro-resolving effects of an oxylipins-containing biomass from Chlamydomonas debaryana in a recurrent colitis model in mice. 36° Congreso de la Sociedad Española de Farmacología (Valencia, 2015).

12. A. Rodríguez-Luna, E. Talero, A. Alcaide, C. de los Reyes, E. Zubía, M. Payá, MC. Terencio, V. Motilva. Glicolípidos aislados de microalgas previenen la hiperplasia epidérmica inducida con TPA en ratones. VII Reunión de Jóvenes Farmacólogos de Andalucía. Comunicación oral (Granada, 2015).

13. A. Rodriguez-Luna, E. Talero, C. de los Reyes, E. Zubía, S. García-Mauriño, V. Motilva. Photoprotective effect of glycosylglycerols and glycolipids from microalgae in HaCaT keratinocytes. 35º Congreso de la Sociedad Española de Farmacología. Póster (Madrid, 2014).

14. A. Rodriguez-Luna, E. Talero, C. de los Reyes, E. Zubía, S. García-Mauriño, V. Motilva . Photoprotective effect of glycosylglycerols and glycolipids from microalgae in HaCaT keratinocytes. VI Reunión de Jóvenes Farmacólogos de Andalucía. Comunicación oral. (Sevilla, 2014).

10. Premios y otros eventos científicos.

- Premio a "Mejor comunicación oral". Efectos del carotenoide F-111 y del polifenol RA-303, solos o en combinación, sobre la producción de ROS y la apoptosis en queratinocitos humanos HaCaT irradiados con UVB. IX Reunión de Jóvenes Farmacólogos de Andalucía. (Sevilla, 2014).

- Reconocimiento a Mejor Expediente Académico de la Primera Promoción del Máster Universitario en Especialización Profesional en Farmacia. Facultad de Farmacia (Sevilla, 2014).

- Finalista al I Premio de la Confederación Empresarial de Oficinas de Farmacia de Andalucía. Facultad de Farmacia (Sevilla, 2014).

- Premio a "Mejor comunicación oral". Photoprotective effect of glycosylglycerols and glycolipids from microalgae in HaCaT keratinocytes. VI Reunión de Jóvenes Farmacólogos de Andalucía. (Sevilla, 2014).

- Comité organizador en IX Reunión de Jóvenes Farmacólogos de Andalucía. (Sevilla, 2017).

- Comité organizador en VI Reunión de Jóvenes Farmacólogos de Andalucía. (Sevilla, 2014).

- Asistencia a Taller sobre "Cómo publicar Trabajos de Investigación". Springer. (Sevilla, 2013).

- Módulo de Farmacología y Farmacoterapia. Comisión de Formación Continuada. (Valencia, 2013).

11. Otros datos.

- Miembro de la Comisión de Jóvenes Farmacólogos de la Sociedad Española de Farmacología. Community manager.

12. Habilidades adicionales.

IT: Windows XP, Microsoft Word, Microsoft Power-Point, Microsoft Excel, bases de datos.

Otros programas: Unycopwin, graphpad instat, prism, Adobe Photoshop. Internet.

Idiomas:

Inglés B1 del Marco Europeo de Referencia para las Lenguas.

Actualmente estudiando "First Certificate in English (FCE)."

12. TÉCNICAS EXPERIMENTALES

Técnicas bioquímicas: Western Blotting, ELISA, inmunohistoquímica, histología, microscopía, citometría de flujo.

Cultivo celular: Modelos *in vitro* de queratinocitos humanos inmortalizados (HacaT) empleando técnicas relacionadas con la radiación UVB, entre otras.

Manipulación animal: Modelos de inflamación en piel de ratón, modelos de hiperplasia epidérmica (Psoriasis) y modelos de inflamación y envejecimiento por estimulación con UVB.

Elaboración de fórmulas farmacéuticas, desarrollo de sistemas de liberación (liposomas).

Técnicas de perfiles de permeación tanto *in vitro* como *ex vivo*.

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ÍNDICE

ABREVIATURAS

A

APC	Células presentadoras de antígenos
ARE	Elemento de respuesta antioxidante
ARN	ARN mensajero
ASC	Apoptosis-attached-speck-like protein containing a CARD

B

BAX	Homólogo pro-apoptótico de BCL-2
BCL-2	Células de linfoma B tipo 2

C

CASP	Caspasa
CBC	Carcinoma Basocelular
CEC	Carcinoma Epidermoide Cutáneo
Ch	Cholesterol
CLSM	Confocal Laser Scanning Microscopy
COX	Ciclooxigenasa
CPNM	Cáncer de Piel No Melanocítico
CXC	Quimiocinas

D

DC	Células dentríticas
DCF	DCF, 2',7'-dichlorofluorescein
DCF-DA	DCF-DA, dichlorofluorescein diacetate
DCFH-DA	DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate
DCm	Células dendríticas mieloides
DCp	Células dendríticas plasmocitoides
Dex	Dexamethasone
DFBN	2,4-dinitrofluorobenceno
DGDGs	Digalactosil-diacilgliceroles
DHA	Ácido Docosahexaenoico
DMBA	Dimetilbenz[a]antraceno
DLQI	Dermatology Life Quality Index
DPA	Ácido Docosapentaenoico

E

EPA	Ácido eicosapentaenoico
EPC	L-α phosphatidylcholine from egg-yolk

F

Flg	Filagrina
FX	Fucoxantina

G

GSH	Glutatión reducido

H

HO-1	Hemoxigenasa-1

I

I. galbana	*Isochrysis galbana*
IFN	Interferón
IL	Interleucina
IMQ	Imiquimod
iNOS	Óxido Nítrico Sintasa inducible

J

Jak	Janus quinasa

K

KEAP	Proteína asociada (Kelch-like ECH-associated protein)

L

LC	Células de Langerhans
LDH	Lactato deshidrogenasa
LPS	Lipopolisacárido

M

MAPK	Map kinasas
MCP-1	Proteína quimioatrayente de monocitos tipo 1
MGDGs	Monogalactosyldiacylglycerols
MGMGs	Monogalactosil-monoacilglicerol
MMP	Metaloproteinasa
MPO	Mieloperoxidasa
MTX	Metotrexato

N

NF-κB	Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
NL	Nanoliposomas
NMSC	Non-melanoma skin cancer
NO	Óxido nítrico
Nrf2	Factor nuclear eritroide 2
NRLP3	NOD-LRR-and pyrin domain-containing 3

P

PASI	Psoriasis Area Severity Index
PdI	Polidispersity index
PGA	Physician Global Assessment
PGs	Prostaglandinas
pKa	Constante de ionización
PKC	Proteína kinasa C
Poli(I:C)	Poliinosínico-policitidílico
PRRs	Receptores de reconocimiento de patrones
PUVA	Psoraleno oral y radiación de la zona afectada con luz UVA

Q

QA	Queratosis actínica

R

RA	Ácido rosmarínico
RMS	Root mean square
RNS	Especies reactivas de nitrógeno
ROS	Especies reactivas de oxígeno

S

SC	*Stratum corneum*
SDC	Sodium deoxycholate
SOD	Superóxido dismutasa
SQAGs	Sulfoquinovosil-diacilgliceroles
SRB	Sulforhodamine B
STAT	Señal de activación y traducción

T

TFG-β	Factor de crecimiento transformante beta
Th1	Células T helper 1
TLR	Receptores Toll like
TNF-α	Factor de necrosis tumoral α
TPA	12-*O*-tetradecanoylphorbol-13-acetate

U

UV	Ultravioleta

V

VEGF	Vascular endothelial growth factor

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RESUMEN

Título: "Estudio de lípidos aislados de microalgas y polifenoles en modelos inflamatorios de piel"
Autora: Azahara M Rodríguez Luna

Resumen:

La piel es el principal órgano que actúa como defensa frente a agresiones externas, protegiendo al organismo de sustancias tóxicas, daño físico, e invasión por patógenos o radiaciones, suponiendo una pieza indispensable del sistema inmunológico. En este sentido, la perturbación de su estructura o función podría estar causada tanto por factores externos, como sustancias tóxicas o radiación ultravioleta (UV), o por factores internos, como pueden ser la predisposición genética, desórdenes inmunitarios u hormonales, o estrés. Como resultado de estas alteraciones, se puede desencadenar una respuesta inflamatoria, una disminución de la respuesta inmune o un daño oxidativo, entre otros.

La exposición crónica de la piel a la luz UV induce la producción de especies reactivas de oxígeno (ROS), así como una respuesta inflamatoria, la cual juega un papel importante en el fotoenvejecimiento, en el desarrollo de lesiones pre-cancerosas, como la queratosis actínica, y en el cáncer de piel. Por otro lado, las enfermedades inflamatorias de la piel tienen un alto impacto en la calidad de vida de los pacientes; una de ellas es la psoriasis, enfermedad crónica mediada por el sistema inmune, de afectación predominantemente cutánea, cuya patogénesis no está clara aún. Hoy en día, las estrategias para la protección de la piel se centran en el uso de compuestos naturales para contrarrestar el estrés oxidativo y la inflamación. En los últimos años, las microalgas han emergido como fuente de compuestos bioactivos, incluyendo lípidos, proteínas, polisacáridos y carotenoides. Estos compuestos han atraído el interés de la industria farmacéutica y cosmética en base a sus actividades como antioxidantes, anti-inflamatorios y anti-carcinogénicos.

En este estudio, hemos evaluado la actividad biológica de ciertos glicolípidos y el carotenoide fucoxantina, aislados de la especie *Isochrysis galbana*, así como del polifenol ácido rosmarínico, en diferentes modelos inflamatorios de piel tanto *in vitro* como *in vivo*, los cuales están desarrollados en los siguientes párrafos.

En primer lugar, hemos evaluado la actividad citotóxica, antioxidante y fotoprotectora de diferentes glicolípidos aislados de

la microalga *Isochrysis galbana,* empleando un modelo *in vitro* de queratinocitos humanos HaCaT irradiados con UVB. El pre-tratamiento de los queratinocitos con compuestos puros con estructuras de monogalactosil-monoacilgliceroles (MGMGs) y digalactosil-diacildigliceroles (DGDGs), así como las fracciones ricas en monogalactosil-diasilgliceroles (MGDGs) o DGDGs redujeron significativamente el daño celular inducido por UVB, mediante la disminución de la liberación de LDH, ROS y la producción de IL-6. Este estudio preliminar nos permitió la selección de los compuestos más activos, que fueron la fracción de glicolípidos **MGDG** y el glicolípido puro **MGMG-A**, para su estudio posterior en modelos *in vivo*(Capítulo 1).

En el primer modelo *in vivo*, se estudiaron la fracción MGDG y el glicolípido puro MGMG-A, en un modelo de hiperplasia epidérmica inducida TPA en ratones Swiss CD-1. Son numerosas las evidencias que muestran que la exposición de la piel a un activador de la proteína kinasa C, como es el 12-O-tetradecanoilforbol-13-acetato (TPA), induce una respuesta pleiotrópica en el tejido, dando lugar al desarrollo de lesiones macroscópicas como descamación y eritema, lo cual se asemeja al fenotipo aparente de la psoriasis. En primer lugar, examinamos los efectos anti-inflamatorios de la aplicación tópica de MGDG y MGMG-A, disueltos en acetona (10 µg/µL, 200 µg por zona), comparándolos con la Dex como compuesto de referencia. Los tratamientos se aplicaron en un área delimitada de 1 cm^2 en el dorso de los ratones y después de 30 minutos, se aplicó tópicamente el TPA (disuelto en acetona a 2 nmol por zona), en las mismas áreas durante 3 días. Nuestros resultados mostraron que el tratamiento con los glicolípidos disminuyó el edema, el espesor de la epidermis y la producción de las citocinas pro-inflamatorias TNF-α, IL-1β, IL-6 y IL-17.

Sin embargo, es sabido que la aplicación tópica de compuestos requiere una adecuada incorporación de las moléculas bioactivas en una fórmula que ofrezca estabilidad, que permita la permeación y que permanezca el suficiente tiempo en la piel. En este sentido, desarrollamos diferentes estudios de permeación *in vitro*, *ex vivo* e *in vivo*, para comparar el perfil de tres formulaciones diferentes (gel, pomada y crema). Los estudios *in vitro* y *ex vivo* se desarrollaron en células de Franz y se completaron con un estudio de permeabilidad *in vivo*, usando gel, pomada y crema con rodamina, lo que permitió evaluar la estabilidad de las formulaciones y las propiedades de permeación de las mismas a través de las capas de la piel. Los estudios de permeabilidad mostraron que la crema era la formulación con el mejor

perfil de permeación. Por tanto, estos resultados nos llevaron a evaluar una crema que contenía la fracción MGDG (MGDG-crema) (0.2 % p/p), en el modelo de hiperplasia en ratón, comenzando esta vez el tratamiento 2 días antes de la primera aplicación de TPA. En concordancia con la actividad anti-inflamatoria demostrada *in vitro* con esta fracción, la aplicación de la crema disminuyó el edema provocado por el TPA, mejoró los daños histopatológicos y redujo el infiltrado celular. Cabe destacar que estos resultados fueron más significativos con la aplicación de la crema que con la fracción disuelta en acetona. Además, esta formulación inhibió la expresión epidérmica de COX-2 de manera similar, alcanzando niveles similares a la Dex. Por tanto, nuestros datos muestran por primera vez el efecto preventivo de los glicolípidos en patologías inflamatorias de la piel como la psoriasis y sugieren que la fracción formulada en crema podría ser una interesante estrategia terapéutica para el tratamiento de esta patología (Capítulo 2).

El siguiente estudio nos condujo a la evaluación del efecto anti-inflamatorio, antioxidante y fotoprotector del carotenoide **fucoxantina** (FX) aislado de *Isochrysis galbana* en modelos tanto *in vitro* como *in vivo*. En primer lugar, la actividad anti-inflamatoria fue evaluada en macrófagos THP-1 estimulados con LPS y en queratinocitos HaCaT estimulados con TNF-α y la actividad antioxidante en queratinocitos HaCaT irradiados con UVB (50 mJ/cm^2). Nuestros resultados mostraron una disminución de las citocinas pro-inflamatorias TNF-α e IL-6, así como un efecto protector de la FX, debido a la reducción de la producción intracelular de ROS y LDH. Estos datos nos llevaron a evaluar el posible efecto preventivo de la FX en dos modelos *in vivo*: un modelo de hiperplasia epidérmica inducida por TPA en ratones Swiss CD-1, y un modelo de eritema agudo inducido por UVB en ratones sin pelo SKH-1, expuestos a una irradiación de 360 mJ/cm^2. Con el objeto de evitar los efectos negativos derivados del uso de disolventes como la acetona y de aumentar la permeabilidad de la FX a través de la piel, se formuló una crema que contenía el carotenoide (FX-crema) para su aplicación a los animales (0.2 % p/p). Los resultados del modelo de TPA mostraron que el pre-tratamiento con la FX-crema efectivamente mejoraba la hiperplasia en ratón inducida por TPA, reduciendo significativamente el edema de la piel, el espesor de la epidermis y la actividad de la MPO, así como la expresión epidérmica de los niveles de COX-2. Por otro lado, los datos obtenidos del modelo de exposición a UVB

demostraron que la FX-crema reducía el eritema inducido por UVB en ratón, así como el edema y la actividad MPO. Además, la crema con el carotenoide inhibió la expresión de COX-2 en la piel expuesta a UVB, así como aumentó la expresión de la proteína antioxidante HO-1, a través de la regulación de la vía del Nrf2.Los resultados obtenidos en todos los estudios realizados con la FX sugieren que este carotenoide, vehiculizado en una formulación tópica para mejorar su permeación, podría suponer una estrategia novedosa para prevenir las exacerbaciones asociadas a la psoriasis y proteger a la piel de la radiación UV(Capítulo 3).

En relación a los estudios con compuestos naturales, las plantas siguen siendo hoy en día uno de los campos más prometedores para el descubrimiento de nuevos compuestos que pueden conducir al desarrollo de fármacos. En este sentido, los polifenoles son uno de los grupos más estudiados; estos compuestos son metabolitos secundarios de las plantas y generalmente, participan en los mecanismos de defensa de éstas contra agresiones externas. Con más de 8.000 estructuras diferentes conocidas, tienen como base común la presencia de uno o más grupos fenólicos. La actividad anti radicales libres de esta familia de compuestos ha sido ampliamente descrita, de hecho son los antioxidantes más potentes que consumimos en nuestra dieta. Sin embargo, la investigación en torno a ciertas propiedades todavía continúa en desarrollo. En este sentido, la actividad anti-inflamatoria es una de las más importantes. Por lo tanto, en nuestro siguiente estudio evaluamos el efecto de un compuesto polifenólico, el ácido rosmarínico (RA) en un modelo de psoriasis inducida por Imiquimod (IMQ). El IMQ es un agonista de los receptores *Toll-like* 7 y 8, cuya aplicación tópica en el dorso de ratones Balb/c durante 6 días, induce una inflamación acompañada de alteraciones típicas de la psoriasis. Debido a que la efectividad del RA aplicado por vía tópica puede ser limitada por su baja liposolubilidad y estabilidad, así como su facilidad para oxidarse o degradarse con la luz, hemos usado nanoliposomas (NL) para encapsular dicho polifenol. El pre-tratamiento tópico con RA en gel y RA encapsulado en NL disueltos en gel (ambos a 0.2 % p/p) 30 minutos antes de la aplicación del IMQ (62.5 mg por zona durante 6 días consecutivos) inhibió el daño macroscópico y el peso del punch obtenido de la piel afectada, sugiriendo la reducción del edema de la piel, la infiltración neutrofílica, la actividad de la MPO y la expresión de citocinas pro-inflamatorias como TNF-α, IL-1β, IL-6 e IL-17. El RA en gel mostró mejores resultados que el RA encapsulado en NL, esto podría ser debido a que la constante de disociación (pka)

del RA a pH fisiológico afecta a la liberación de este compuesto desde los NL al medio celular, y consecuentemente, reduce su actividad biológica. En este sentido, al confirmar que la estructura química del RA influye en la actividad final de la formulación, sería necesaria una encapsulación específica para este tipo de compuestos. Contribuyendo con estos resultados al uso de estos sistemas en la industria farmacéutica, puesto que suponen una esperanzadora herramienta como sistemas de liberación de fármacos vía tópica, debido a su efectividad para favorecer la permeación y llevar el fármaco hasta ciertas zonas de la piel (Capítulo 4).

Por último, evaluamos un posible sinergismo entre los principales compuestos empleados en esta tesis, FX y RA. Para ello, se ensayaron en un modelo de queratinocitos HaCaT irradiados con UVB (100 mJ/cm^2) con el objetivo de elucidar su mecanismo de acción y determinar si una mezcla de los mismos (5 µM de FX + 5 µM de RA) podría mejorar sus actividades biológicas. En este estudio se determinó la viabilidad celular, el ciclo celular, y la inducción de apoptosis, así como se evaluaron mediadores implicados en la activación del inflamasoma. La mezcla de estos compuestos demostró un mejor efecto fotoprotector y antioxidante que los compuestos por separado, disminuyendo la muerte celular, con el consecuente aumento de la viabilidad celular, y reduciendo la apoptosis y los niveles de ROS inducidos por la acción del UVB. Además, la mezcla moduló la respuesta inflamatoria disminuyendo la expresión de los componentes del inflamasoma como son el NLRP3 y ASC, y aumentando la expresión de los niveles de la proteína antioxidante HO-1, mediante la regulación de la vía del Nrf2(Capítulo 5).

En conclusión, nuestros estudios *in vitro* e *in vivo* han demostrado los efectos beneficiosos de varios productos naturales, entre los que destaca la FX y el RA, en enfermedades de base inflamatoria, asociadas tanto a la exposición UVB como a patologías mediadas por el sistema inmune como la psoriasis. Sus mecanismos de acción podrían estar envueltos en la prevención del estrés oxidativo a través de la activación de la vía Nrf2 y la reducción de la inflamación mediante la regulación del inflamasoma y otras citocinas relacionadas con la vía del NF-κB. Por esta razón, estos compuestos podrían suponer moléculas prometedoras para su posible incorporación en productos para el cuidado de la piel como por ejemplo protectores solares, cremas anti-edad o regeneradoras, o formulaciones tópicas para el tratamiento de patologías inflamatorias como la psoriasis.

SUMMARY

Title: "Study of lipids from microalgae and polyphenols in skin inflammatory models"

Author: Azahara M Rodríguez Luna

Abstract:

Skin is the organ that acts as principal defence against external environment factors, protecting the organism from harmful substances, mechanical damage, pathologic invasion and radiations. It is well known that skin is an indispensable piece of the immune system. The disturbance of skin structure and its functionality could be caused by external factors such as toxic compounds or ultraviolet (UV) radiation, or internal factors including genetic predisposition, immune and hormone disorders, or stress. These perturbations could trigger the inflammatory process, a lower immune response or an oxidative stress, among others.

Chronic exposure of the skin to UVB radiation induces reactive oxygen species (ROS) production and inflammatory responses, which play a pivotal role in photo-aging, and in the development of pre-cancerous lesions as actinic keratosis or skin cancer. Inflammatory skin diseases have a significant impact on the quality-of-life of patients; one of them is psoriasis, considered as a common immune-mediated inflammatory skin disorder, whose pathogenesis is unclear. Nowadays, strategies for skin protection comprise the use of a variety of natural compounds to counteract oxidative stress and inflammation. In last years, microalgae have emerged as a source of bioactive compounds, including lipids, proteins, polysaccharides and carotenoids. These compounds have attracted the interest of the pharmaceutical and cosmetical industry based on their antioxidant, anti-inflammatory and anti-carcinogenic activities.

In this study, we have evaluated the effect of some glycolipids and the carotenoid fucoxanthin, isolated from *Isochrysis galbana*, as well as the polyphenol rosmarinic acid in different *in vitro* and *in vivo* skin inflammatory models, which are explained in the next paragraphs.

Firstly, we assessed the cytotoxic, antioxidant and protective activities of different glycolipids obtained from the microalga *Isochrysis galbana*, by using an UVB-irradiated HaCaT human keratinocytes *in vitro* model. Pre-treatment of HaCaT keratinocytes

with pure monogalactosylmonoacylglycerols (MGMGs) and digalactosyldiacylglycerols (DGDGs) as well as fractions containing monogalactosyldiacylglycerol (MGDGs) or DGDGs markedly attenuated UVB-induced cell damage through reduction of LDH release, ROS levels and IL-6 production. This preliminary study let us to select the most active compounds: the fraction of glycolipids **MGDG** and the pure glycolipid **MGMG-A** in order to perform the *in vivo* models (Chapter 1).

In the first *in vivo* study, the fraction of glycolipids **MGDG** and the pure glycolipid **MGMG-A** were evaluated on a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin hyperplasia model in Swiss CD-1 mice. Accumulating evidence has demonstrated that exposure of skin to the protein kinase C activator TPA induces a pleiotrophic tissue response and promotes macroscopic lesions as peeling and edema, mimicking an apparent psoriasis phenotype. Firstly, we evaluated the anti-inflammatory effects of topical application of **MGDG** and **MGMG-A** dissolved in acetone (10 µg/µL, 200 µg per site), in the TPA model. For this study, we also used the reference agent dexamethasone (Dex) dissolved in acetone (10 µg/µL, 200 µg per site). The treatments were topically administered to the skin surface of the backs in an area of 1 cm^2. After 30 min, TPA (2 nmol per site, dissolved in acetone) was topically applied to the same areas during 3 days. Pre-treatment with acetone-dissolved glycolipids reduced skin edema, epidermal thickness and pro-inflammatory cytokines production (TNF-α, IL-1β, IL-6 and IL-17) in the tissue. Nevertheless, it is well known that the topical application requires a suitable incorporation of these bio-compounds into a carrier that offers stability, permeation enhancer properties, and remaining more time in the skin. In this regard, we performed different *in vitro*, *ex vivo* and *in vivo* permeation studies, to compare the profile of three formulations (gel, ointment and cream). The *in vitro* and *ex vivo* studies were developed in Franz cells, and were completed with an *in vivo* permeability study, by using rhodamine-containing cream, ointment or gel, to check the stability and determinate the permeation properties of the formulation through skin layers. Permeation studies showed that the cream formulation had the best permeability profile; thus, a MGDG-containing cream (0.2 % w/w) and a Dex-cream (0.2 % w/w) were evaluated on the hyperplasia murine model starting the pre-treatment 2 days before the first TPA application. MGDG-cream application attenuated TPA-induced skin edema, improved histopathological features and reduced the inflammatory cell infiltrate. In addition, this formulation inhibited epidermal expression of COX-2 on a similar way than dexamethasone.

These results suggest that a MGDG-containing cream could be an emerging therapeutic strategy for the treatment of inflammatory skin pathologies as psoriasis (Chapter 2).

The next study aimed to evaluate the *in vitro* and *in vivo* anti-inflammatory and photo-protective effects of the carotenoid **Fucoxanthin** (FX) isolated from *Isochrysis galbana*. In a first step, the anti-inflammatory activity was studied in LPS-stimulated THP-1 human macrophages and TNF-α-stimulated HaCaT human keratinocytes. The antioxidant activity was performed in UVB-irradiated HaCaT cells (50 mJ/cm^2). Our results showed the reduction of pro-inflammatory mediators as TNF-α and IL-6 as well as the photo-protective effects of FX, by reducing ROS and LDH intracellular production in the cell models assayed. In a second step, we evaluated the anti-inflammatory effects of a FX-containing cream (0.2 % w/w) on a TPA-induced epidermal hyperplasia model in Swiss CD-1 mice, as well as on a UVB-induced acute erythema model in hairless SKH-1 mice (360 mJ/cm^2). The results showed that the pre-treatment with the FX-cream effectively ameliorated TPA-induced hyperplasia in mice, by reducing skin edema, epidermal thickness, MPO activity and COX-2 expression levels. Moreover, we demonstrated that FX-cream reduced UVB-induced erythema in mice through down-regulation of COX-2 and iNOS protein expression as well as up-regulation of the antioxidant HO-1 protein levels through Nrf-2 signalling pathway. These findings suggest that FX, administered in a topical formulation, could be a novel approach for preventing psoriatic exacerbations and protecting skin against UV radiation (Chapter 3).

On the other hand, the study of natural products derived from plants remains today one of the most promising field for the discovery of new compounds that may lead to drugs development. In this line, polyphenols are one of the most studied groups. These compounds are plant secondary metabolites generally involved in defense mechanisms against external aggression. Over 8.000 structural variants are known, being the common structural base the presence of one or more phenolic rings. The anti-free radical activity of polyphenols is widely described; in fact, they are the most potent antioxidants in our diet. However, the investigation of new properties still continues, and in this regard, the anti-inflammatory activity is one of the most important. Therefore, in our next study we assayed the effects of a polyphenolic compound, the **Rosmarinic acid** (RA) in a murine Imiquimod (IMQ)-induced psoriasis-like skin inflammation model. IMQ is a Toll-

like receptor 7 and 8 agonist, which, topically applied to the back of the Balb/c female mice, induces skin inflammation accompanied by structural features typical of psoriasis. Since the effectiveness of topical application of RA can be limited by its low liposolubility, stability, photo-oxidation or photo-degradation, we used nano-liposomes to encapsulate this polyphenol. Topical treatment with RA-gel and RA-loaded nanoliposomes in gel (0.2 % w/w) 30 min prior to IMQ application (62.5 mg per site for 6 consecutive days) inhibited macroscopic damage and the skin punch weight, suggesting an inhibition of skin edema, neutrophilic infiltration, MPO activity and the expression of pro-inflammatory cytokines as TNF-α, IL-1β, IL-6 and IL-17. RA-gel showed better results than RA-loaded nanoliposomes in gel. This could be due to that the dissociation constant (pK_a) of RA at physiologic pH affected the liberation of this compound from nanoliposome to cell medium and, consequently, reduced its biological action. In stating that the chemistry structure of RA affects the formulation activity, would be necessary a specific NL elaboration for this kind of molecules. Contributing with these results to use of these systems in the pharmaceutical industry, since suppose hopeful vehicles for topical drug delivery systems due to their effectiveness to enhancing skin permeation and to delivering drug in skin specific areas(Chapter 4).

Finally, we evaluated a possible synergism between the main compounds assayed in this thesis, FX and RA. For that purpose, these products were tested in an UVB-irradiated HaCaT human keratinocytes model at the dose of 5 µM, studying their mechanism of action and evaluating if a mixture of these compounds (5 µM of FX + 5 µM of RA) may improve the individual biological activity. Cell viability, cell cycle arrest, apoptosis induction and inflammasome mediators have been assessed in this study. The mixture has shown a better photo-protective and antioxidant effect than the individual compounds, by reducing cell death and the consequent increase of cell viability diminishing apoptosis and ROS production induced by UVB exposition in pre-treated cells. Furthermore, the mixture modulated inflammatory response by diminishing the expression of inflammasome components as NLRP3 and ASC, in addition to increase the expression of the antioxidant HO-1 protein levels through Nrf-2 signalling pathway (Chapter 5).

In conclusion, our *in vitro* and *in vivo* studies have demonstrated the beneficial effects of some natural products,

specially **fucoxanthin** and **rosmarinic acid** in inflammation, associated with UVB exposure and psoriasis. Their mechanisms of action could be related with the prevention of oxidative stress through of activation of Nrf2 pathways as well as with the reduction of inflammation via reduction of inflammasome components and other cytokines involved in NF-κB pathway. For this reason, these compounds could be promising molecules for their incorporation in many skin care products such as sun protectors and anti-aging creams, as well as for the treatment of inflammatory skin pathologies as psoriasis.

STUDY OF LIPIDS FROM MICROALGAE AND POLYPHENOLS IN INFLAMMATORY SKIN MODELS

UVB MODELS

PSORIASIS MODELS

CHAPTER 1 — LIPIDS FROM MICROALGAE

Study of cytotoxic, antioxidant and protective activities of different glycolipids and carotenoids obtained from *Isochrysis galbana*, employing an UVB-irradiated HaCaT human keratinocytes *in vitro* model

CHAPTER 2 — GLYCOLIPIDS

The anti-inflammatory activity of glycolipids (MGDG and MGMG-A) was studied in LPS-stimulated THP-1 macrophages and TNF-α-stimulated HaCaT keratinocytes

The fraction of glycolipids MGDG and the pure glycolipid MGMG-A, was selected to their evaluation on TPA-induced skin hyperplasia model in Swiss CD-1 mice

CHAPTER 3 — FUCOXHANTIN

The anti-inflammatory and photo-protective effect of the carotenoid fucoxanthin (FX) both *in vitro* and *in vivo* models. Firstly, the anti-inflammatory activity was studied in LPS-stimulated THP-1 macrophages and TNF-α-stimulated HaCaT keratinocytes, and the anti-oxidant activity in UVB-irradiated HaCaT cells (50 mJ/cm2)

Evaluation of the preventive effects of FX-cream on UVB-induced acute erythema model in hairless SKH-1 mice (360 mJ/cm2)

Evaluation of the preventive effects of FX-cream on TPA-induced epidermal hyperplasia in Swiss CD-1 mice

CHAPTER 5 — FUCOXHANTIN AND ROSMARINIC ACID AND THEIR MIXTURE

Finally, the main compounds employed in this thesis as FX and RA were evaluated in an UVB-irradiated HaCaT human keratinocytes *in vitro* model to elucidate their mechanism of action and evaluate if a mixture of these compounds might improve their biological activities

CHAPTER 4 — ROSMARINIC ACID

Furthermore, we studied the topical effect of RA-loaded liposomes in the murine Imiquimod-induced psoriasis-like skin inflammation model

INTRODUCCIÓN

BLOQUE A: PIEL, INFLAMACIÓN Y PATOLOGÍAS

1. PIEL

1.1 Anatomía

La piel es un órgano complejo integrado por células, tejidos y elementos de la matriz extracelular que median una importante función de barrera contra agentes patógenos, agresiones mecánicas o químicas, cambios de temperatura, o radiación ultravioleta (UV). Cabe destacar su función reguladora de los fluidos corporales, modulando la pérdida transcutánea de agua, el mantenimiento del equilibrio térmico y la transmisión de información externa que accede al organismo por el tacto, la presión, la temperatura y los receptores del dolor. Se trata del órgano de mayor tamaño, siendo su superficie media de 2 m^2, según el peso y la altura del individuo. Tiene un espesor medio de 2 mm y representa el 6 % del peso corporal. Estructuralmente, la piel se encuentra dividida en tres capas de tejido. Desde el exterior hacia el interior, se encuentran (I) la epidermis, (II) la dermis y (III) la hipodermis o tejido subcutáneo (Figura 1). Mientras que la epidermis, y su capa más externa, el estrato córneo, constituyen una gran parte de la barrera física que proporciona la piel, la dermis y la hipodermis son las responsables de la integridad estructural de la piel como un todo.

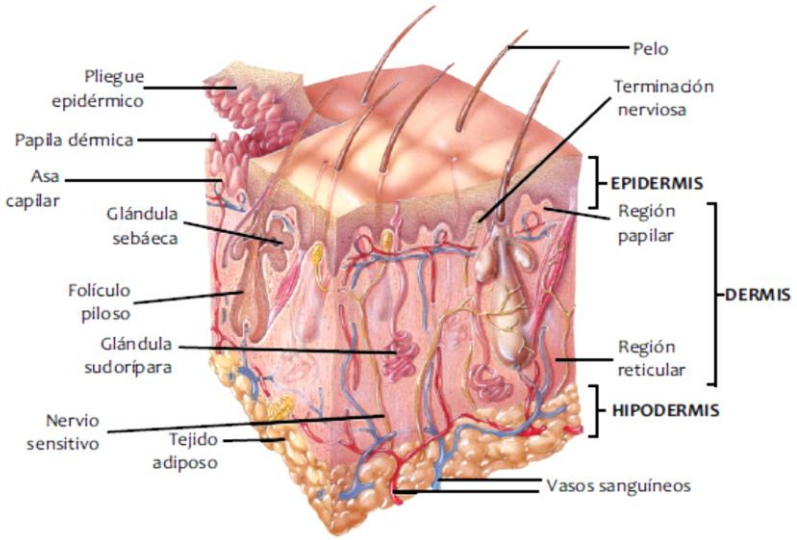


Figura 1: Corte transversal de piel y tejido subcutáneo. Imagen adaptada de (Tortora and Derrickson, 2013)

1.1.1. Epidermis

La epidermis se caracteriza por ser un epitelio plano poliestratificado y queratinizado que cubre la totalidad de la superficie corporal. Se trata de una estructura que se renueva continuamente y que da origen a estructuras derivadas denominadas apéndices (unidades pilosebáceas, uñas y glándulas sudoríparas). Es la capa que presenta mayor número de células, teniendo un espesor variable, de 0,1 - 2 mm según la zona del cuerpo. La mayor parte de las células en la epidermis son queratinocitos que están organizados en cuatro estratos o capas, que desde el exterior al interior corresponderían con: (I) estrato córneo (*stratum corneum*), (II) granuloso (*stratum granulosum*), (III) espinoso (*stratum spinosum*) y (IV) basal (*stratum basale*). En las zonas en la que la epidermis presenta un mayor grosor, cuenta con cinco capas al aparecer el estrato lúcido (*stratum lucidum*), el cual se encuentra situado entre la capa córnea y la granular.

La mayor parte de células que componen la epidermis son queratinocitos, células capaces de sintetizar queratina, una proteína estructural, insoluble en agua y que presenta gran resistencia frente a cambios de pH y temperatura. Los queratinocitos se van diferenciando progresivamente a partir de células basales proliferativas, adheridas a la membrana basal epidérmica, hasta que terminan su diferenciación formando ya parte del estrato córneo queratinizado, el cual es la capa más externa de la piel. Entre estos queratinocitos, se encuentran intercalados otros tipos celulares como son los melanocitos (sistema pigmentario), células de Langerhans (sistema inmune) y células de Merkel (sistema nervioso). Otras células como los linfocitos, no suelen aparecer en la piel sana y, cuando aparecen, lo hacen de forma transitoria debido a que actúan en presencia de antígenos desencadenando la respuesta inmune cuando es necesario.

Estrato basal

Los queratinocitos constituyen el 80 % del total de las células de la piel. El proceso de diferenciación de un queratinocito o queratinización, consta de modificaciones morfológicas y eventos metabólicos programados genéticamente, cuyo fin es un queratinocito muerto terminalmente diferenciado (corneocito). En la capa basal es donde se localizan principalmente las células mitóticamente activas de la epidermis, las cuales ocuparán el espacio de las células erosionadas de la capa córnea.

Estrato espinoso

La forma, estructura y propiedades moleculares de las células espinosas están relacionadas con su posición dentro de la epidermis. A medida que se van diferenciando, se van desplazando hacia la epidermis superior y se vuelven progresivamente más aplanadas, desarrollando gránulos laminares. Las células espinosas retienen las queratinas estables que se producen en la capa basal, pero no sintetizan nuevo ARN mensajero (ARNm) que dé lugar a estas proteínas, excepto en trastornos hiperproliferativos. En patologías como la psoriasis, la queratosis actínica (QA) o la reparación de heridas, se produce una disminución del ARNm de las proteínas de queratinas estables, viéndose favorecida la síntesis de queratinas propias de queratinocitos hiperproliferativos, lo cual provoca una discontinuidad en la diferenciación normal de las siguientes capas epidérmicas, granulosa y estratificada.

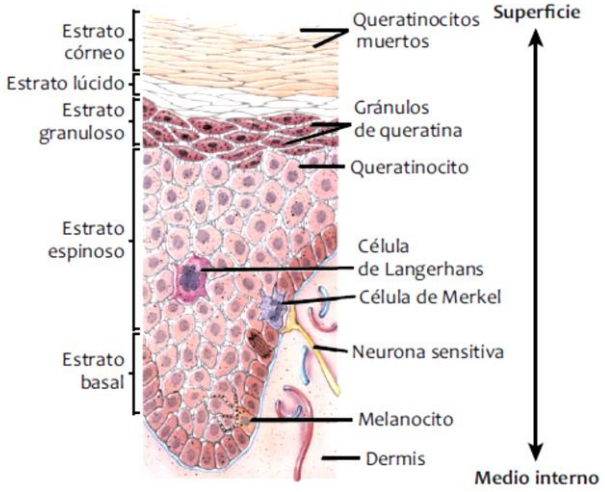


Figura 2: La epidermis consiste en un epitelio escamoso estratificado. Imagen adaptada de (Tortora and Derrickson, 2013)

Estrato granuloso

Se denomina así por los gránulos queratohialinos basófilos, precursores de la queratina que se acumula en el citoplasma en forma de gránulos, adquiriendo la morfología típica de esta capa y siendo aquí donde se comienza a formar la envoltura de las células queratinizadas. La etapa final de la diferenciación de las células granulosas incluye la destrucción de las mismas, transformándose en corneocitos diferenciados.

Estrato córneo

La completa diferenciación celular de las células granulosas da lugar a capas apiladas de células sin núcleo, queratinizadas y aplanadas que forman el estrato córneo. Esta capa aporta a la piel la protección mecánica y actúa como barrera frente a la pérdida de agua y a la invasión de sustancias. El estrato lúcido y el estrato córneo están compuestos de células muertas, estrechamente empaquetadas y

rodeadas de lípidos. Estas capas protegen las más internas y son finalmente eliminadas por descamación conforme nuevos queratinocitos las van reemplazando. En condiciones fisiológicas, esta progresión tiene una duración de 28 días y va acompañada de cambios fundamentalmente en el contenido celular, que dan lugar a las diferentes capas visibles al microscopio (Figura 2).

Debido a que el ciclo celular típico de un queratinocito tiene una duración aproximada de 6 días, no todas las células de la capa basal están activas a la vez. Sin embargo, en determinadas condiciones patológicas, como la psoriasis (Perera et al., 2012) o algunos tipos de Cáncer de Piel No Melanocítico (CPNM) como puede ser el Carcinoma Epidermoide Cutáneo (CEC), se produce una hiperproliferación de queratinocitos normales y atípicos que rompen la membrana basal e invaden la dermis subyacente formando islotes o cordones de células atípicas, con una tendencia variable a la queratinización (Xiang et al., 2017).

1.1.2. Dermis

La dermis es un sistema complejo y vascularizado que sirve de soporte y alimento a la epidermis. Se encuentra formada principalmente por tejido conectivo fibroelástico, en el que se localizan las redes vasculares y nerviosas. Mientras que las células de la epidermis son numerosas e íntimamente asociadas, las de la dermis están separadas por un complejo conglomerado de material extracelular como el colágeno, la elastina y otras fibras extracelulares que dotan de fuerza estructural y flexibilidad a la piel. Las células principales de la dermis son los fibroblastos, pero también existen células del sistema inmune, terminaciones nerviosas y vasos sanguíneos encargados de irrigar tanto la dermis como la epidermis (Figura 1). Histológicamente, la dermis se divide en dos capas: la papilar (*stratum papillare*) y la reticular (*stratum reticulare*). Esta estructura es la que proporciona a la piel la elasticidad y capacidad de adaptarse a movimientos y cambios de volumen. Además, la dermis interactúa con la epidermis para mantener la unión entre ambas capas, e interviene en la reparación y remodelación de la piel después de sufrir heridas.

1.1.3. Hipodermis

El límite entre la dermis reticular profunda y la hipodermis es la transición de un tejido conjuntivo dérmico predominantemente

fibroso a un tejido subcutáneo fundamentalmente adiposo. Aunque anatómicamente son diferentes, estructural y funcionalmente se encuentran integrados mediante nervios, vasos y apéndices epidérmicos. Los folículos pilosos en crecimiento activo, se extienden hacia la grasa subcutánea, encontrándose también a esta profundidad las glándulas sudoríparas. La masa principal de la hipodermis se encuentra formada por adipocitos organizados en lóbulos definidos por tabiques de tejido conjuntivo fibroso. El tejido subcutáneo o hipodermis actúa como reservorio de energía, aislante térmico y protector mecánico frente a golpes (Cordero, 1996; Wolff, et al., 2009).

2. INFLAMACIÓN

El proceso inflamatorio o inflamación es un concepto antiguo, acuñado por primera vez en el siglo I por Cornelius Celso, que describe este proceso como la conjunción de cuatro puntos cardinales: enrojecimiento, hinchazón, calor y dolor. Así, el desarrollo de una enfermedad inflamatoria se definía como un desequilibrio de estos cuatro puntos (Majno, 1975)(Majno, G, Joris I, 2004). Posteriormente, se añadió a la definición la pérdida de función. Actualmente, el proceso inflamatorio agudo se considera una respuesta que tiene lugar en los tejidos vascularizados para defender al hospedador y mantener la homeostasis tisular, considerándose un proceso activo y altamente regulado que conlleva una resolución programada del mismo.

Tradicionalmente, se ha considerado que el proceso inflamatorio puede eliminarse mediante el catabolismo de los mediadores pro-inflamatorios. Sin embargo, ésta es sólo una fase del proceso de resolución de la inflamación, ya que, una vez las células inmunes o polimorfonucleares (PMNs) han cumplido su misión, deben eliminarse de forma controlada y efectiva, debido a que pueden ocasionar daños si permanecieran infiltradas en el tejido. Para la resolución completa de la inflamación, estas células inflamatorias deben ser devueltas nuevamente al torrente sanguíneo (proceso no bien conocido hasta el momento) o bien se produce una muerte local de éstas, para ser retiradas, mediante fagocitosis, por los macrófagos. Tras esta fagocitosis, parte de estos macrófagos saldrían de la zona inflamada vía linfática y otros morirían por apoptosis (Serhan et al., 2007). Si estos acontecimientos transcurren de esta forma, la inflamación aguda se resolverá sin causar excesivos daños tisulares, impidiendo que la inflamación persista provocando las consecuentes complicaciones, como una inflamación crónica o fibrosis del tejido.

Pueden ser diversos los estímulos que causen una lesión tisular, bien de tipo exógeno o endógeno, dando lugar al desarrollo de un proceso inflamatorio, por lo que el tipo y el grado de respuesta inflamatoria dependerán tanto de la naturaleza del agente que la provoque, como de la persistencia del mismo. En este sentido se pueden desarrollar o exacerbar diferentes patologías inflamatorias, de tipo inmune, como la psoriasis o las ocasionadas por un agente externo como es el eritema cutáneo por exposición al sol, el cual puede degenerar en lesiones precancerosas y cáncer de piel.

2.1 Psoriasis

La psoriasis es una enfermedad inflamatoria crónica de la piel, de carácter autoinmune, cuyas manifestaciones clínicas son eritema, aumento del espesor de la piel y descamación cutánea. Las afectaciones cutáneas suelen aparecer en forma de placas eritematosas que frecuentemente cursan con prurito e incluso dolor. Aunque puede afectar a cualquier región corporal, las zonas más afectadas son los codos y las rodillas, además de la zona sacra, el cuero cabelludo o las uñas (Perera et al., 2012) (Figura 4). Clínicamente, esta enfermedad se puede clasificar en lesiones pustulosas y no pustulosas. Existen varios tipos de psoriasis, pero las más comunes se encuentran dentro de las no-pustulosas, como son la psoriasis en gota, la artritis psoriásica y la psoriasis *vulgaris*, que es el fenotipo más común, constituyendo el 80-90 % de los pacientes psoriásicos (Sarac G, Koca T T, 2016).

La psoriasis es una patología crónica que afecta al 2-4 % de la población mundial, presentando carácter recurrente, con periodos intermitentes de exacerbación y remisión. Actualmente, su etiología es desconocida, pero se conoce su carácter multifactorial donde se combinan factores tanto genéticos (hereditarios), como ambientales (alcohol, tabaco, infecciones, estrés, fármacos) que pueden favorecer su aparición, así como su desarrollo y exacerbación. El pronóstico de los pacientes con psoriasis no suele afectar a la salud general del paciente, es sólo en las manifestaciones más graves en las que se requieren tratamientos sistémicos. Sin embargo, resulta de gran importancia el impacto en la calidad de vida de los mismos, ya que al tratarse de una enfermedad que puede resultar visualmente impactante, en muchos casos puede llegar a condicionar la vida socio-profesional y emocional del paciente (Sala et al., 2016).

2.1.1. Fenotipo y características histopatológicas de la psoriasis

La psoriasis *vulgaris*, o también conocida como psoriasis en placas, se caracteriza por la presencia simétrica de placas de 1-10 cm^2 perfectamente delimitadas (Figura 3). Son placas escamosas, blancas o plateadas que cubren superficies variables del cuerpo, pudiendo experimentar sangrado al ser extirpadas, fenómeno que se conoce como signo de Auspitz (Bernhard, 1997). De tal modo, un paciente psoriásico presenta zonas afectadas (piel lesional) y zonas libres no afectadas (piel no lesional). La evaluación de la gravedad del paciente se lleva a cabo mediante una escala establecida llamada PASI (del inglés *Psoriasis Area Severity Index*) que tiene en cuenta el eritema, el endurecimiento y la descamación de las lesiones en diferentes zonas del cuerpo, así como el área que ocupa cada una de ellas. Los valores de la escala se encuentran entre 0, asociado a la ausencia de enfermedad y 72, valor atribuido a la máxima gravedad (Hägg et al., 2017).

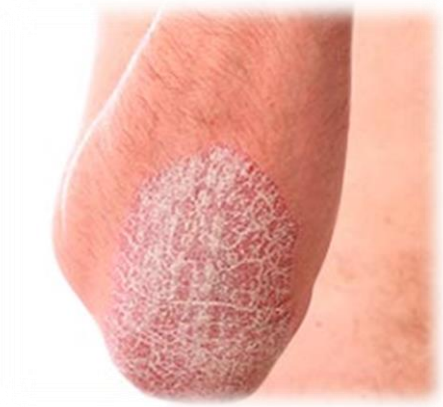


Figura 3: Imagen de una lesión en placa, forma más común de psoriasis.

Los principales rasgos histológicos que se desarrollan en una placa psoriásica están relacionados con las siguientes respuestas inmunológicas (Figura 4) (Bos et al., 2005):

- Aumento del espesor de la epidermis, debido a la prematura maduración de queratinocitos y, como consecuencia, la incompleta cornificación, lo que resulta en la ausencia de núcleo en las células del estrato córneo, fenómeno conocido como paraqueratosis.
- Anormal incremento en el recambio de los queratinocitos basales, que causa el aumento del espesor de la epidermis (acantosis) y la elongación de las crestas epiteliales hacia la dermis (papilomatosis), debido al estado hiperproliferativo de los queratinocitos. Además, existe una pérdida de la capa granular (hipogranulosis).
- Aumento de la vascularización de la lesión, debido a la formación de nuevos vasos, causado por factores angiogénicos (como el factor de crecimiento vascular endotelial (*vascular endothelial growth factor* (VEGF)). Se manifiesta con el signo de Auspitz, cuando la descamación se produce por rotura accidental o es quirúrgicamente extraída.

- Infiltración celular, compuesta por células dentríticas (DC) y linfocitos CD4+ T helper (Th), los cuales se encuentran principalmente en la dermis y células CD8+ T, localizadas en la epidermis (Perera et al., 2012).

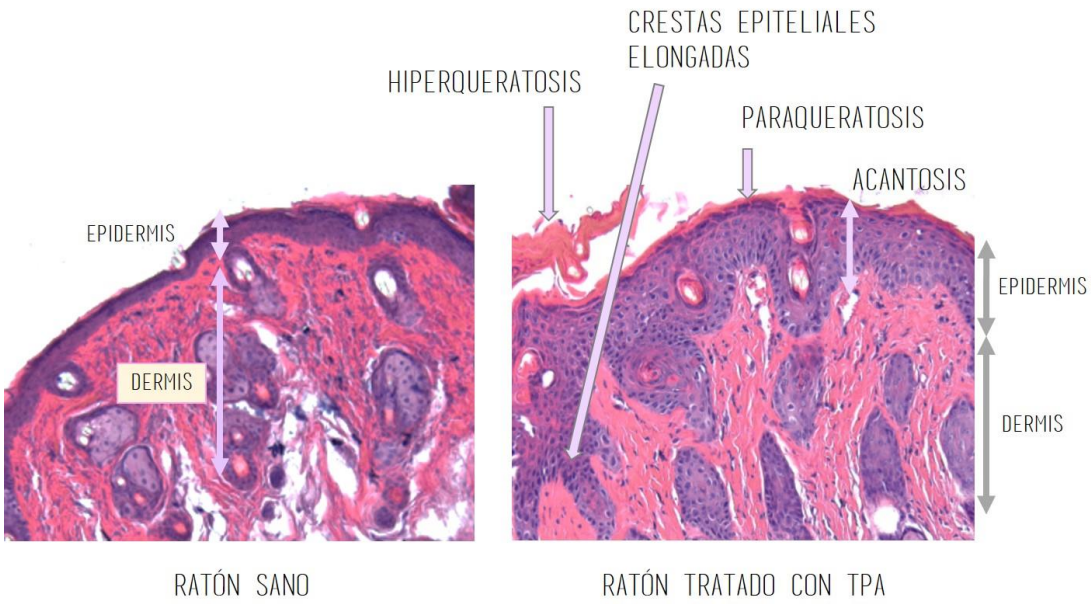


Figura 4: Cambios histológicos observados en la piel de un ratón con hiperplasia epidérmica frente a la de un ratón sano. La imagen de la derecha se relaciona con los cambios histológicos característicos de la psoriasis. En ella se observa una hiperplásica epidérmica en ratones tratados con acetato de 12-*O*-tetradecanoilforbol-13 (TPA), en los que se destaca: el engrosamiento del estrato córneo (hiperqueratosis) y la presencia de queratinocitos provistos de núcleo en las capas más superficiales (paraqueratosis) en una epidermis significativamente engrosada (acantosis) cuyas crestas se elongan hacia la dermis. Aspectos característicos ausentes en la piel de un ratón sano. Las imágenes proceden de cortes histológicos a 7 µm, teñidos con hematoxilina y eosina. Aumento óptico original 100X. Adaptado de (Arasa, 2014a).

2.1.2. Inmunopatogénesis de la psoriasis. Inicio de la enfermedad y tipos celulares.

Es complicado definir de forma exacta la patogénesis de la psoriasis, debido a su carácter multifactorial (Li et al., 2017). Debido a ello, son varios los modelos que se plantean en relación a su fisiopatología, aunque se conoce que, en presencia de un agente desencadenante (ambiental o genético), la primera línea de defensa son los queratinocitos, que junto a las células del sistema inmune, causan el desarrollo y mantenimiento de la inflamación en esta

patología (Costa et al., 2017). Durante muchos años, a este grupo de células se le consideraba el principal responsable de la patogénesis de la psoriasis debido a la morfología tan característica de la epidermis en pacientes psoriásicos, como la hiperplasia, la acantosis, la paraqueratosis, la hiperqueratosis y a su ausencia en zonas sanas sin placas. Debido a esto, se ha cuestionado si la lesión en la barrera epidérmica era la causa o la consecuencia de la inflamación. Ha sido en los últimos años y con los avances de las terapias, cuando se han ampliado los objetivos terapéuticos buscando diferentes dianas, siendo en la actualidad el sistema inmune (citocinas pro-inflamatorias) el principal eje sobre el que giran todos los tratamientos (Hobbs et al., 2017). Aun así, este grupo de células sigue siendo uno de los más afectados y cuando se estresan, liberan el péptido antimicrobiano LL-37, el cual puede formar complejos LL-37-ADN, que a su vez, activan receptores Toll like (TLR)-9 dando lugar a la activación de las células dendríticas plasmocitoides (respuesta inmune innata) (Lande et al., 2007). Las células dendríticas plasmocitoides (DCp) son un tipo de células dendríticas (DC) que, junto a las células de Langerhans (LC) y células dendríticas mieloides (DCm), son las encargadas de enlazar la respuesta inmune innata con la adaptativa, actuando como células presentadoras de antígenos (APC). Las DCp actúan liberando grandes cantidades de interferón (IFN) tipo I, el cual junto a factor de necrosis tumoral α (TNF-α), interleucinas (IL)-6 e IL-1β activan a las DCm, las cuales actúan como punto de unión entre la inmunidad innata y adaptativa. Este tipo de células constituyen una importante fuente de citocinas pro-inflamatorias como la interleucina-23 (IL-23) o la IL-12, las cuales promueven la diferenciación de células T en células T efectoras como células T helper 1 (Th1), Th17 y células T citotóxicas (Mahil et al., 2016). El papel de estas citocinas se considera clave en la patogénesis de la psoriasis, ya que están implicadas tanto en el mantenimiento como en la producción de las células Th1, principalmente mantenidas por IL-12, así como en el mantenimiento de las Th17 de cuya diferenciación se encarga la IL-23 (Hanley and Yiu, 2017).

Posteriormente, las DC activadas y las citocinas pro-inflamatorias recirculan hacia la piel y ejercen efectos sobre otros tipos celulares: queratinocitos, leucocitos, neutrófilos, células endoteliales y células musculares lisas. De esta forma, al estimular la proliferación de queratinocitos, se produce un gran número de factores de crecimiento endotelial, lo que favorece la angiogénesis, desencadenando un aumento de la vascularización y reactivando de nuevo

la inflamación (Figura 5) (Dubois Declercq and Pouliot, 2013). Otras células también presentes en las lesiones psoriásicas son los macrófagos. Son células fagocíticas con capacidad APC que producen IL-1β, IL-6, IFNγ, Óxido Nítrico Sintasa inducible (iNOS) y la proteína quimioatrayente de monocitos tipo 1 (MCP-1), además presentan un papel muy importante por ser los principales productores de TNF-α (Gatzka, 2017). La relevancia de sus funciones se ha ido confirmando con estudios en modelos animales, en los que, tras la supresión de este tipo celular, se ha observado la mejora de dicha patología (Wang et al., 2009; Leite Dantas et al., 2016).

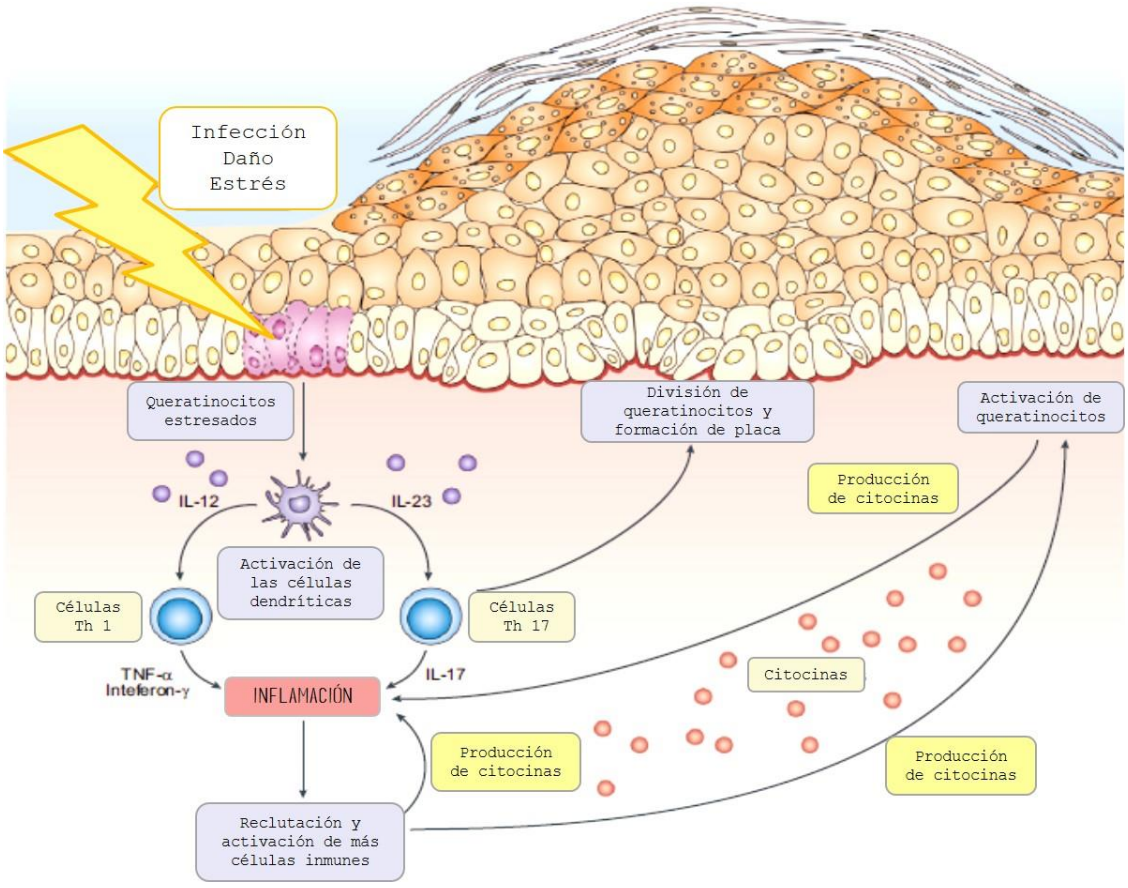


Figura 5: Esquema resumido de la inmunopatogénesis de la psoriasis. En la fase de inicio los queratinocitos se estresan y activan las células dendríticas plasmocitoides (pDC) y éstas posteriormente a las DC dérmicas, las cuales migran a los ganglios linfáticos y promueven la diferenciación de células T *naive* a células Th1 y Th17. Éstas se dirigen al tejido afectado y liberan los distintos mediadores inflamatorios, entre los que se encuentran numerosas citocinas como interleucina (IL)-17 y factor de necrosis tumoral alfa (TNF-α), las cuales provocan la diferenciación aberrante de los queratinocitos, exacerbando aún más el proceso inflamatorio y produciendo ciclos de

retroalimentación positiva que dan lugar a la formación de la placa psoriásica (Arasa, 2014b). Imagen adaptada de (Nestle et al., 2009).

2.1.3. Citocinas implicadas en la psoriasis

Como se ha mencionado anteriormente, las citocinas tienen un papel importante en la patogénesis de la psoriasis. De hecho, los tratamientos actuales se basan en su bloqueo terapéutico mediante anticuerpos específicos, que permiten la atenuación de los síntomas. A continuación, se describen las citocinas implicadas en la psoriasis y su papel en esta patología.

2.1.3.1. Factor de necrosis tumoral alfa (TNF-α)

El TNF-α es una citocina que puede ser producida por diferentes tipos de células como macrófagos, linfocitos T, queratinocitos, DCs, *natural killer*, entre otras (Zaba et al., 2011). Se trata de una de las principales citocinas asociadas a la inmunidad innata, presentando un papel muy importante en la psoriasis debido a su detección a altas concentraciones en lesiones psoriásicas (Chaudhari et al., 2001). Sus efectos están mediados por diferentes receptores (TNFR), los cuales son unos 30 miembros que se clasifican en tipos 1 y 2. TNFR1 se expresa en todos los tejidos, mientras que TNFR2 es inducible y se expresa principalmente en células endoteliales o neuronas (Faustman and Davis, 2010). La presencia de TNF-α, y la consecuente activación de sus receptores desencadena la cascada inflamatoria tal y como se ha descrito anteriormente, generando la amplificación y mantenimiento de la respuesta inflamatoria en la placa psoriásica (Eissner et al., 2004). En este sentido, induce la expresión de otras citocinas, quimiocinas, moléculas de adhesión y factores de transcripción, como el factor nuclear de las cadenas ligeras κ de células B activadas (NF-κB). El NF-κB es esencial para la producción de enzimas tales como la iNOS o la Ciclooxigenasa (COX), que generan especies reactivas de oxígeno (ROS) y prostaglandinas (PGs) siendo a su vez responsable de la regulación de la expresión génica de distintas citocinas como IL-8, IL-6 o el propio TNF-α, por lo que de forma general juega un papel importante en el desarrollo de la respuesta inflamatoria (Hayden and Ghosh, 2011). La implicación del TNF-α en el desarrollo de la psoriasis está avalada desde hace años por el desarrollo de los principales fármacos biológicos anti-TNF-α empleados en la terapéutica como son Infliximab, Adalimumab y Etanercept (Wcisło-Dziadecka et al., 2016).

2.1.3.2. Eje Interleucina-12/Interleucina-23

Actualmente, una de las estrategias terapéuticas para la psoriasis se centra en el bloqueo del eje IL-23/Th17. La IL-23 es una citocina heterodimérica compuesta por una subunidad p19 (IL-23A) y una subunidad p40 que es común a la IL-12 (IL-12/23p40), y es producida por DC y macrófagos generalmente (Nograles K E, Davidovici B, 2011). Mientras que la IL-12 promueve la diferenciación de las células T a Th1, provocando el incremento de citocinas pro-inflamatorias, la IL-23 potencia la formación de células Th17, las cuales secretan IL-17 e IL-22. La activación de esta cascada pro-inflamatoria da lugar a la proliferación de queratinocitos, y a la angiogénesis y migración de nuevas células inmunes hacia zonas de la piel lesionadas (Nestle et al., 2009). Así, la IL-23 se considera un estímulo para la producción de IL-17, de ahí el objetivo terapéutico de inhibir el eje IL-12/IL-23 (Ustekinumab, Briakinumab)(Dziadecka et al., 2016)(Girolomoni et al., 2017).

2.1.3.3. Interleucina-1β, Interleucina-6 e Interleucina-8.

Las interleucinas IL-1β, IL-6 y la IL-8 también están fuertemente implicadas en el desarrollo de la psoriasis.

La IL-1 es producida por las DC de la piel, y expresada de forma constitutiva en queratinocitos (IL-1α e IL-1β en su forma inactiva) (Yano et al., 2007). Esta expresión se encuentra incrementada en ciertas situaciones o patologías, por lo que se considera una de las citocinas iniciadoras de los procesos inflamatorios en piel (Groves et al., 1996). Además, está implicada en el crecimiento y diferenciación de los queratinocitos (Maas-Szabowski et al., 2001). Son numerosas las evidencias que muestran la relación entre la IL-1 con la patogénesis de la psoriasis (Wei et al., 1999; Mee et al., 2006; Onderdijk et al., 2015). Desde hace años se ha observado que tanto las terapias utilizadas de forma efectiva en psoriasis, como retinoides o glucocorticoides (Lee et al., 1991), así como el empleo de nuevos compuestos (Sun et al., 2013; Carrenho et al., 2015), tienen efectos visibles sobre la disminución de la IL-1. Esta citocina es una de las responsables de activar células de la inmunidad innata (linfocitos, neutrófilos, y macrófagos), las cuales estimulan la producción de TNF-α, IL-6 o IL-8 (Pietrzak et al., 2008). Además, se conoce que el aumento de la producción de TNF-α, unido a la síntesis de IL-1 provoca un incremento de la PGE_2 que conlleva al aumento de la expresión de

COX-2, siendo éste característico en lesiones psoriásicas (Schirmer et al., 2010).

Otra citocina pro-inflamatoria presente de forma constitutiva en la piel sana es la IL-6, cuyos niveles se encuentran aumentados tanto en las biopsias como en el plasma de pacientes con psoriasis (Lowes et al., 2014). Es una de las primeras citocinas de la inmunidad innata cuya síntesis aumenta junto a la IL-1β y el TNF-α y, su importancia radica en su capacidad de iniciar la diferenciación de células T a Th17 y bloquear las funciones de las células T (Kupetsky et al., 2013). La IL-8 es un miembro de la familia de las quimiocinas (CXC) que estimula la quimiotaxis y desgranulación de los neutrófilos, induce la angiogénesis y ejerce gran influencia sobre la función de los queratinocitos (Pietrzak et al., 2008). La IL-8 se sintetiza por una gran cantidad de células implicadas en el proceso inflamatorio, como los monocitos, las células endoteliales y con un papel destacado, los queratinocitos (Ozawa et al., 2005). Su principal papel lo lleva a cabo activando a los neutrófilos, los cuales están involucrados en la activación de las células T y en la proliferación de los queratinocitos (Li et al., 2003). En psoriasis, los neutrófilos se encuentran principalmente en la epidermis y, cuando se activan por diferentes CXC, aumentan los niveles de citocinas pro-inflamatorias como la IL-17 (Figura 6) (Mahil et al., 2016). Además, esta acumulación de neutrófilos va acompañada de la liberación de grandes cantidades de IL-8, lo que se relaciona con el desarrollo de patologías inflamatorias crónicas, tales como la psoriasis (Tuschil et al., 1992). Se ha observado que en modelos animales de inflamación aguda, anticuerpos neutralizantes de la IL-8 reducen considerablemente la función de los neutrófilos y resuelven la inflamación. Por lo tanto, la neutralización de la actividad de la IL-8 representa una estrategia terapéutica para enfermedades inflamatorias crónicas (Pietrzak et al., 2008; Wu et al., 2017).

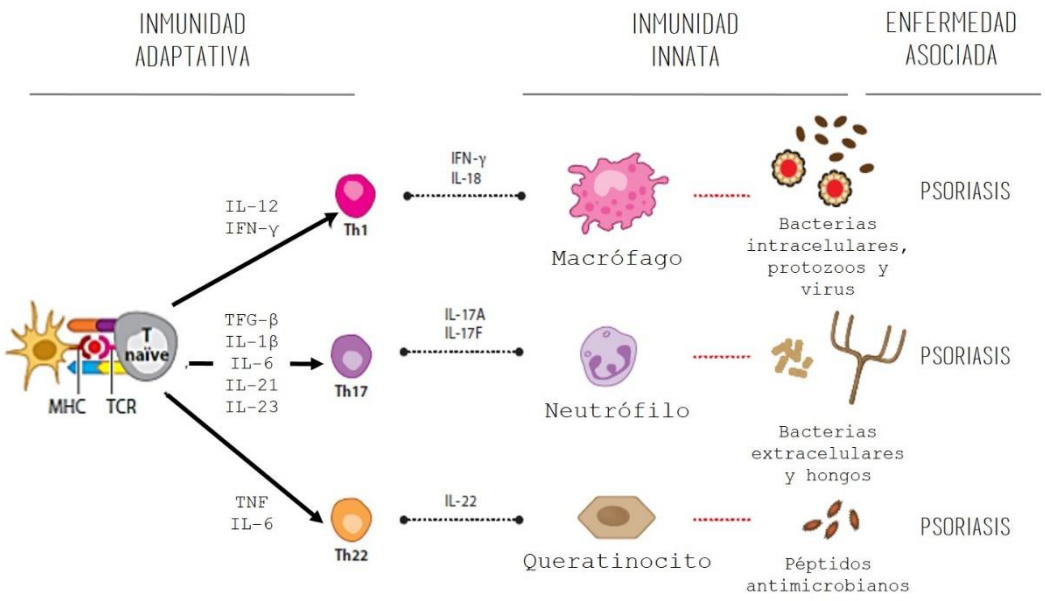


Figura 6: Esquema resumen de células y citocinas implicadas en la inmunopatogénesis de la psoriasis. Abreviaturas: MHC, complejo mayor de histocompatibilidad; TCR, receptor de células T; IL, interleucina; IFN-γ, interferón gamma; TFG-β, factor de crecimiento transformante beta; TNF, factor de necrosis tumoral. Adaptado de (Perera et al., 2012).

2.1.4. Farmacoterapia

En la actualidad, no se dispone de ningún tratamiento curativo para la psoriasis, por lo tanto, el fin de los tratamientos asignados para esta patología es paliar los síntomas y disminuir la gravedad y extensión de las lesiones. Existen una gran variedad de terapias que incluyen los tratamientos tópicos o fototerapia, seguidos de los tratamientos sistémicos y los fármacos biológicos en las formas más severas. La elección del tratamiento se realiza utilizando herramientas de medida validadas como el grado y la extensión de las lesiones (Psoriasis Area Severity Index (PASI)), la valoración global por parte del médico (Physician Global Assessment (PGA)) y el índice de calidad de vida dermatológica (Dermatology Life Quality Index (DLQI)). Debido a la naturaleza crónica de estos tratamientos, aparecen inconvenientes como el coste elevado, las reacciones adversas o las comorbilidades que pueden limitar su elección y/o uso de largos periodos de tiempo (Sala et al., 2016). En este sentido, el primer escalón terapéutico empleado es el tratamiento tópico, que se basa en el uso de corticoides, queratolíticos, antralinas (ditranol), alquitranes y derivados de la vitamina D (calcipotriol) o de la vitamina A (retinoides), algunos de los cuales pueden combinarse con

fototerapia con luz UVA o UVB (Almutawa et al., 2015) (Warren et al., 2016).

Actualmente, la primera línea de tratamiento son los corticoides (Figura 7), tanto en monoterapia como en tratamiento complementario en la terapia sistémica. Tienen propiedades anti-inflamatorias, antimitóticas, apoptóticas, vasoconstrictoras e inmunomoduladoras. Se clasifican en 4 clases según su potencia anti-inflamatoria: (I) leve, (II) moderadamente potente, (III) potente, y (IV) muy potente, siendo imprescindible el conocimiento de su clasificación para su aplicabilidad (Uva et al., 2012). Se sabe que los corticoesteroides tienen una tolerabilidad favorable cuando se usan en diferentes rangos terapéuticos, pero la continua exposición a los mismos está asociada a problemas tanto cutáneos (atrofia y aclaramiento de la piel o dermatitis) como sistémicos (supresión del eje hipotálamo-hipofisario, originando insuficiencia suprarrenal), los cuales se relacionan de forma directa con la potencia del corticoide (Gastaldello et al., 2017). Por todo ello, y teniendo en cuenta el carácter crónico de los tratamientos, su aplicación conlleva una implicación importante por parte del paciente, en términos de seguridad y/o eficacia (Queille-Roussel et al., 2015).

En relación a la fototerapia, que consiste en la exposición de la piel a longitudes de onda de luz UV bajo supervisión médica, la más empleada es la PUVA. Ésta resulta de la combinación de un Psoraleno oral (metoxaleno) y la radiación de la zona afectada con luz UVA (Hemne et al., 2017). Aunque es muy común recomendar la exposición solar natural de forma controlada, la exposición a luz UVB bajo supervisión médica y dirigida ha sido desarrollada para evitar la exposición de la piel sin lesiones. Este tipo de terapia permite aplicar mayores dosis de UVB sobre las lesiones, lo que mejora considerablemente el aclaramiento de las placas psoriásicas (Almutawa et al., 2015).

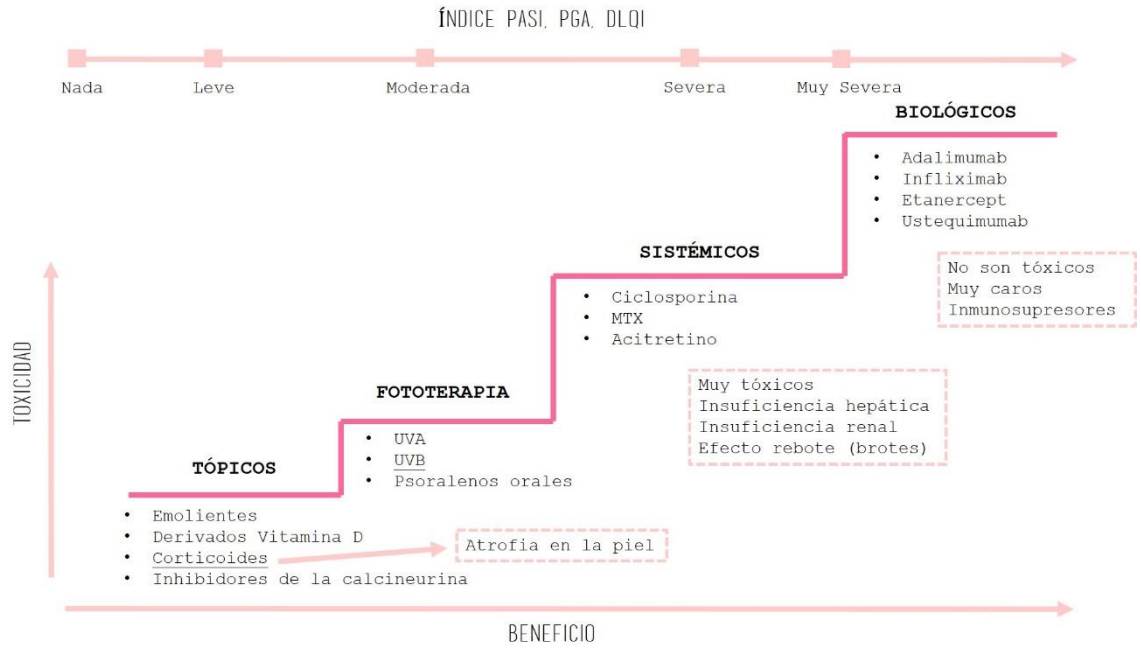


Figura 7: Esquema de los diferentes tratamientos empleados en la psoriasis según la severidad del paciente. La elección del tratamiento se realiza utilizando herramientas de medida validadas como el grado y la extensión de las lesiones (Psoriasis Area Severity Index (PASI)), la valoración global por parte del médico (Physician Global Assessment (PGA)) y el índice de calidad de vida dermatológica (Dermatology Life Quality Index (DLQI)). Abreviaturas: UV, ultravioleta; MTX, Metotrexato.

Si fracasan los tratamientos anteriormente citados, se recurre a la terapia por vía sistémica, siendo numerosos los fármacos empleados. Los inmunosupresores, como el metrotexato o la ciclosporina A, eran las principales elecciones en las formas más severas, sin embargo, se han ido sustituyendo debido a su elevada toxicidad y eficacia moderada, que comprometía, en gran medida, la vida de los pacientes más graves (Visser and van der Heijde, 2009; Delyon et al., 2015; Fink et al., 2017).

En los últimos años, hemos sido testigos de una auténtica revolución en la farmacoterapia de la psoriasis respecto al uso de agentes biológicos, que han permitido el tratamiento efectivo de las formas más severas de la enfermedad. En la actualidad, disponemos de siete agentes biológicos aprobados; Alefacept (Anti-CD2), Etanercept, Infliximab y Adalimumab (Anti-TNF-α), Ustekinumab (Anti-IL-12/IL-23), Brodalumab e Ixekizumab (Anti-IL-17A) (Kazemi et al., 2017). Además, hay otros fármacos que se encuentran en las fases más avanzadas de los ensayos clínicos como son: Secukinumab (anti-IL-17A), Briakinumab (anti IL-12/IL-23), Guselkumab o Tildrakizumab (Anti-IL-23) (Dong and

Goldenberg, 2017) (Hanley and Yiu, 2017). Sin embargo, la mayoría de los pacientes no se beneficiarán de estos nuevos tratamientos, al menos a corto plazo; el elevado coste de las terapias biológicas, la vía de administración parenteral, y el riesgo de aparición de reacciones adversas graves, limita el uso de este tipo de agentes en los casos leves-moderados, que suponen el 90 % de los pacientes diagnosticados (Feldman et al., 2016). Por lo que, pese a los grandes avances logrados en las terapias sistémicas, los tratamientos tópicos empleados rutinariamente han permanecido invariables durante los últimos 20 años.

2.1.4.1. Nuevos horizontes

La búsqueda de nuevas moléculas sencillas, aptas para el tratamiento de las formas menos agresivas de la enfermedad, supone un área de especial interés dentro de la actual farmacoterapia de la psoriasis. Durante el desarrollo de esta tesis, nos centraremos en la búsqueda de nuevos agentes terapéuticos de origen natural, con los que elaboraremos diferentes fórmulas galénicas para su aplicación vía tópica.

En este sentido, la aparición de nuevos sistemas de administración de fármacos abre nuevas puertas al tratamiento de enfermedades dermatológicas. En los últimos años, se ha incrementado el interés por la utilización de las vesículas lipídicas para el desarrollo de formulaciones basadas en liposomas y estructuras coloidales como sistemas portadores de fármacos activos. La utilidad fundamental de estos vehículos radica en conseguir formulaciones eficaces y seguras que mejoren la calidad de vida del paciente, basándose en una mejora en la penetración a través de la piel y la acumulación de fármaco en los diferentes estratos. La capacidad hidratante de las vesículas lipídicas y sus interacciones con los lípidos de la piel, son la posible razón de la mejora en el transporte cutáneo de los fármacos. El desarrollo de nuevos sistemas de liberación basados en sistemas lipídicos se presenta como una alternativa capaz de ejercer un control sobre el comportamiento fisicoquímico, en general, y biofarmacéutico, en particular, de los compuestos (Gonzalez-Rodriguez et al., 2007) (González-Rodríguez and Rabasco, 2011). Otra ventaja adicional de estos sistemas estriba en su capacidad para encapsular moléculas tanto de naturaleza lipófila como hidrófila (Maestrelli et al., 2005). Estos sistemas han sido ampliamente evaluados como potenciales sistemas portadores de fármacos

para su uso en terapéutica, por su capacidad de alterar las propiedades farmacocinéticas y reducir la toxicidad de los fármacos que se incorporan en ellos (Maestrelli et al., 2006). La aplicación de formulaciones de liposomas por vía tópica ha sido propuesta como una de las más relevantes y con mayor eficacia en el tratamiento de trastornos dermatológicos. Estas formas de administración, gracias a los fosfolípidos que contienen como componentes mayoritarios, así como a sus características estructurales, han permitido encapsular numerosos polifenoles en liposomas, con aplicaciones en el tratamiento del cáncer de piel o infecciones vaginales (Jøraholmen et al., 2015). En esta línea, la administración de fármacos a través de liposomas o similares por vía tópica para paliar los síntomas de la psoriasis, ya está siendo investigada (Zhang et al., 2014; Moghddam et al., 2016; Kumar et al., 2016; Doppalapudi et al., 2017).

2.2 Efectos de la radiación UV en la piel

2.2.1. Radiación ultravioleta

La luz solar es un elemento vital para los seres humanos, pero a la vez es fuente de radiaciones que dañan la piel, proceso conocido como fotodaño. La exposición al sol de forma excesiva a lo largo del tiempo predispone, por acción acumulativa, a la aparición de afecciones dermatológicas de tipo estéticas y de poca gravedad como el fotoenvejecimiento, la hiperpigmentación o las arrugas, además de dar lugar a otras consecuencias más severas como el cáncer de piel.

El espectro solar está integrado principalmente por la radiación UV, que se divide en UVC (100-280 nm), UVB (280-315 nm) y UVA (315-400nm), así como la luz visible (400-770 nm) y los rayos infrarrojos (> 700 nm). De todas ellas, la luz UVC es bloqueada por la capa de ozono, mientras que la UVB y UVA son las que atraviesan las diferentes capas de la atmósfera y entran en contacto con la piel (Figura 8) (Rass and Reichrath, 2008).

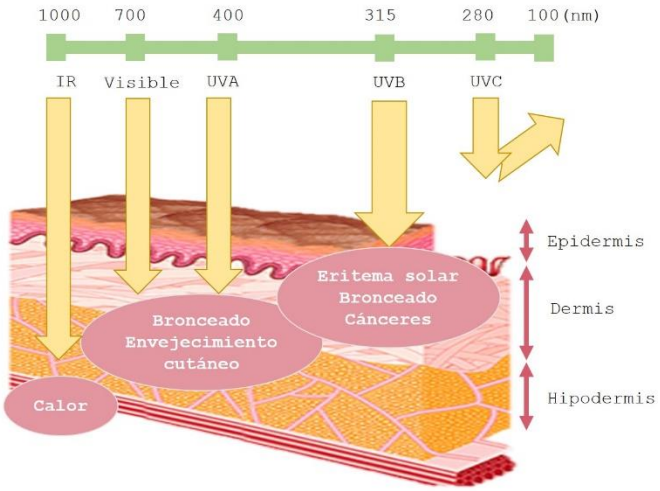


Figura 8: Efectos de la radiación solar en la piel según la longitud de onda (nm). Abreviaturas: IR, infrarrojo; UV, ultravioleta.

Los efectos nocivos de la radiación UV sobre la piel son ampliamente conocidos, aunque su mecanismo de acción no lo es tanto (Figura 9). Los UVA corresponden al 95 % de la radiación UV y la piel recibe 1000 veces más UVA que UVB, ya que están presentes todo el día. Estas radiaciones penetran hasta la dermis profunda, producen bronceado de corta duración, fotoenvejecimiento, cáncer de piel, reacciones por fotosensibilidad medicamentosa y daño en la retina (Matsumura and Ananthaswamy, 2004). Sin embargo, aunque de la radiación UVB llega sólo un 10 % a la piel, es más energética que la UVA y se concentra a las horas con más intensidad solar, entre las 10 y 16 horas. El UVB penetra desde las capas más superficiales de la piel hasta la capa basal de la epidermis, donde genera ROS y de nitrógeno (RNS) nocivas. Es la principal responsable de la quemadura solar y el bronceado prolongado; también favorecen el envejecimiento cutáneo, la inflamación, la inmunosupresión cutánea y el cáncer de piel (Duncan et al., 2009).

La radiación UVC es absorbida por la capa de ozono y son muy peligrosos en cuanto a su acción eritematógena y cancerígena. En los últimos años, en los que cada vez son más palpables los efectos del cambio climático, se ha notado una gran disminución de la capa de ozono, lo que ha permitido un incremento de la infiltración de rayos UVC hacia la superficie terrestre afectando, entre otros aspectos, al riesgo de sufrir lesiones cancerígenas (Schuch and Menck, 2010; Lucas et al., 2015).

Es importante destacar que los datos más recientes tanto epidemiológicos, clínicos, como los estudios en modelos animales, apuntan a la radiación UV solar como elemento clave inductor de inflamación. Esta condición, establecida de manera permanente, pero de bajo nivel, de tal manera que no provoca manifestaciones clínicas alarmantes, favorece la aparición de lesiones como la QA, incrementa el envejecimiento celular y facilita el desarrollo de cáncer de piel (Zhan et al., 2016).

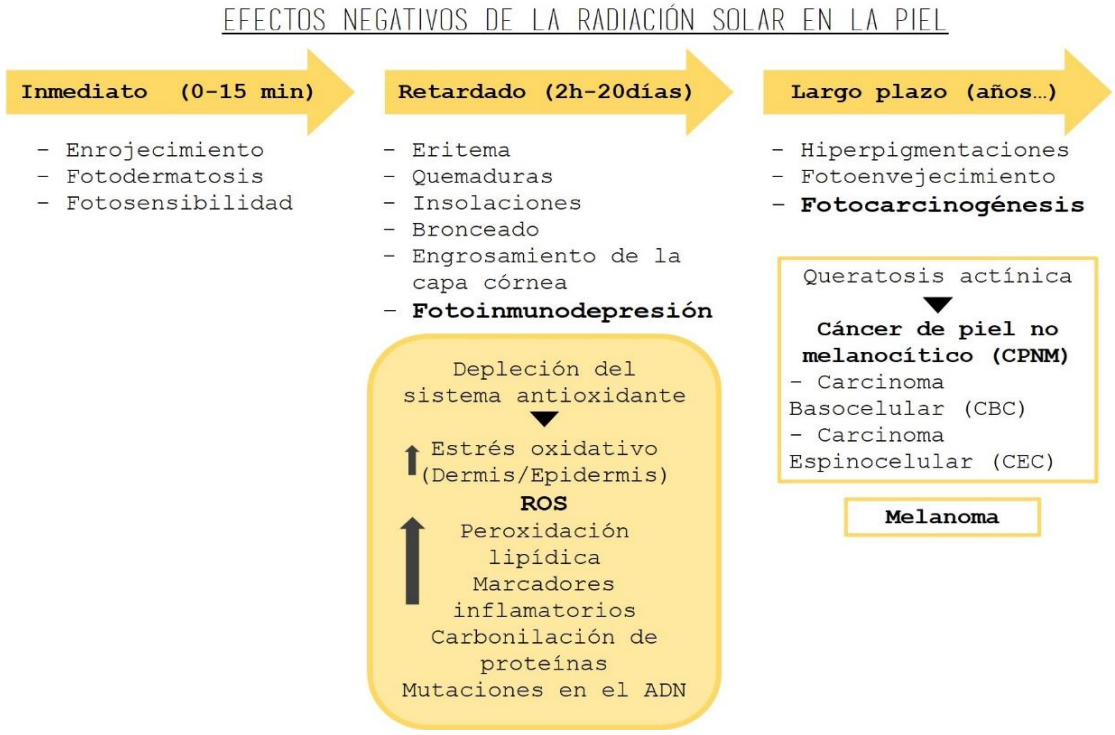


Figura 9: Esquema resumen de cómo afecta la exposición solar a lo largo del tiempo. Abreviaturas: ROS, especies reactivas de oxígeno; CPNM, Cáncer de piel no melanocítico; CBC, Carcinoma Basocelular; CEC, Carcinoma espinocelular.

2.2.2. Estrés oxidativo e inflamación

Es sabido que el estrés oxidativo tiene una gran implicación en enfermedades crónicas con base inflamatoria tales como las enfermedades cardiovasculares (Mahmoud et al., 2017), la diabetes (Yang et al., 2017), las enfermedades de la piel (Caddeo et al., 2016a) o el cáncer (Saha et al., 2017). Entre los mecanismos moleculares que podrían explicar cómo un proceso inflamatorio permanente afecta a los tejidos, destaca el estrés oxidativo mantenido en el tiempo. Esta situación genera una sobreproducción de ROS, que se traduce en un desajuste de la respuesta inflamatoria, provocando un daño tisular. En el caso de enfermedades cutáneas, la exposición a luz

UV puede causar un aumento de ROS, lo que facilitaría el desarrollo de un proceso inflamatorio en la zona afectada.

Las ROS, o también llamadas radicales libres de oxígeno, son una familia de moléculas generadas, transformadas y consumidas en el organismo como consecuencia de la vida aerobia y se producen principalmente a partir de las reacciones metabólicas. Las principales ROS que participan en estas reacciones son el anión superóxido ($[O_2]\cdot^-$), el peróxido de hidrógeno (H_2O_2), el ácido hipocloroso (HOCl), el singulete de oxígeno ($^1O_2$), el radical hidroxilo ($[OH]\cdot$), y diferentes hidroperóxidos (ROOH). La principal fuente de O_2^- es la mitocondria o el retículo endoplasmático, seguidos de otros orgánulos como peroxisomas y fagosomas, los cuales median reacciones catabólicas de oxidación para obtener la energía para el organismo a través de reacciones de oxidación-reducción (redox) (Bergendi et al., 1999)(Chang, 2012).

En condiciones fisiológicas existe un delicado equilibrio entre la producción de ROS y su eliminación mediante los sistemas de defensa antioxidantes de las propias células (equilibrio redox). Si este equilibrio es alterado hacia el aumento de ROS, se produce lo denominado como estrés oxidativo. Esto puede deberse a varias causas (Lushchak, 2014):

- Incremento en los niveles endógenos de ROS, o de compuestos exógenos que entran en auto-oxidación acoplada a la producción de ROS.
- Inactivación de la producción de enzimas antioxidantes y disminución de antioxidantes intracelulares de bajo peso molecular como el ácido ascóbico (Vitamina), tocoferol (vitamina E), carotenoides, etc.

En condiciones fisiológicas, los niveles de ROS se encuentran dentro de unos parámetros estables. Sin embargo, estos niveles pueden aumentar fuertemente, de modo que si los sistemas antioxidantes son capaces de eliminar el exceso de ROS y volver a las condiciones homeostásicas, se trataría de una situación de "estrés oxidativo agudo". Por el contrario, si el sistema antioxidante no es capaz de contrarrestar estos niveles, el aumento en la concentración de ROS podría tender a ser más estable en el tiempo y modificar ciertos componentes celulares, por lo que estaría distorsionando la homeostasis, dando lugar a un "estrés oxidativo crónico", situación

que ocurre en las patologías como la psoriasis o la queratosis actínica (Figura 9).

En cuanto a la radiación UV, se sabe que la exposición a la misma induce la respuesta inflamatoria con activación de células del sistema inmunitario con capacidad de generar ROS, como los neutrófilos (Wölfle et al., 2014). Estas ROS son también producidas por efecto directo de la radiación, pero en cualquier caso, desencadenan una serie de acontecimientos especialmente dañinos para la piel, incluyendo la producción de peróxidos lipídicos que favorecen la destrucción de la porción hidrofóbica de la membrana, y la generación de hidroperóxidos cíclicos altamente tóxicos por su implicación en la disfunción de la mitocondria y la afectación de órganos como el hígado.

Actualmente, se trabaja en dilucidar los mecanismos moleculares capaces de explicar cómo un estado inflamatorio permanente afecta a los tejidos. Además del daño oxidativo, el componente inflamatorio supone la activación de varias vías de señalización intracelular reguladas por cinasas, como el activador mitógeno de proteína quinasa (MAPK) (Choi et al., 2017), la janus quinasa (JAK), y la señal de activación y traducción (STAT). Consecuentemente, se produce la activación de factores de transcripción como el NF-κB (Bosch et al., 2015), coordinándose así la producción de numerosos mediadores inflamatorios, incluyendo citocinas y quimiocinas (TNF-α, IL-1, IL-6, IL-8, IL-10)(Terui and Tagami, 2000), PGs producidas por la actividad enzimática coordinada de la COX y de la prostaglandina sintetasa de membrana (mPGES-1)(Tang et al., 2017), así como óxido nítrico (NO), sintetizado por la enzima iNOS (Wu et al., 2017). Además, se produce la activación de factores de transcripción, que regulan la expresión de genes antioxidantes, como el factor nuclear eritroide 2 (Nrf2), entre otros.

2.2.3. Vías de señalización

2.2.3.1. Respuesta antioxidante

Como se ha comentado anteriormente, el organismo dispone de moléculas y enzimas antioxidantes cuya síntesis se encuentra regulada por el factor Nrf2, el cual determina la señal de la respuesta antioxidante (Furue et al., 2017).

Bajo condiciones fisiológicas, Nrf2 se encuentra en el citoplasma y su función es regulada por la formación del complejo Nrf2-KEAP1-CUL3. Cuando se produce la unión entre KEAP1 y Nrf2, su actividad queda inhibida y se produce la ubiquitinización de Nrf2 gracias a CUL3. Sin embargo, cuando se disparan los niveles de ROS, se produce la oxidación de una cisteína residual en KEAP1, de modo que cambia su conformación y se produce la disociación de Nrf2 del complejo. En esta situación, Nrf2 se activa y transloca al núcleo iniciando la transcripción de genes anti-inflamatorios como la HO-1 (Gęgotek and Skrzydlewska, 2015; Marchev et al., 2017) (Figura 10).

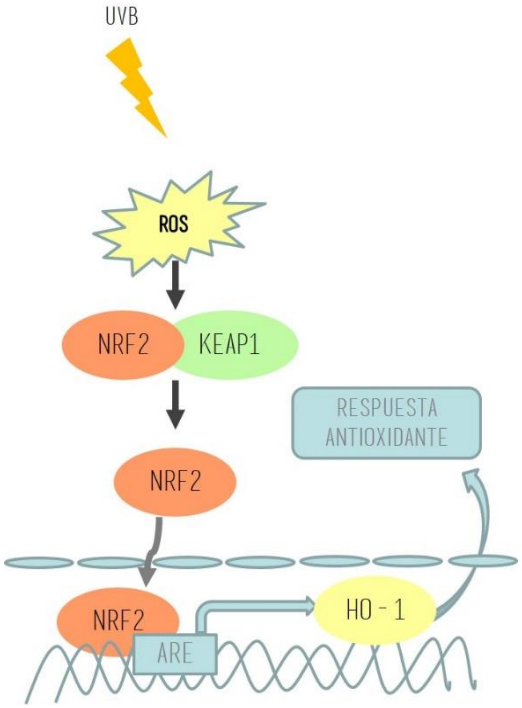


Figura 10: Esquema de activación del complejo Nrf2. Abreviaturas: ROS, especies reactivas de oxígeno; Nrf2, factor nuclear eritroide 2; KEAP, proteína asociada (Kelch-like ECH-associated protein); ARE, elemento de respuesta antioxidante; HO-1, hemo-oxigenasa 1.

2.2.3.2. Activación del inflamasoma

Los queratinocitos, tras la exposición a la radiación UV, activan el sistema inmune innato, a través de receptores de reconocimiento de patrones (PRRs), entre los que se encuentran los receptores tipo NOD (NLR). La activación puede ser llevada a cabo por patógenos (hongos, bacterias, virus), moléculas generadas en la interacción con el patógeno (ATP o glucosa extracelular, liberados por las células dañadas), y agentes ambientales irritantes, como la radiación UVB. En este sentido, son varios los autores que afirman que ROS y el daño en la mitocondria son responsables de la activación de ciertos receptores como son concretamente los del tipo NRLP3 (NOD-LRR-and pyrin domain-containing 3) (Zhou et al., 2010) (Sorbara and Girardin, 2011). Algunos de estos receptores forman un complejo multiproteico, denominado inflamasoma, siendo NLRP3 el mejor caracterizado hasta la fecha, aunque otras subunidades como NLRP1 están siendo estudiadas en la actualidad con interés. Este complejo está implicado en la activación de la caspasa-1 y posterior maduración

proteolítica de las citocinas pro-inflamatorias IL-1β e IL-18 (Ahmad et al., 2017).

En reposo, NRLP3 está asociado al retículo endoplásmico; tras la estimulación se relocaliza hacia el espacio perinuclear y recluta al adaptador proteico ASC y a la pro-caspasa-1. Este complejo multiproteico resulta en la activación de la caspasa-1, responsable de la activación de dianas citoplasmáticas como son las citocinas pro-inflamatorias IL-1β e IL-18 (Figura 11) (Haitao Guo, Justin B. Callaway, 2015). Sin embargo, hasta la fecha no se conoce con exactitud el mecanismo de activación de NRLP3. En este sentido, existen evidencias que demuestran que la mitocondria tiene un papel clave en este proceso, ya que es la principal productora de ROS activadores del inflamasoma (Gan et al., 2016). Debido a su asociación con numerosas enfermedades inflamatorias, el inflamasoma tipo NRLP3 ha despertado un gran interés; sin embargo, son escasos estudios en piel que relacionan la activación del inflamasoma con la inflamación inducida por exposición a luz UVB (Faustin B, 2008; Yoon et al., 2015; Sollberger et al., 2015; Chen et al., 2016; Hasegawa et al., 2016; Ahmad et al., 2017).

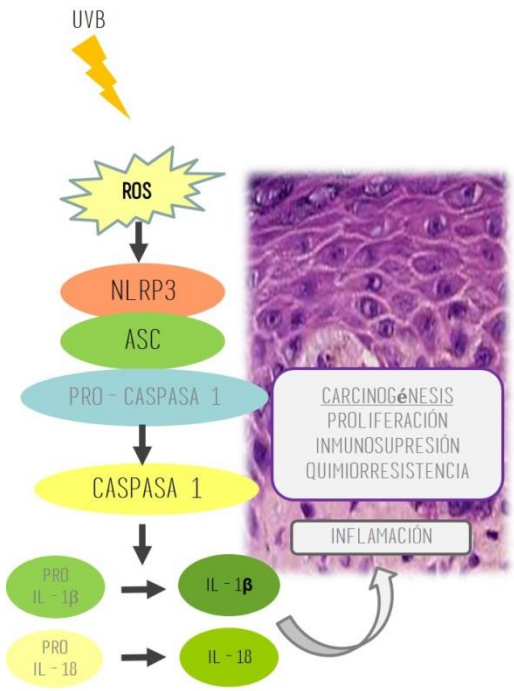
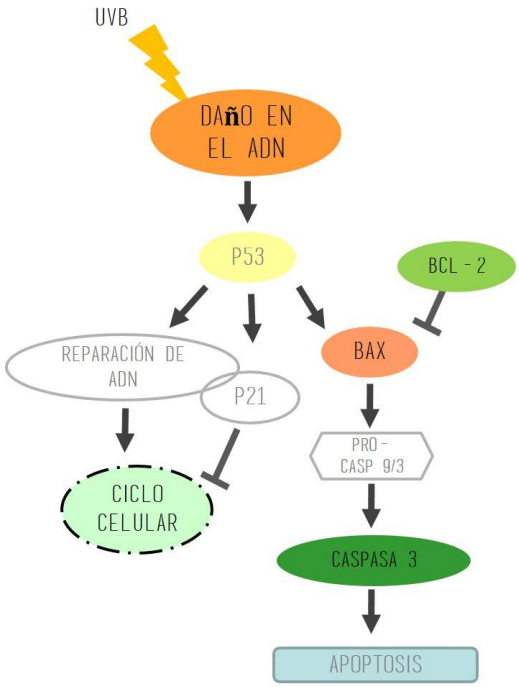


Figura 11: Esquema de activación del inflamasoma. Abreviaturas: ROS, especies reactivas de oxígeno; NLRP3, Receptor de tipo NOD-LRR-and pyrin domain-containing 3; ASC, adaptador proteico; IL, interleucina.

2.2.3.3. Apoptosis y ciclo celular

Es sabido que la exposición de las células de la piel a luz UV causa la pérdida de la integridad de la membrana celular, daño en el ADN y respuestas celulares como apoptosis e inflamación (Jeayeng et al., 2017). La apoptosis es un término que se estableció para nombrar a la muerte celular programada (Kerr et al., 1972). Se trata de un proceso en el que participan multitud de señales bioquímicas, sobre las cuales destaca la actividad de las caspasas (Figura 12). Durante

esta respuesta, la degradación que sufre la célula es aislada del medio extracelular por la membrana, la cual permanece impermeable durante este proceso. Sin embargo, cuando el daño es mayor o persiste en el tiempo, se produce una alteración de dicha membrana perdiendo su integridad, de modo que el contenido celular puede salir al espacio extracelular y comenzar la respuesta inflamatoria, este fenómeno es conocido como necrosis (Oropesa-Ávila et al., 2017).



Siendo ampliamente conocido el papel del UVB como generador de ROS y su consecuente inducción de inflamación, inmunosupresión y apoptosis (Salucci et al., 2013), en los últimos años se ha establecido una relación entre la activación del inflamasoma y la iniciación de distintas formas de muerte celular programada, como es el caso de la piroptosis, que se caracteriza por la lisis celular y la activación de la respuesta inflamatoria (Sharma and Kanneganti, 2016).

Figura 12: Esquema de activación del proceso de apoptosis. Abreviaturas: UVB, ultravioleta B; BCL-2, células de linfoma B tipo 2; BAX, homólogo pro-apoptótico de BCL-2; Casp, caspasa.

Ciclo celular

Cuando las células son expuestas de forma repetida a luz UV, se puede producir un daño en el ADN, que suele ser irreparable. Estas alteraciones genéticas pueden causar un desajuste en el control del ciclo celular, lo cual llevaría a la división descontrolada de estas células alteradas. Cuando una célula acumula daños genéticos que le impiden su correcta función en el organismo, se activa el proceso de apoptosis controlada para eliminar las células dañadas, evitando así la división de células propensas a acumular mutaciones y que puedan generar un cáncer a largo plazo. En este sentido, una exposición crónica a luz UV puede conllevar a una excesiva muerte celular provocando un daño permanente en el tejido (Strozyk and Kulms, 2013). De forma similar, una exposición aguda provoca una alteración en la dinámica del ciclo celular, de modo que la radiación UV induce una

disminución del número de células que se encuentran en la fase G0/G1. Mientras que el resto de fases, S y G2, no suelen verse muy alteradas. En este sentido, la subpoblación de la fase sub-G1, suele verse aumentada tras la radiación UV, lo cual se relaciona con un aumento de la apoptosis (Figura 13). Por este motivo, es de gran interés la búsqueda de compuestos que protejan a las células de la luz UV y mantengan un ciclo celular normal en el caso de exposiciones agudas a radiación UV (Ascenso et al., 2016).

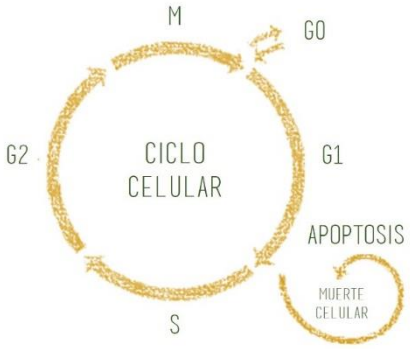


Figura 13: Ciclo celular y apoptosis. Podemos distinguir varias fases: G0. Fase de diferenciación; G1. Fase de pre-síntesis; S. Fase de síntesis de material biológico celular; G2. Fase de post-síntesis; M. Mitosis. En la apoptosis se activan las caspasas, las cuales inducen la condensación de cromatina y la fragmentación de ADN, produciéndose la destrucción nuclear y citoplasmática, y por último la fagocitosis por macrófagos (Burgués Gasión et al., 2005).

2.2.4. Fotoenvejecimiento, lesiones preneoplásicas y cáncer de piel

Para que ocurra una reacción fotoquímica en la epidermis, es necesario que la radiación UV sea absorbida por un cromóforo, iniciándose así una serie de reacciones que afectan a numerosos procesos bioquímicos (Lucas et al., 2015). La radiación UVB es la principal responsable del daño en el ADN, produciendo principalmente, su oxidación y por tanto, la rotura de alguna de sus cadenas, por lo que son numerosos los estudios que consideran que el ADN es un importante cromóforo para el desarrollo del eritema (Tewari et al., 2012). Las reacciones oxidativas pueden también modificar proteínas y lípidos, produciendo, en consecuencia, cambios en la funcionalidad de las estructuras afectadas. De esta manera, las fotolesiones de ADN localizadas en la epidermis, por acción del UVB, son el escenario clave para el desarrollo de mutaciones y en consecuencia la aparición de tumores en la piel (Young et al., 1998).

Clínicamente, el fotoenvejecimiento se caracteriza por sequedad, pigmentación, laxitud, arrugas y eritema. El daño tisular se refleja en alteraciones funcionales y estructurales, con una base inflamatoria que afecta a componentes de la matriz extracelular (colágeno, elastina y glicosaminoglicanos) y provoca la activación de enzimas proteolíticas, fundamentalmente metaloproteinasas, que suman sus efectos sobre la matriz extracelular (Lephart, 2016). Actualmente, las

estrategias dermatológicas para la prevención del fotoenvejecimiento son varias, pero fundamentalmente van dirigidas a la hidratación de la piel para compensar la pérdida de agua, y a la utilización de compuestos pantalla para el bloqueo de la penetración de la radiación, y de otros que puedan favorecer la vascularización de la piel o la reestructuración de componentes dañados (Blume-Peytavi et al., 2016).

Lesiones preneoplásicas y cáncer de piel

Según la Organización Mundial de la Salud, se estima que en las últimas décadas el cáncer de piel ha ido incrementando de forma alarmante, apareciendo entre 2 y 3 millones de casos referidos al tipo no melanoma y 132.000 casos de melanoma en todo el mundo cada año. De esta manera, en el mundo uno de cada tres casos de cáncer corresponde a cáncer de piel, siendo la exposición a la luz UV el principal factor de riesgo para su desarrollo ("WHO | Skin cancers," 2017). A pesar de ser uno de los tipos de cáncer que más fácilmente se podría prevenir, el número de casos detectados, especialmente del tipo no melanoma (CPNM), está en aumento. En este sentido, la exposición solar se asocia con el desarrollo del 65 % de los casos de melanoma, y casi del 90 % de los CPNM, que a su vez comprenden el carcinoma de células basales y el CEC, conocido también como carcinoma de células escamosas o espinocelular (Zink et al., 2016). Se sabe que la evolución de determinadas lesiones epidérmicas precancerosas pueden transformarse en lesiones clínicamente invasoras, con capacidad metastásica (Martorell-Calatayud et al., 2013). Entre ellas destaca la QA y su evolución hacia CEC. La QA es una lesión hiperqueratósica que afecta normalmente a la población anciana que ha estado expuesta de manera frecuente al sol durante largos periodos de su vida. Si estas lesiones preneoplásicas no se tratan, pueden degenerar en CEC, por lo que es recomendable iniciar su tratamiento en un estadio precoz (Figura 14) (Howell and Ramsey, 2017).

El tratamiento actual de esta patología se puede agrupar en dos categorías: (I) la terapia lesional, la cual se emplea directamente sobre una lesión aislada, e incluye crioterapia y cirugía, y (II) la terapia de campo que abarca una mayor área de la piel y suele tratar varias lesiones en conjunto. En esta

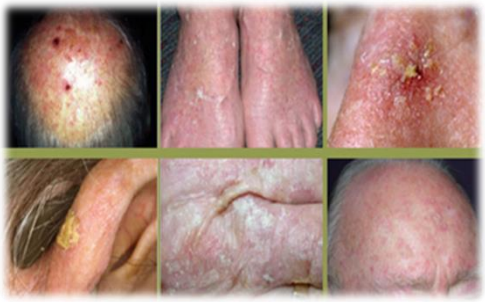


Figura 14: Imágenes de diferentes lesiones de Queratosis actínica.

última, se incluyen fármacos aplicados por vía tópica como ingenol mebutato al 0,05%, imiquimod 5%, diclofenaco 3%, 5-fluoracilo 0,5%, así como la terapia fotodinámica. De forma general, los pacientes están insatisfechos con el tratamiento de esta patología, debido al impacto físico y psicológico que ocasiona, lo cual se traduce en una disminución de su calidad de vida (Khanna et al., 2017).

Los estudios epidemiológicos indican que la QA aparece por una sobre-exposición acumulativa a la radiación UV, teniendo una mayor incidencia en personas más pálidas (fototipo I/II). Por estas razones, entre los principales factores de riesgo se encuentran: el sexo, se da más frecuentemente en hombres que en mujeres, la edad avanzada, el color de la piel (fototipo) y la inmunosupresión (Soyer et al., 2015). Además, el riesgo de sufrir este tipo de lesiones se ve aumentado en personas que viven en zonas en las que tienen gran exposición solar a lo largo de todo el año, como es el caso de los países del trópico, árido y mediterráneo (Schuch and Menck, 2010). Si a ello le sumamos que la piel no está preparada para soportar altas radiaciones y que cada vez la peligrosidad de las radiaciones es mayor debido a la disminución de la capa de ozono por el cambio climático, se favorece la frecuencia para la aparición de un cáncer de piel. Adicionalmente, existen situaciones patológicas que incrementan el riesgo de sufrir cáncer de piel, como es la inmunosupresión. Debido al relevante papel del sistema inmunitario en la patogenia del cáncer, personas sometidas a trasplantes de órganos y que reciben fármacos inmunodepresores, así como pacientes que tienen tratamientos inmunosupresores para tratar patologías como la psoriasis, tienen un riesgo elevado de sufrir cáncer de piel, particularmente del tipo CEC (Peleva et al., 2017).

En resumen, la producción excesiva de factores pro-inflamatorios conduce a un estado inflamatorio crónico, el cual a su vez aumenta el riesgo de tumorigénesis. Por lo tanto, la búsqueda de estrategias farmacológicas que controlen esta respuesta crónica de la piel a la radiación solar podría reducir la incidencia de cáncer (Maru et al., 2014). En este sentido, compuestos anti-inflamatorios y antioxidantes de origen natural capaces de retrasar el fotoenvejecimiento de la piel, así como, compuestos capaces de reducir las manifestaciones clínicas de la psoriasis, o que impidan el desarrollo frecuente de recidivas, son excelentes estrategias alternativas o complementarias a la terapéutica actual. No obstante, carotenoides como el β-caroteno (Stahl and Sies, 2012a), la luteína (Horváth et al., 2015a), o la fucoxantina (Urikura et al., 2011) y polifenoles como el resveratrol

(Caddeo et al., 2016b), la taxifolina (Kim et al., 2015), o el ácido rosmarínico (Fernando et al., 2016), han recibido especial atención por sus efectos beneficiosos para la salud humana, debido a sus propiedades antioxidantes, anti-inflamatorias y antitumorales (Chinembiri et al., 2014; Działo et al., 2016; Costa et al., 2016).

BLOQUE B. MOLECULAS EN ESTUDIO

3. MICROALGAS COMO FUENTE DE MOLÉCULAS BIOACTIVAS

En base a la necesidad de nuevas alternativas terapéuticas para procesos inflamatorios, la quimioprevención con compuestos naturales está en auge en estos tiempos. La quimioprevención es un viejo concepto que consiste en el uso de medicamentos, vitaminas, o alimentos funcionales para reducir el riesgo de desarrollar una enfermedad (Sporn, 2011). En este sentido, alternativas de interés surgen sobre la idea de que en cáncer la prevención de la inflamación sería de máximo provecho (Singh et al., 2014). Del mismo modo, compuestos anti-inflamatorios capaces de retrasar el fotoenvejecimiento de la piel serían excelentes estrategias alternativas o complementarias a la terapéutica actual.

Tradicionalmente, los compuestos bioactivos procedentes de plantas han sido estudiados extensamente en el desarrollo de sustancias con actividades terapéuticas (Cragg et al., 2014). Sin embargo, en los últimos años, la necesidad de nuevas moléculas ha proporcionado numerosos estudios con compuestos procedentes de invertebrados o de microorganismos marinos. El mundo de las microalgas está poblado por sorprendentes especies que modulan sus capacidades metabólicas para adaptarse a un entorno en constante cambio. Las microalgas son organismos primigenios formados por una membrana lipídica que protege, con una serie de estructuras, a unas moléculas más o menos complejas, y a una maquinaria que les sirve para sobrevivir y perpetuarse. Tal y como comentábamos anteriormente, la investigación en la última década, ha llevado al descubrimiento de nuevos e interesantes compuestos de origen marino, acuñándose el término “productos naturales marinos”. En este sentido, las microalgas son las que centran gran parte de la actividad de nuestro trabajo debido al alto y variado contenido en moléculas bioactivas, entre las que se incluyen ácidos grasos poli-insaturados de cadena larga (PUFAs), polisacáridos, glicolípidos, o carotenoides. Las microalgas

han demostrado ser una fuente alternativa a sus homólogos terrestres de productos con uso farmacológico. Sus singulares características las hacen idóneas para obtener diferentes productos que han demostrado tener potente actividad biológica (Talero et al., 2015).

Se ha demostrado que muchas de estas estructuras moleculares pueden ser potencialmente usadas como fármacos en el tratamiento de enfermedades como el cáncer, la inflamación crónica, infecciones y desórdenes neurológicos (Granado-Lorencio and Hernández-Alvarez, 2016; Ruiz-Torres et al., 2017; Valcarcel et al., 2017). En la Figura 15 se recogen los principales componentes de las microalgas, los cuales destacan por su actividad antioxidante y algunos en su mayoría ant-inflamatoria y anticancerígena (Talero et al., 2015).

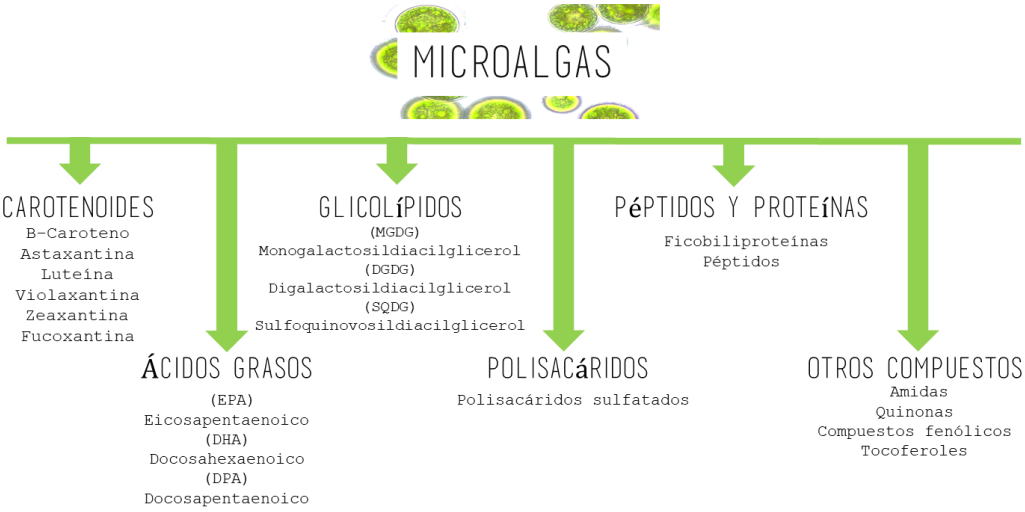


Figura 15: Principales compuestos obtenidos de las microalgas.

En la presente Tesis Doctoral se han estudiado varios compuestos que se encuentran dentro del grupo de los glicolípidos, así como se ha profundizado en la actividad biológica de un carotenoide aislado de microalgas como es la fucoxantina.

3.1 Glicolípidos

Resultan de la unión de un lípido y un hidrato de carbono, y se caracterizan por formar parte de las membranas tilacoidales de los orgánulos fotosintéticos de plantas, algas eucariotas y cianobacterias (Nakamura and Li-Beisson, 2016).

3.1.1. Estructura

Los glicerolglucolípidos están formados por un azúcar y un glicérido, normalmente PUFAs ω-3, unidos mediante enlace glucosídico, siendo los principales los galactolípidos y sulfolípidos (Dominguez, 2013). Dentro de los galactolípidos, en los que el hidrato de carbono es la galactosa unida al glicérido por enlace tipo-β, se diferencian los monogalactosil-diacilgliceroles (MGDGs) y digalactosil-diacilgliceroles (DGDGs). Los sulfolípidos son similares, pero presentan un grupo funcional de azufre en el hidrato de carbono, se unen al glicérido por enlace tipo-α y se nombran como sulfoquinovosil-diacilgliceroles (SQAGs)(Figura 16) (Colombo et al., 2013).

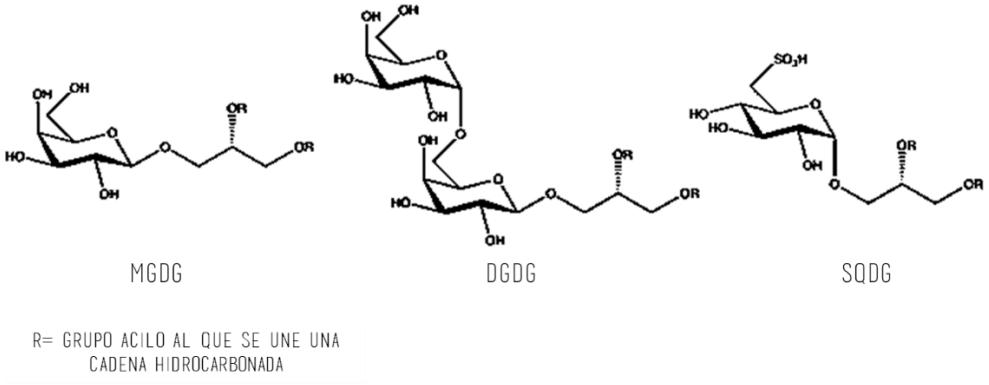


Figura 16: Estructuras de los glicolípido MGDG, DGDG, SQDG. Abreviaturas: MGDG, monogalactosil-diacilgliceroles; DGDG, digalactosil-diacilgliceroles; SQAG, sulfoquinovosil-diacilgliceroles (Plouguermé et al., 2014).

3.1.2. Actividad biológica

Debido a su alto porcentaje lipídico, la principal aplicación de las microalgas es la obtención de biofuel, estableciéndose como una fuente con alto potencial para suponer una alternativa a las fuentes actuales (Yamane et al., 2013; Paniagua-Michel et al., 2014; Navarro López et al., 2016). Sin embargo, en los últimos años, han supuesto una revolución innovadora como fuente de ingredientes funcionales en alimentación, debido a su alto contenido en lípidos, proteínas e hidratos de carbono. Debido a su alta tasa de generación y requerimientos nutricionales simples, las microalgas pueden ser cultivadas a gran escala, bien en condiciones naturales o artificiales mediante el uso de fotorreactores, suponiendo en los últimos años, microorganismos de gran interés para la explotación biotecnológica en las industrias química, alimentaria y farmacéutica.

En relación a la actividad biológica que desempeñan, los glicolípidos han destacado principalmente por su función como antitumorales y anti-inflamatorios. Para los primeros estudios con

glicolípidos de microalgas se utilizó *Gymnodinium mikimotoi,* microalga perteneciente al género de las Dinoflageladas, especie que destacó por su contenido en MGDG y DGDG, y por su actividad hemolítica, la cual fue asociada con la actividad citotóxica que se producía en ciertas especies de peces al ingerir dicha microalga (Parrish et al., 1998). Sin embargo, estos compuestos se distinguen por su actividad anticancerígena, la cual ha sido ampliamente demostrada en estos años. En este sentido, determinados glicolípidos con estructura monogalactosil-monoacilglicerol (MGMG), MGDG, DGDG y SQDG han destacado por su capacidad de inhibir a la ADN-polimerasa y la topoisomerasa II, por lo cual se les ha atribuido el papel de inmunosupresores y agentes antitumorales (Matsumoto et al., 2000; Maeda et al., 2005). Numerosos estudios han demostrado su efecto anti-tumoral en diferentes líneas celulares, destacando su selectividad por células tumorales, sin afectar a las células sanas, sugiriendo que este tipo de estructuras moleculares podrían tener gran potencial como agentes anti-tumorales (Matsui et al., 2009). Estudios realizados en diferentes líneas tumorales, incluyendo tejidos gástricos (Murakami et al., 2003; Mizushina et al., 2003; Kuriyama et al., 2005), melanoma (Maeda et al., 2005), colon (Hossain et al., 2005) y cérvix (Maeda et al., 2007a)(Maeda et al., 2007b), sirvieron como precedente para realizar ensayos con animales a los que se les administraron preparados con MGDG por vía oral para estudiar la progresión de tumores en colon. Se observó el papel antitumoral, debido a la inhibición de la angiogénesis y a la acción anti-proliferativa de células tumorales por un aumento de la apoptosis (Maeda et al., 2008; Maeda et al., 2013).

Los glicolípidos de microalgas comienzan a tener un papel importante para su aplicación en patologías inflamatorias gracias a los estudios de Banskota et al. 2013b, en los que demostraron su capacidad de interferir en los mecanismos de acción del óxido nítrico (NO). En este sentido, extractos de *Tetraselmis chui* y *Palmaria palmata* mostraron un efecto inhibitorio de la producción del NO inducida por LPS en macrófagos RAW254.7, así como una disminución de la actividad de la enzima iNOS, en el caso de *P. palmata* (Arjun H. Banskota et al., 2013; Banskota et al., 2014).

3.1.3. Estudios con glicolípidos en piel

Los efectos de los glicolípidos aislados de microalgas frente a inflamación y cáncer de piel, así como sus mecanismos de acción, han

sido ampliamente estudiados, empleando tanto modelos *in vitro* como experimentos con animales. En este sentido, Colombo et al. 2000 investigaron varios glicoglicerolípidos sintéticos con el fin de valorar su actividad frente a la incidencia de tumores de piel. Para ello, utilizaron un modelo en el que el tumor se inició con dimetilbenz[a]antraceno (DMBA) y se continuó con TPA durante 20 semanas, observándose progresivamente el aumento en la incidencia de papilomas. El tratamiento tópico con este tipo de compuestos, redujo tanto la incidencia (número de ratones que expresaron tumores), así como la multiplicidad (número de tumores por animal), en comparación con el grupo control sin tratamiento. Sería más adelante, cuando evaluarían el mecanismo de acción de dichos compuestos con experimentos en fibroblastos estimulados con el agente promotor de tumores TPA, confirmando la disminución de la traslocación de la proteína kinasa C (PKC) (Colombo et al., 2011), proteína relacionada con procesos de crecimiento celular e implicada en la señalización de mecanismos vinculados a la carcinogénesis (Foster et al., 2012). Posteriormente, estos autores avanzaron en su investigación sintetizando nuevos glicolípidos con el fin de buscar estructuras que presentasen una mejor respuesta en los modelos tumorales de piel estudiados (Colombo et al., 2013).

En relación con la actividad anti-inflamatoria, se han desarrollado modelos de inflamación en oreja y pata de ratón, en los que diferentes compuestos MGDG y DGDG fueron aplicados por vía tópica disueltos en etanol. La actividad anti-inflamatoria de un compuesto MGDG aislado de un alga azul, *Phormidium sp* ETS-05, destacó en comparación con los agentes usados de referencia, betametasona, en el caso del modelo de inflamación en oreja, e indometacina, empleado en el modelo en pata de ratón, planteando el posible mecanismo de acción del mismo por su actuación sobre la COX-2 e iNOS (Bruno et al., 2005). Con respecto a la inflamación inducida por UVB, existen pocos trabajos que hayan evaluado la actividad de estos compuestos en este tipo de modelos. Se ha confirmado la actividad anti-inflamatoria de una fracción aislada del alga parda *Sargassum fulvellum,* por su supresión de enzimas como la COX-2, iNOS o PGE_2, cuya síntesis es inducida tras la exposición a UVB, tanto en modelos *in vitro* con queratinocitos humanos inmortaizados HaCaT como en modelos con animales empleando ratones Balb/c (Lee et al., 2013).

3.2 Carotenoides

Los carotenoides son pigmentos naturales biosintetizados por todos los organismos fotosintéticos y algunos hongos y bacterias no fotosintéticas. Estos pigmentos se encuentran ampliamente repartidos entre los seres vivos, estimándose una producción anual de más de 100,000.000 toneladas, destacando la fucoxantina, procedente de diversas especies de microalgas, seguida de los tres carotenoides presentes en las hojas verdes como son la luteína, violaxantina y neoxantina. El reparto de los carotenoides en las plantas no presenta un patrón único; así, en verduras se concentran en mayor proporción luteína, β-caroteno, violaxantina y neoxantina, mientras que en frutos encontramos en mayor medida xantofilas, aunque hay excepciones como el tomate en el que el licopeno es el carotenoide mayoritario (Meléndez-Martínez et al., 2004). Respecto al reino animal, son incapaces de sintetizar carotenoides, por lo que deben incluirlos en su dieta. En el caso de los animales, estos carotenoides son los responsables del color característico de ciertas especies (como es el caso del salmón, cangrejo o gamba). Sin embargo, los humanos los incorporamos a nuestra dieta y son útiles, en nuestro organismo, por su función como precursores de compuestos esenciales, como la vitamina A. Los carotenoides que encontramos en mayor proporción en nuestro organismo son el β-caroteno y α-caroteno, licopeno, luteína, zeaxantina y α- y β-criptoxantina, los cuales, tras ser ingeridos, son transportados hasta la piel y acumulados en las capas de la epidermis. La cantidad de los pigmentos en el organismo variará en función del aporte dietético de cada individuo, así como de la biodisponibilidad de los mismos en los alimentos (Fernández-García, 2014). Entre las numerosas fuentes de carotenoides, las microalgas son consideradas uno de los principales reservorios. Por esta razón, las microalgas no sólo se emplean para aislar compuestos puros, sino que se posicionan como un potencial ingrediente para su incorporación en alimentos funcionales debido a sus múltiples actividades biológicas descritas (Matos et al., 2017).

3.2.1. Estructura

Los carotenoides son compuestos con estructura isoprenoide, que contienen una serie de dobles enlaces conjugados, lo que conforma el grupo cromóforo. La presencia de este grupo, además de ser el responsable de los colores amarillos, naranjas o rojos, les proporciona la capacidad de captar la energía luminosa, en el caso de organismos fotosintéticos, para transferirla a las clorofilas de los sistemas fotosintéticos donde se transforma, durante la fotosíntesis,

en energía química (Meléndez-Martínez et al., 2007). Estos compuestos se pueden clasificar en dos subgrupos atendiendo a su composición química: los carotenos y las xantofilas.

Los carotenos son hidrocarburos lineales o cíclicos en uno o ambos lados de la cadena. Los más importantes son α- y β-caroteno y licopeno, destacando por su papel en la síntesis de vitamina A, esencial para determinadas funciones biológicas. Respecto a las xantofilas, son derivados oxigenados, y destacan la astaxantina, luteína o fucoxantina, cuya aplicabilidad es fundamentalmente como antioxidantes y colorantes.

3.2.2. Actividad biológica

Numerosas evidencias indican los efectos beneficiosos de los carotenoides para la salud humana. Si bien se conocen por su papel antioxidante y anti-inflamatorio, sus acciones se relacionan con su capacidad para modular la expresión de diferentes genes. Los efectos de algunos carotenoides se relacionan con su actividad provitamina A (β-caroteno y β-criptoxantina); sin embargo, otros carotenoides, que no presentan esta actividad, también presentan claros efectos beneficiosos sobre la salud. Son numerosas las propiedades biológicas que se les han atribuido a estos compuestos debido al efecto que ejercen en patologías crónicas como la ateroesclerosis (Howard and Thurnham, 2017), trastornos metabólicos (dislipemias) (Deng et al., 2017), enfermedades cardiovasculares (Chung et al., 2017), diabetes mellitus (Roohbakhsh et al., 2017), patologías neurodegenerativas (Kim et al., 2017), y patologías oculares como la degeneración macular asociada a la edad (Bernstein et al., 2016). Además, presentan una importante actividad anti-tumoral, siendo numerosos los estudios que la avalan en diferentes líneas celulares, recientemente revisados en Hoshyar and Mollaei 2017.

3.2.3. Estudios con carotenoides en piel

Los carotenoides siempre han destacado por su papel anti-oxidante debido a su estructura, lo cual se ha relacionado con el papel fotoprotector frente al daño solar (Stahl and Sies, 2012b). Numerosos estudios, tanto *in vitro* como *in vivo,* atribuyen a los carotenoides un papel fotoprotector, planteando diferentes mecanismos de acción. Yoshihisa et al. 2014, observaron que la astaxantina disminuyó la expresión de COX-2 e iNOS, los niveles de citocinas pro-inflamatorias IL-1β y TNF-α, y ciertos marcadores apoptóticos, en

queratinocitos HaCaT irradiados con UVB. Otros autores utilizaron queratinocitos humanos primarios irradiados y observaron que la astaxantina atenuaba la producción de transglutaminasa, un factor que regula la queratinización de la piel, interfiriendo en diferentes vías que confluyen en la activación de NF-κB (Terazawa et al., 2015). De manera similar, la luteína, en células HaCaT irradiadas con luz UV, redujo la actividad de la metaloproteinasa MMP-9 y los niveles de TNF-α, confirmándose así la actividad anti-inflamatoria y sugiriéndola como un potente remedio para patologías inflamatorias de la piel (Oh et al., 2013).

Por otro lado, la luteína y la zeaxantina, incorporadas a la dieta de ratones SKH-1 sin pelo y somentidos a irradiación UVB, mostraron un descenso significativo en la respuesta cutánea edematosa, entre otros parámetros, demostrando su capacidad de generar una respuesta anti-inflamatoria y antiproliferativa ante la exposición a esta radiación (González et al., 2003). Más recientemente, modelos *in vivo* con astaxantina, han puesto de manifiesto su papel anti-inflamatorio, reduciendo la síntesis de citocinas pro-inflamatorias características de un modelo de dermatitis en ratón (Yoshihisa et al., 2016). La astaxantina también destaca por su papel fotoprotector, atenuando varios parámetros asociados al fotodaño como son la pérdida de agua transepidermal o la aparición de arrugas, en un modelo de ratones SKH-1 irradiados con UVA, a los que se administraba en la dieta, astaxantina obtenida de la microalga *Haematococcus pluvialis* (Komatsu et al., 2017). Siguiendo esta línea, Cooperstone et al. 2017, evaluaron el efecto protector de una dieta con tomate frente a otra libre del mismo, en un modelo de tumores de piel inducidos por UVB. Los resultados mostraron que los animales que consumían tomate, con alto contenido en licopeno seguido de fitoeno, presentaban menor número de tumores en comparación con los que tenían una dieta exenta de tomate.

Por estas razones, los carotenoides, principalmente licopeno, β-caroteno y luteína son considerados compuestos de interés por su aplicabilidad en la industria cosmética, de modo que son incorporados en formulaciones fotoprotectoras debido a su demostrada actividad biológica (Corinaldesi et al., 2017).

3.3 β- caroteno

El β-caroteno es el carotenoide más conocido y consumido por los humanos ya que se encuentra en numerosas frutas y verduras. En

relación a las fuentes marinas, se halla principalmente en especies de microalgas pertenecientes a los géneros *Dunaliella* y *Haematococcus*. El β-caroteno se convierte en el principal precursor de la vitamina A, ya que su estructura simétrica (Figura 17) es capaz de dividirse en dos moléculas de *trans*-retinol, convirtiéndose así en una importante fuente de esta vitamina A (Grune et al., 2010).

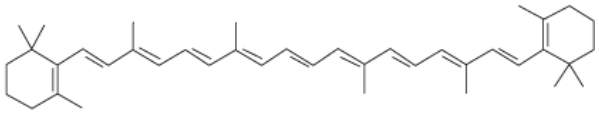


Figura 17: Fórmula estructural plana del β-caroteno (Meléndez-Martínez et al., 2004).

Algunos de sus efectos antioxidantes y anticancerígenos se asocian a esta transformación en retinol, molécula que interviene y controla numerosos procesos como embriogénesis, crecimiento, proliferación celular, apoptosis, visión ocular, formación de huesos, metabolismo y procesos inmunitarios (Chelstowska et al., 2016; Moreb et al., 2017; Milani et al., 2017). Los primeros estudios *in vitro* analizaron su potencial antioxidante, mostrando que reducía la inactivación inducida por UV de enzimas antioxidantes como la catalasa y la SOD. Este pigmento, entre otras de sus funciones, ha demostrado prevenir la expresión de HO-1 en fibroblastos irradiados con UVA, evitándose así mecanismos que desencadenan la respuesta oxidante (Trekli et al., 2003). En esta línea, estudios en fibroblastos pre-tratados con un extracto rico en β-caroteno, y posteriormente estresados con H_2O_2, mostraron un incremento en la viabilidad celular, así como una significativa reducción de la cantidad de ROS, la peroxidación lipídica y el daño en el ADN, lo cual se acompañó de una mejora en la funcionalidad de la mitocondria, aumentando la capacidad regenerativa de las células tras la exposición al estímulo pro-oxidante (Giampieri et al., 2014).

La actividad anti-inflamatoria y antioxidante del β-caroteno también se ha puesto de manifiesto en modelos *in vivo*. En este sentido, los efectos de este pigmento han sido evaluados en modelos de inflamación en oreja, mostrando una reducción del edema, así como de la actividad de la enzima MPO (Horváth et al., 2015b). La capacidad protectora de este compuesto también se demostró en un modelo de inflamación en piel de ratón inducida por ozono, reduciendo mediadores pro-inflamatorios tales como TNF-α, COX-2, e iNOS, así como marcadores

de estrés oxidativo como HO-1 (Valacchi et al. 2009). Además, se han llevado a cabo estudios en humanos que reflejan que la suplementación con este carotenoide previene el edema inducido por UVB, debido al efecto que ejerce sobre la peroxidación lipídica (Lee et al., 2000). En esta línea, un estudio en mujeres de más de 50 años demostró que la ingesta diaria de β-caroteno mejoró la presencia de arrugas, elasticidad y daño en el ADN inducido por la radiación UVB, lo que sugiere que este compuesto previene y repara las alteraciones asociadas al fotodaño (Cho et al., 2010). En estudios relacionados con CPNM, se ha descrito que pacientes con niveles reducidos de este pigmento en suero, debido a que su ingesta dietética es menor, presentan una mayor incidencia de desarrollar tumores de tipo no melanoma (Kune et al., 1992). En este sentido, se determinó que la aplicación de este compuesto por vía tópica evitó la disminución de los niveles totales de carotenoides en piel tras la exposición a radiación infrarroja, ejerciendo un efecto protector en voluntarios sanos irradiados, en comparación con los sujetos tratados solo con el vehículo (Darvin et al., 2011).

3.4 Fucoxantina

La fucoxantina es uno de los carotenoides más abundantes, constituyendo más del 10 % de la producción total de estos pigmentos en la naturaleza. Se caracteriza por su color naranja, se encuentra principalmente en algas pardas, macro y microalgas y diatomeas, y pertenece al grupo de las xantofilas (Chuyen and Eun, 2017). Respecto a su estructura, incluye un enlace aleno, inusual en carotenoides, y grupos funcionales oxigenados como epóxido, hidroxilo, carbonilo y carboxilo (Figura 18), responsables de su actividad antioxidante (Peng et al., 2011).

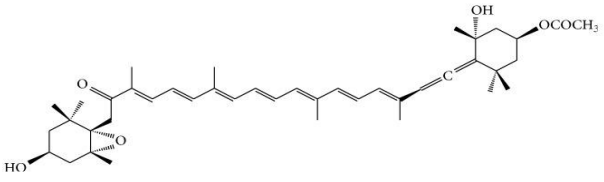


Figura 18: Estructura química de la fucoxantina (Zhang et al., 2015).

Debido a su carácter antioxidante, se le han atribuido actividades relacionadas con el secuestro de radicales libres (*radical scavenging*) (Sachindra et al., 2007), efectos anti-inflamatorios (Kim et al., 2010), anti-obesidad (Beppu et al., 2013), protector en

hipertensión e hiperglicemia (Hosokawa et al., 2010) y en el síndrome metabólico (Nishikawa et al., 2012), e incluso efectos anti-fibróticos en modelos de fibrosis pulmonar *in vitro* (Ma et al., 2017).

Este carotenoide también destaca por su papel fotoprotector y antiedad, actividades sobre las que se centran la mayoría de los estudios hasta el momento, debido al interés que presenta para su uso en la industria farmacéutica y cosmética (Pallela et al., 2010). En este sentido, la fucoxantina ha demostrado un papel importante como antioxidante y fotoprotector en diferentes modelos *in vitro*. Este pigmento aislado de la especie *Sargassum siliquastrum* mostró una actividad fotoprotectora reduciendo la producción intracelular de ROS, el daño celular y la apoptosis en fibroblastos humanos irradiados con UVB (Heo and Jeon, 2009). De forma similar, la fucoxantina redujo los niveles de ROS en queratinocitos HaCaT estresados mediante la adicción de H_2O_2 (Zheng et al., 2013). Con el objetivo de elucidar el mecanismo de acción de este compuesto, Zheng et al. 2014 evaluaron su papel antioxidante en queratinocitos humanos HaCaT, observando un aumento de la expresión de enzimas como la glutatión sintetasa y la subunidad catalítica ligasa glutamato-cisteína, las cuales se encuentran ligadas a la síntesis de glutatión reducido (GSH). Además, se estableció una relación directa con la translocación nuclear y la fosforilación de Nrf2, vía PI3K/Akt, lo que condujo a la recuperación de la glutatión reducida por UVB.

El efecto antiedad de la fucoxantina ha sido evaluado experimentalmente, empleando ratones sin pelo. Así, el pre-tratamiento tópico con fucoxantina, aislada de *Undaria pinnatifida,* durante 10 semanas, en el dorso de estos ratones irradiados con UVB redujo el número de arrugas, mejoró la hipertrofia, y disminuyó la expresión de VEFG y MMP-13 en la epidermis (Urikura et al., 2011). Otros autores han confirmado que la aplicación tópica y oral de fucoxantina, suprimió la síntesis y expresión de receptores melanogénicos, así como la expresión de COX-2 y la actividad de la tirosinasa en ratones sin pelo irradiados con UVB, provocando un efecto anti-melanogénico y anti-pigmentario (Shimoda et al., 2010). Más recientemente, la fucoxantina ha despertado el interés por el efecto ejercido sobre marcadores como la filagrina (Flg), una proteína con un papel fundamental en la cornificación de la piel y que se encuentra claramente afectada cuando la piel es expuesta a la radiación UV. De esta manera, se ha planteado que el efecto protector de la fucoxantina podría estar relacionado con la inducción de Flg, lo que promovería la

formación de la barrera de la piel alterada por la radiación UV (Matsui et al. 2016).

4. POLIFENOLES

Los polifenoles son los antioxidantes más abundantes en nuestra dieta, encontrándose de manera generalizada en las frutas, las verduras, los cereales, el aceite de oliva, las legumbres secas y el chocolate, así como también en bebidas, como el té, el café y el vino. Algunos polifenoles son específicos de determinados alimentos como por ejemplo las flavononas en cítricos, y otros, como la quercetina, se pueden encontrar en un gran número de plantas. Numerosos factores medioambientales como la luz, el grado de madurez o el grado de conservación pueden afectar al contenido total de polifenoles, además del procesado de los alimentos (Del-Castillo-Alonso et al., 2016). Estos compuestos son metabolitos secundarios de plantas y, por lo general, están involucrados en el mecanismo de defensa de las mismas debido a sus características antibióticas y antifúngicas (Gil Hernańdez and Sańchez de Medina Contreras, 2010), aunque algunos son indispensables para las funciones fisiológicas vegetales.

4.1 Estructura y clasificación

Con más de 8.000 variantes estructurales, su característica general es la presencia de anillos aromáticos, con uno o más restos hidroxilo. Los polifenoles se dividen en varias clases según el número de anillos fenólicos que contienen y los elementos estructurales que se unen entre ellos. Así, se pueden establecer 5 grandes grupos: ácidos fenólicos, estilbenos, lignanos, alcoholes fenólicos y flavonoides. Nos centraremos en los ácidos fenólicos, grupo en el que se sitúa el compuesto en estudio.

4.1.1. Ácidos fenólicos

Son compuestos polifenólicos no flavonoides que se pueden dividir en dos tipos principales, (I) los derivados del ácido benzoico (gálico, vanilíco, catéquico, elágico y siríngico) y (II) los derivados del ácido cinámico (*p*-cumárico y cafeico). Si bien las frutas y las verduras contienen numerosos ácidos fenólicos libres, en granos y semillas se encuentran a menudo en la forma unida (Chandrasekara and Shahidi, 2010). Estos ácidos fenólicos sólo pueden ser liberados o hidrolizados por hidrólisis ácida o alcalina, o por enzimas (Tsao, 2010). Con el objetivo de focalizar el contenido, nos

centraremos en los ácidos hidroxicinámicos, los cuales no suelen presentarse en forma libre sino que se encuentran esterificados en la pared celular vegetal, y por lo tanto, poseen una baja solubilidad. Los principales representantes son el ácido ferúlico, el ácido cumárico y el ácido cafeico.

4.1.1.1. Ácido Rosmarínico

El ácido rosmarínico (RA) resulta de la esterificación de una molécula de ácido cafeico y otra de ácido 3,4-dihidrofeniláctico (Figura 19). Fue aislado por primera vez como compuesto puro en 1958 por dos químicos italianos (Scarparti and Oriente, 1958), y se trata del segundo derivado del ácido cafeico más frecuente en el reino de las plantas (Simanaviciute et al., 2017).

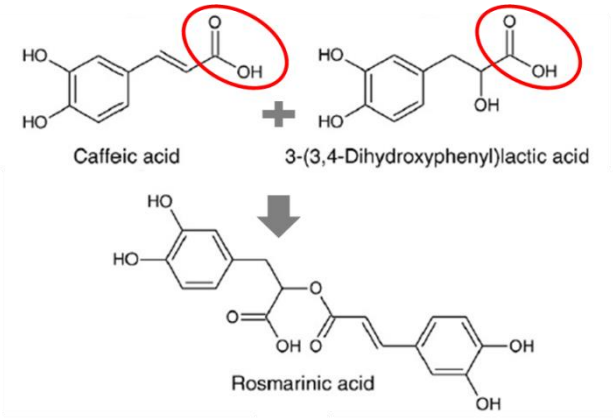


Figura 19: Esquema adaptado de la formación del RA (Adomako-Bonsu et al., 2017).

El RA se aísla principalmente de *Rosmarinus officinalis* (romero), de la familia de las lamiáceas, aunque también se puede encontrar en la mayoría de las especias y hierbas incluyendo la salvia, el bálsamo de limón, el orégano, la hierbabuena, el tomillo, la albahaca y la mejorana. Se utiliza en muchas cocinas para condimentar comidas, pero también es conocido por sus importantes actividades biológicas (Petersen, 2013).

4.1.1.2. Actividad biológica del Ácido Rosmarínico

Durante años, numerosos estudios han evaluado las actividades biológicas de este compuesto con el objeto de ser utilizado en la defensa de las plantas frente a patógenos y herbívoros (Petersen and Simmonds, 2003), debido a su actividad antimicrobiana (Moreno et al., 2006; Ekambaram et al., 2016). Son varias las revisiones sobre sus

actividades biológicas, con numerosas citas bibliográficas que acreditan su importante papel y su prospectiva como agente terapéutico. La mayoría de sus cualidades se atribuyen a la capacidad de captar radicales superóxidos e hidroxilo, así como inhibir lipoproteínas de baja densidad y la hemólisis, entre otras. Debido a su demostrada actividad antioxidante como captador de ROS (Amoah et al., 2016), son cada vez más los autores que centran la aplicación de este compuesto en patologías concretas, como es el caso de la hepatofibrogénesis relacionada con la alta producción endógena de ROS (Lu et al., 2017), o el pterigión, un tumor ocular en el que se desarrolla un crecimiento excesivo del tejido conjuntivo (Chen et al., 2017). Este compuesto también ha destacado por su actividad anti-inflamatoria, ya que actúa inhibiendo la expresión de proteínas como la COX, PGE-2, IL-1β, MMP2 y NO, entre otras, mecanismo de acción que junto a la actividad antioxidante, ha servido para atribuirle el perfil de molécula hepatoprotectora (Nunes et al., 2015), además de tener aplicaciones en patologías como el dolor neuropático (Ghasemzadeh Rahbardar et al., 2017). Recientemente, Moore et al. 2016, han revisado el papel antitumoral que presenta esta molécula en modelos *in vitro* de células tumorales de colon, pulmón, páncreas, ovario y leucemia, así como en modelos animales de tumores de hígado, colon, próstata, leucemia y tumores de piel inducidos por DMBA. La disminución de la viabilidad de las células tumorales expuestas a RA se ha relacionado con procesos de inducción de apoptosis, intervención en el ciclo celular, activación de mecanismos antioxidantes vía Nrf2 y efectos anti-inflamatorios relacionados con la inhibición de COX e IL-1, entre otros. Cabe destacar, que en esta familia de compuestos, la vía de administración afecta a la bioestabilidad y biodisponibilidad del compuesto. Es decir, cuando estas moléculas se administran por vía oral sufren un fuerte metabolismo, de modo que el responsable de la acción no tendría por qué ser la molécula administrada, sino que podría ser un metabolito, los cuales una vez transformados pueden acumularse en plasma, piel o hígado según la estructura resultante, de ahí la importancia de analizar el compuesto responsable de la acción (Yamada et al., 2006).

4.1.1.3. Estudios con ácido rosmarínico en piel

Las propiedades terapéuticas de RA también se han evidenciado en modelos experimentales de piel, destacando su actividad fotoprotectora tanto *in vitro* como *in vivo* frente a la radiación UVB. Esta actividad ha sido evaluada en modelos *in vitro* donde RA es capaz de aumentar la

viabilidad celular en queratinocitos HaCaT, disminuyendo la apoptosis estimulada por la radiación UVB, además de incrementar la actividad de ciertas enzimas anti-oxidantes como la SOD, catalasa y a la activación de la vía Nrf2, deprimidas por la acción del UVB, lo que provoca un aumento de HO-1 (Fernando et al., 2016). En este mismo modelo celular, se evaluó el efecto fotoprotector tanto de un extracto de *Prunella vulgaris* rico en RA como del RA puro, observándose un menor daño en la membrana celular mediante la disminución de la liberación de lactato deshidrogenasa (LDH), así como una reducción de la citocina IL-6 y de marcadores pro-apoptóticos como la caspasa 3 y 9 en células tratadas con dichos compuestos (Vostálová et al., 2010). En otro estudio más reciente, se ha observado la capacidad de este polifenol de disminuir la expresión de IL-6, IL-8 y MCP-1, e incrementar la expresión de IL-10, disminuyendo por tanto la respuesta inflamatoria generada en células HaCaT tras la exposición a UVB (Lembo et al., 2014).

Diferentes estudios en animales irradiados con UV confirman la actividad antioxidante y fotoprotectora de RA. Así, la aplicación por vía tópica del ácido cafeico y su derivados, el RA entre ellos, en ratones sin pelo irradiados con UVA, generó una disminución en los niveles de ROS, acción que fue confirmada en el mismo modelo administrando el cafeico vía oral. Tal y como se ha comentado en el apartado anterior, en este estudio se puso de manifiesto la importancia de la vía de administración de los compuestos (Yamada et al., 2006). Tras estudiar su actividad antimutagénica y radioprotectora, y con los antecedentes previos de actividad antioxidante, Sánchez-Campillo et al. 2009, decidieron determinar si el RA tenía efecto en la melanogénesis, observando un incremento de melanina en células de melanoma B16 tratadas con RA. Este resultado sumado a su actividad antioxidante, situaban a esta molécula como un posible agente fotoprotector. Esto fue confirmado *in vivo*, empleando un modelo de irradiación crónica con UVA durante 33 semanas, irradiando tres veces a la semana, con un total de 100 sesiones, en el que se administraba diariamente RA al 2 % en la comida y bebida. Los resultados mostraron la ausencia o disminución en el grado de displasia en los animales que recibieron el RA. A este respecto, los últimos estudios llevados a cabo se han realizado con un extracto de *Thymus vulgaris,* una hierba tradicional cuyo contenido es rico en RA, el cual se aplicó por vía tópica al 1 y 5 %, en animales sin pelo irradiados con UVB a una intensidad de 100 mJ/cm^2 durante 5 semanas. En este estudio se observó una disminución significativa en la formación de arrugas, el enrojecimiento y el engrosamiento de la epidermis,

además de un incremento en el contenido de las fibras de elastina y colágeno tipo I. La aplicación tópica del extracto rico en RA disminuyó de forma significativa los niveles de MMP-1 estimulados por UVB, cuya acción es la degradación del colágeno tipo I, y aumentó el porcentaje de fibras de colágeno y elastina, así como los niveles TGF-β1. El mecanismo de acción de este extracto estuvo relacionado con la activación de la vía Nrf2 y la inhibición de la vía de las Map kinasas MAPK/AP1, estudiado en un modelo *in vitro* de fibroblastos (Sun et al., 2016).

Respecto a la actividad anti-inflamatoria de RA se han llevado a cabo ensayos en líneas celulares donde este polifenol ha inhibido la expresión de genes relacionados con la vía del factor NF-κB en fibroblastos estimulados con TNF-α (Lee et al., 2006). Más recientemente, se evaluó el efecto del pre-tratamiento con el RA en queratinocitos primarios estimulados con ácido poliinosínico-policitidílico poli(I:C). Este compuesto genera una respuesta inmune en la que los queratinocitos producen altos niveles de TLR-3, lo cual provoca una fuerte respuesta inflamatoria, comparable a aquella generada en la piel psoríasica. Los resultados evidenciaron que el RA disminuyó la secreción y expresión de citocinas pro-inflamatorias como IL-1β, TNF-α, IL-6, IL-8, sugiriendo de este modo la posible aplicación del polifenol en el tratamiento de patologías inflamatorias como la psoriasis (Zhou et al., 2016).

En estudios experimentales, RA ha demostrado su actividad anti-inflamatoria en un modelo de dermatitis atópica en ratón, inducida por 2,4-dinitrofluorobenceno (DFBN). La administración peritoneal del RA disminuyó la producción de las citocinas IL-4 e IFN-γ, la infiltración de células inflamatorias como linfocitos y los niveles de IgE (Jang et al., 2011). Osakabe et al. 2004, evaluaron la actividad anti-carcinogénica de un extracto de *Perilla frutescens,* constituido en un 68 % de RA, en diferentes modelos de inflamación en piel. Desarrollaron un modelo tumoral inducido por TPA/DMBA, en el que observaron que el grupo de ratones tratados con el extracto, presentaba una menor incidencia de papilomas. Además, evaluaron dicho extracto en un modelo de inflamación en oreja inducida por TPA. Los resultados mostraron que el extracto inhibió la expresión de moléculas de adhesión, disminuyó el peso del edema y la producción de IL-8 así como los niveles de COX-2, PGE_2 y LTB_4, y la expresión de un marcador característico de la proliferación de queratinocitos (8OH-dG). En base a estos antecedentes, RA tiene gran potencial para el tratamiento de numerosas patologías, aunque su uso tiene algunos inconvenientes como

son su baja liposolubilidad, alta biotransformación (Baba et al., 2004), o reducida estabilidad por su rápida oxidación, ocasionada por la luz o el calor, dificultando, por tanto, su aplicación (Nunes et al., 2015). En este sentido, cada vez son más los autores que plantean alternativas para vehiculizar estos compuestos con el objetivo de proteger su estructura y/o aumentar su permeabilidad a través de la piel (Stelmakienė et al., 2015)(Ezzat et al., 2016).

OBJETIVOS

En base a la necesidad de encontrar nuevas alternativas terapéuticas en el tratamiento de la psoriasis, la queratosis actínica o el cáncer de piel, la quimioprevención con compuestos naturales está tomando un importante papel en los últimos años. Así, compuestos anti-inflamatorios capaces de reducir las manifestaciones clínicas de la psoriasis o de impedir el desarrollo frecuente de recidivas son excelentes alternativas complementarias a la terapéutica actual. Del mismo modo, la búsqueda de estrategias encaminadas a retrasar el fotoenvejecimiento de la piel para prevenir el desarrollo de lesiones pre-cancerosas presenta un gran interés en la actualidad. En los últimos años, las microalgas han emergido como fuente de compuestos bioactivos, incluyendo lípidos, proteínas, polisacáridos y carotenoides. Estos compuestos han atraído el interés de la industria farmacéutica y cosmética en base a sus actividades como antioxidantes, anti-inflamatorios y anti-carcinogénicos.

Por todo ello, el **objetivo general** de esta Tesis Doctoral es valorar la potencial actividad biológica (antioxidante, anti-inflamatoria y fotoprotectora) de compuestos lipídicos aislados de las microalgas *Isochrysis galbana* y *Micractinium pusillum* (glicolípidos y carotenoides principalmente) y del polifenol ácido rosmarínico (RA), así como evaluar su uso para el tratamiento de diversas patologías de la piel con base inflamatoria, en diversos modelos experimentales.

Los objetivos específicos planteados para esta Tesis Doctoral fueron los siguientes:

1. Evaluar la actividad anti-inflamatoria, anti-oxidante y foto-protectora de una serie de glicolípidos aislados de las microalgas *I. galbana* y *M. pusillum,* en un modelo *in vitro* de queratinocitos humanos HaCaT irradiados con UVB.

2. Desarrollar, en colaboración con el Departamento de Farmacia y Tecnología Farmacéutica de la Facultad de Farmacia de la US, fórmulas farmacéuticas óptimas para la aplicación tópica de una fracción de glicolípidos (MGDG), fucoxantina (FX) y ácido rosmarínico (RA).

3. Estudiar el efecto preventivo de un glicolípido puro (MGMG-A), así como de una fracción enriquecida en glicolípidos (MGDG) de *I. galbana*, tanto disuelta en acetona como formulada en crema, en un modelo experimental de hiperplasia epidérmica inducida por TPA en ratones.

4. Evaluar las propiedades farmacológicas de la FX, aislada de *I. galbana*, incorporada a una formulación en crema, en el modelo de hiperplasia epidérmica inducida con TPA en ratón, así como en un modelo de eritema cutáneo inducido por una exposición aguda a radiación UVB en ratón.

5. Estudiar los efectos anti-inflamatorios del RA en un modelo experimental de psoriasis inducida por imiquimod en ratón, empleando una formulación basada en nanoliposomas.

6. Evaluar los posibles mecanismos de acción anti-inflamatoria y anti-oxidante de la FX y del RA, así como una posible sinergia entre ambos, en queratinocitos humanos HaCaT irradiados con luz UVB.

OBJECTIVES

Given the necessity to find new therapeutics alternatives for psoriasis treatment, actinic keratosis or skin cancer, the chemoprevention with natural products is playing an important role in last years. Thus, anti-inflammatory compounds that reduce clinical manifestations of psoriasis or prevent the recurrences are excellent alternatives to complement the current treatments. Moreover, the use of therapeutic strategies for delaying the photoaging and preventing the development of pre-cancerous lesions is currently being investigated. In last years, the microalgae have emerged as source of bioactive compounds including, lipids, proteins, polysaccharides and carotenoids. These compounds have attracted the interest of pharmacy and cosmetic industry due to their antioxidant, anti-inflammatory and anti-carcinogenic activities.

For these reasons, the overall objective of this Doctoral Thesis is to assess the potential biological activity (antioxidant, anti-inflammatory and photoprotective) of lipid compounds isolated from the microalgae *Isochrysis galbana* and *Micractinium pusillum* (glycolipids and carotenoids) and the polyphenol rosmarinic acid (RA), as well as to evaluate their use for the treatment of inflammatory skin pathologies, in different experimental models.

This work has been focused on the following specific objectives:

1. To evaluate the anti-inflammatory, antioxidant and photoprotective effects from a variety of galactolipids isolated from the microalgae *M. pusillum* and *I. galbana*, using an *in vitro* model of UVB-irradiated HaCaT human keratinocytes.

2. To develop, in collaboration with the Department of Pharmacy and Pharmaceutical Technology of Faculty of Pharmacy of Seville, pharmaceutical formulations to improve the topical application of a fraction of glycolipids (MGDG), fucoxanthin (FX) and rosmarinic acid (RA).

3. To study the preventive effect of a pure glycolipid (MGMG-A), and an enriched fraction of glycolipids (MGDG) from *I. galbana*, such dissolved in acetone as formulated in cream, on the 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced epidermal hyperplasia in mice.

4. To evaluate the pharmacological properties of a FX, from *I.galbana*, loaded in a cream formulation, on the TPA-induced hyperplasia model in mice, as well as on the UVB-induced erythema model in hairless mice.

5. To study the anti-inflammatory effect of RA on an imiquimod-induced psoriasis-like model in mice, employing a formulation elaborated with nanoliposomes.

6. To evaluate the potential anti-inflammatory and antioxidant action mechanisms of FX and RA, as well as a possible synergy between them, on UVB-exposed HaCaT human keratinocytes.

CAPÍTULO I

GLYCOLIPIDS FROM *MICRACTINIUM PUSILLUM* AND *ISOCHRYSIS GALBANA* AND THEIR PHOTOPROTECTIVE EFFECT ON UVB-IRRADIATED HUMAN HACAT KERATINOCYTES.

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Abstract

Chronic exposure of the skin to ultraviolet B (UVB) radiation induces reactive oxygen species (ROS) production and inflammatory responses, which play a crucial role in actinic keratosis and skin cancer. Nowadays, strategies for skin protection comprise the use of natural compounds to counteract oxidative stress and inflammation. In this line, galactolipids have shown anti-inflammatory and anti-tumor properties in different skin models. This study was aimed to evaluate the anti-inflammatory effects on macrophages THP-1 and the protective mechanisms against UVB-induced cell damage in HaCaT human keratinocytes of a variety of glycolipids isolated from the microalgae *Micractinium pusillum* and *Isochrysis galbana*. The structures of the isolated monogalactosylmonoacylglycerols (MGMG), and digalactosyldiacylglycerols (DGDG) were defined by spectroscopic analyses (NMR, HRMS, and MS/MS). TNF-α production was evaluated on LPS-stimulated THP-1 macrophages. LDH enzyme activity, intracellular ROS levels and IL-6 production were measured on UVB-irradiated HaCaT cells. Seven MGMGs and six DGDGs displaying a variety of C16 and C18 acyl chains were obtained from the microalgae and subjected to bioactivity assays. Most of these compounds significantly reduced TNF-α levels in LPS-stimulated THP-1 cells. Pre-treatment of HaCaT keratinocytes with pure MGMGs and DGDGs and fractions containing MGDGs or DGDGs markedly attenuated UVB-induced cell damage through reduction of LDH release, ROS levels and IL-6 production. The present study confirms the anti-inflammatory properties of a variety of glycolipids in macrophages and reports for the first time the photoprotective activity of these compounds in HaCaT keratinocytes, suggesting their potential use as novel agents for the prevention of sun exposure-induced skin damage.

Keywords: microalgae; HaCaT; UVB; glycolipids; MGMG; DGDG.

Abbreviations: MGMG, monogalactosylmonoacylglycerol; MGDG, monogalactosyldiacylglycerol DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; UV, ultraviolet radiation; LDH, lactate dehydrogenase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha; LPS lipopolysaccharide; PMA, Phorbol 12-myristate 13-acetate; IL-6, Interleukin 6; DCF-DA, dichlorofluorescein diacetate.

Introduction

Skin is considered the largest organ in the body and represents the first barrier that protects us from the deleterious effects of solar ultraviolet (UV) radiation, which is the main cause for skin cancer (Ramos-e-Silva and Jacques, 2012)). Development of skin cancer depends on the cumulative amount and form of the UV radiation, as well as on the skin type of the individual exposed. Solar UV radiation consists of UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm). Compared with UVA, UVB is less abundant but is more energetic causing dermal change besides affecting epidermal function (Rass and Reichrath, 2008). Direct absorption of UVB to the epidermis may induce damage in epidermal keratinocytes by the production of pro-inflammatory cytokines, chemokines, prostaglandins and reactive oxygen species (ROS) (Divya et al., 2015). An imbalance between the cellular production of ROS and antioxidant defence mechanisms may be defined as oxidative stress (Kim, 2016). Oxidative stress produces oxidative damage of DNA, proteins and lipids, contributing to photoaging and many degenerative processes as actinic keratosis (AK). AK is a common skin lesion associated with long-term exposure to UV radiation, which is characterized by dysplastic keratinocyte lesions confined to the basal epidermal layer of the skin. If untreated, up to 10% of AKs can undergo malignant transformation and progress to squamous cell carcinoma (SCC) (Goldenberg and Perl, 2014). Since sunscreens can provide an insufficient photoprotection, the use of antioxidant and anti-inflammatory compounds incorporated to sunscreen formulations may be a useful strategy for a higher prevention of skin photodamage (Kuo et al., 2015). Nevertheless, nowadays the composition of sunscreens and cosmetic products is highly regulated due to the alarm generated by the environmental risk of organic UV filters (Sobek et al., 2013). The high consumption worldwide of UV filters is leading to their elevated detection in various environmental places as sea water, wastewater, tap water, sediments and biota, among others (Li et al., 2007; Kameda et al., 2011; Li et al., 2017).

Marine algae have been reported to be an interesting natural source of bioactive compounds, including fatty acids, carotenoids, proteins,

polysaccharides, and glycolipids, among others (Lordan et al., 2011). With regard to glycolipids, the major glycolipids in algae, as in other photosynthetic organisms, are the galactolipids (Gurr et al., 2002). The two main classes of galactolipids are the monogalactosyldiacylglycerols (MGDGs) and the digalactosyldiacylglycerols (DGDGs). The MGDGs consists of a D-galactopyranose linked to the *sn*-3 position of a 1,2-diacylglycerol, while the DGDGs contain two galactose units bonded to the 1,2-diacylglycerol. In addition, some studies have described the isolation of minor amounts of monogalactosylmonoacylglycerols (MGMGs) (13,14) which are formed by deacylation of MGDGs. Another class of glycolipids is formed by the sulfoquinovosyldiacylglycerols (SQDGs), which contain a 6-sulfoquinovose (6-deoxy-6-sulfo-D-glucopyranose) linked to the *sn*-3 position of a 1,2-diacylglycerol (Gurr, M.I., Harwood, J.L., Frayn, 2002). Within an organism each glycolipid class, MGDG, DGDG, and SQDG, include an array of compounds which differ among them by the length and/or the unsaturation of the acyl chains on the glycerol moiety. It has been previously reported that several galactolipids have *in vitro* and *in vivo* anti-inflammatory and antitumoral activities in different skin experimental models (Colombo et al., 2000; Bruno et al., 2005). Thus, topical pre-treatment with mixtures of MGDGs, DGDGs or SQDGs obtained from a blue-green alga inhibited the croton-oil-induced ear edema as well as the carrageenan-induced paw edema, with MGDGs being the most potent anti-inflammatory glycolipids (Bruno et al., 2005). Furthermore, we have recently tested the anti-inflammatory activity of galactosylglycerides and galactosylceramides isolated from *Isochrysis galbana*, which showing significant activity as anti-inflammatory agents inhibiting the production of the pro-inflammatory cytokine TNF-α in lipopolysaccharide-stimulated human THP-1 macrophages (De los Reyes et al., 2016). Therefore, more research is necessary to demonstrate the anti-inflammatory/protective effects of galactolipids from *I. galbana*. The aim of the present study was to evaluate the anti-inflammatory effects in THP-1 macrophages, as preliminary screening, and the protective mechanisms against UVB-induced cell damage in HaCaT human keratinocytes of a variety of galactolipids isolated from the microalgae *Micractinium pusillum* and *Isochrysis galbana*.

Methods

Microalgal biomass

Dried biomass derived from cultures of the microalgae *M. pusillum* and *I. galbana* was provided by the Department of Biotechnology of the

Instituto Tecnológico de Canarias (Santa Lucía, Gran Canaria, Canary Islands, Spain). Stocks of *M. pusillum* and *I. galbana* are maintained at the Microalgae Collection of the Instituto Tecnológico de Canarias under the codes ITC-10 and ITC-Iso-01, respectively.

Extraction and isolation of glycolipids

The dried biomass of *M. pusillum* (40 g) was extracted with acetone-methanol (MeOH) (1:1, v/v, 3L) at room temperature. After filtration, the solvent was evaporated under reduced pressure and the resulting extract (8.5 g) was subjected to column chromatography (30 x 5.5 cm) on silica gel (Merck, 63-200 □m) using as eluents *n*-hexane/diethyl ether (1:1, v/v, 2 L), diethyl ether (1.5 L), $CHCl_3$/MeOH (9:1, v/v, 1.5 L), $CHCl_3$/MeOH (8:2, v/v, 1.5 L), and MeOH (2 L). The fraction eluted with $CHCl_3$/MeOH (8:2, v/v), namely fraction C (1.1 g), was separated over C18 SPE cartridges (Supelco DSC18, 5 x 1 g) previously conditioned with MeOH/H_2O (90:10, v/v, 2 mL) using as eluents mixtures of MeOH/H_2O (90:10, 95:5, and 99:1, v/v, 15 mL each). The resulting fractions, C_1, C_2, and C_3, were repeatedly separated by reversed phase HPLC using as eluents MeOH/H_2O 93:7, 90:10, and 95:5, v/v, respectively, to obtain four pure MGMGs and six DGDGs. The HPLC separations were performed on a LaChrom-Hitachi apparatus equipped with LiChrospher 100 RP-18 (Merck, 250 x 10 mm, 10 □m) and Synergi Fusion-RP (Phenomenex, 250 x 4.6 mm, 5 □m) columns, using a differential refractometer RI-71.

The extraction of the dried biomass of *I. galbana* with acetone/MeOH and the separation of the extract to obtain the fractions containing glycolipids has already been described (De los Reyes et al., 2016). In brief, the lipid extract was subjected to silica gel column chromatography and the fraction eluted with $CHCl_3$/MeOH 8:2, v/v (fraction C, 3.0 g) was further separated over a silica gel column to obtain fractions C_1–C_6. The fractions C_2 and C_4 were purified on C18 SPE cartridges to yield the fractions I-MGDG (788 mg), I-DGDG (113 mg), respectively (De los Reyes et al., 2016). During the separation of fraction C_3 (420 mg) by C18 SPE cartridges, the elution with MeOH/H_2O (90:10, v/v) yielded a mixture of MGMGs (224 mg). This mixture was repeatedly separated by reversed phase HPLC using MeOH/H_2O, 98:2, v/v, to obtain three pure MGMGs. An aliquot of the fraction C_6 (228 mg) was purified by C18 SPE cartridges eluted with MeOH to yield the fraction I-SQDG (176 mg).

Structural determination of glycolipids

The glycolipids isolated from *M. pusillum* and *I. galbana* were subjected to spectroscopic analyses (NMR, HRMS, and MS/MS). NMR spectra were recorded on an Agilent-500 spectrometer using CD_3OD or $CDCl_3$ as solvent. Chemical shifts were referenced using the corresponding solvent signals [δ_H 3.30 and δ_C 49.0 for CD_3OD, δ_H 7.26 and δ_C 77.0 for $CDCl_3$]. COSY, HSQC, and HMBC experiments were performed using standard Agilent pulse sequences. HRMS were performed on a SYNAPT 2G (Waters) spectrometer equipped with an electrospray interface (ESI) and a quadruple-time-of-flight (QTOF) analyser. UPLC-MS analyses were performed by using an Acquity UPLC H-CLASS system coupled to a HRMS SYNAPT 2G (Waters).

Cell culture

The THP-1 human monocytic leukemia cell line and HaCaT human keratinocytes were obtained from the American Type Culture Collection (ATCC, USA). THP-1 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. HaCaT keratinocytes were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Both cell lines were grown in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cell viability assay

Viability of THP-1 macrophages and HaCaT cells upon exposure to pure glycolipids or fractions was determined by the sulforhodamine B (SRB) assay (Skehan et al., 1990). SRB is a microplate colorimetric endpoint assay that counts the viable cells by staining their cellular protein content. In brief, for differentiation into macrophages, THP-1 cells (10^4 cells/well) were incubated with Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO) (0.2 µM) (for 3 days in 96-well plates and HaCaT cells were seeded into 96-well plates in the growth medium at 10^4 cells/well. The compounds were dissolved by 1% DMSO in RPMI 1640 or DMEM medium and were added to the cells at the final concentrations range of 10-100 µM per well for pure compounds and 10-100 µg/mL for fractions. After 24, 48 and 72 h of incubation, the cells were fixed with 50 µL of 50% trichloroacetic acid at 4°C for 1 h and washed with distilled water. Then, the cells were stained with 50 µl of 0.4% SRB solution in 1% acetic acid for 30 min at room temperature. After incubation, the SRB solution was poured off and the plates were washed with 1% acetic acid. The plates were air-dried and 100 µl of 10 mM Tris-base solution was added into each well to solubilize the cell-

bound dye. The absorbance at 492 nm was determined in a microplate spectrophotometer (Sinergy HT, Biotek®, Bad Friedrichshall, Germany).

Determination of TNF-α production

Monocytes were differentiated in 96-well plates (0,2 µM PMA, 72 h) at 10^4 cells/well. Then, the medium was removed, the cells were washed with phosphate buffered saline (PBS, 4°C), and incubated for 1h with pure compounds (10, 30 and 50 µM). Dexamethasone (1 µM) was used as positive reference compound. All the solutions were prepared by dilution of stock solutions in DMSO with the appropriate amounts of fresh medium. The highest concentration of each treatment used in the assay contained DMSO 0.1% (v/v). Subsequently, the inflammatory response was induced by addition of lipopolysaccharide (LPS, 1 µg/mL). Controls contained medium with equivalent amounts of solvent compared to treatments, and were incubated with and without LPS. After 24 h incubation, supernatant fluids were collected and stored at -80°C until tumor necrosis factor alpha (TNF-α) measurement. Commercial enzyme-linked immunosorbent assay (ELISA) kit (Diaclone GEN-PROBE, France) was used to quantify TNF-α according to the manufacturer's protocol.

UVB irradiation of HaCaT Keratinocytes

HaCaT keratinocytes were exposed to UVB radiation, as previously described (Hou et al., 2015). Briefly, the cells were grown to confluence prior to UVB irradiation. The cells were treated with different concentrations of pure compounds (10, 30 and 50 µM) and fractions (10, 30 and 50 µg/mL) for 1 hour before UVB exposition. Then the culture medium was replaced with PBS, and exposed to a single dose of UVB radiation at 50 mJ/cm^2 for 1 min. The UVB source was a CL-1000M UV Crosslinker (UVP, Upland, CA, USA), which was used to deliver an energy spectrum of UVB light (280-315 nm; peak intensity, 302 nm). After UVB irradiation, the cells were supplied with fresh culture medium and incubated for 24 hours.

Analysis of intracellular LDH activity

One method of assaying loss of cellular membrane integrity is monitoring the release of cytosolic enzyme lactate dehydrogenase (LDH) into the culture medium (Verhulst et al., 1998). Cells were seeded at a density of $5x10^5$ cells/well in 6-well plates. After being incubated for 24 h, cells were treated with pure compounds (10, 30 and 50 µM) and fractions (10, 30 and 50 µg/mL) for 1 h and then, were exposed to 50 mJ/cm^2 of UVB. After 24 h, the culture medium was collected and

centrifuged at 1,500 rpm for 5 min in order to obtain a cell-free supernatant. Then, the cells were scraped in cold PBS and sonicated for 10 min to ensure the cell membrane broke down to release the total amount of LDH. Finally, cell lysates were centrifuged at 1,500 rpm for 5 min. The assay is based on the conversion of lactate to pyruvate in the presence of LDH with the concomitant oxidation of NADH. The formation of NAD^+ from the above reaction results in a change in absorbance at 340 nm. Aliquots of medium, cell lysates and reagents were mixed in a 96-well plate and the absorbance was registered using a microplate spectrophotometer system (Sinergy HT, Biotek®, Bad Friedrichshall, Germany). LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content and results were represented as the percentage (%) of change in activity compared with the untreated control cells.

Intracellular ROS scavenging activity

The dichlorofluorescein diacetate (DCF-DA) assay was used to detect intracellular ROS levels in HaCaT keratinocytes (Wang and Joseph, 1999). For detection of ROS in UVB-treated cells, cells were seeded at a density of $1x10^4$ cells/well in 96-well plates. A non-irradiated plate was seeded at the same concentration of cells to use it as a negative control. Twenty-four hours after plating, cells were treated with pure compounds (10, 30 and 50 µM) and fractions (10, 30 and 50 µg/mL). After 1 h, cells were exposed to 50 mJ/cm^2 of UVB, and incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) solution (5 mg/mL) in PBS for 30 min (Hyun et al., 2013). Then, the medium was discarded and the cells were washed with PBS. The fluorescence of the 2',7'-dichlorofluorescein (DCF) product was determined using a fluorescence plate reader (Sinergy HT, Biotek®, Bad Friedrichshall, Germany) at 485 nm for excitation and 535 nm for emission.

Determination of IL-6 production

HaCaT cells ($5x10^5$ cells/well) were seeded in 6-well plates. After 24 h, cells were treated with the different concentrations of pure compounds (10, 30 and 50 µM) and fractions (10, 30 and 50 µg/mL) for 1 h and then, were exposed to 50 mJ/cm^2 of UVB. After being incubated for 24 h, supernatant fluids were collected and stored at -80 °C until IL-6 measurements. Controls contained medium with equivalent amounts of solvent compared to treatments, and were incubated with and without UVB radiation. Commercial enzyme-linked immunosorbent assay (ELISA) kits (Diaclone GEN-PROBE, France) were used to quantify IL-6 according to the

manufacturer's protocol. The absorbance, at 450 nm, was read by a microplate reader. To calculate the concentration of IL-6 (pg/mL), a standard curve was constructed using serial dilutions of cytokine standards provided with the kit.

Statistical analysis

All values in the figures, tables and text are expressed as arithmetic means ± standard error of the mean (S.E.M.). Data were evaluated with GraphPad Prism® Version 5.00 software (SanDiego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni test. P values of <0.05 were considered statistically significant.

Results

Isolation of glycolipids from the biomass of the microalgae *M. pusillum* and *I. galbana*

The extraction of each microalgal biomass with acetone/methanol (1:1, v/v) followed by silica gel column chromatography led to obtain the glycolipids in the fractions eluted with CHCl3/MeOH. Further separation of the glycolipid fraction of *M. pusillum* by SPE and HPLC allowed the isolation of the MGMGs 1 (246.6 mg), 2 (40.8 mg), 3 (10.1 mg), and 4 (20.0 mg), as well as the DGDG 5 (180.7 mg), an inseparable mixture of 6 and 7 (68.2 mg) and the DGDGs 8 (40.5 mg), 9 (31.8 mg), and 10 (29.2 mg).

The separation of the glycolipid fraction of *I. galbana* by silica gel column chromatography led to the obtention of fractions enriched in different types of glycolipids, as determined by thin layer chromatography and NMR. The purification of selected fractions by SPE cartridges allowed the obtention of fractions containing MGDGs (fraction I-MGDG), DGDGs (fraction I-DGDG) (De los Reyes et al., 2016), SQDGs (fraction I-SQDG) and MGMGs. Further separation of this fraction by HPLC led to the isolation of the compounds 4 (7.4 mg), 11 (62.2 mg), 12 (38.6 mg), and 13 (20.2 mg).

Structure determination of glycolipids

The structures determined for the MGMGs 1-4 and 11-13 isolated from *M. pusillum* and *I. galbana*, respectively, are shown in Table 1. The isolated MGMGs contain C16 and C18 acyl chains. The spectroscopic data obtained for compounds 1 (Kwon et al., 1998), 2 (Banskota et al., 2013),

4 (Rho et al., 1996), 11 (Hiraga et al., 2008), and 13 (Kwon et al., 1998) matched those described in the literature. To the best of our knowledge, this is the first report of the isolation and spectroscopic data (supplementary material) of the MGMGs 3 and 12. The structures determined for the DGDGs 5-10 isolated from *M. pusillum* are shown in Table 2. The isolated DGDGs contain C16 and C18 acyl chains. The spectroscopic data obtained from compounds 5 (Morimoto et al., 1995), 8 (Morimoto et al., 1995) and 9 (Reshef et al., 1997) matched those described in the literature. To the best of our knowledge, this is the first report of the isolation and spectroscopic data (supplementary material) of the MGMGs 6/7 and 10. The fractions I-MGDG and I-DGDG were mixtures of MGDGs and DGDGs, respectively, displaying C14, C16, and C18 acyl chains as previously described (De los Reyes et al., 2016).

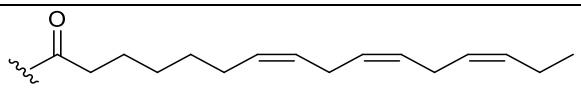
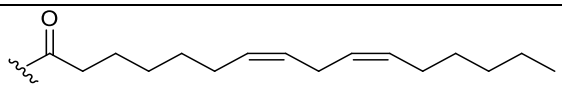
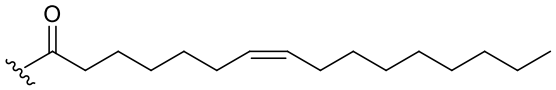
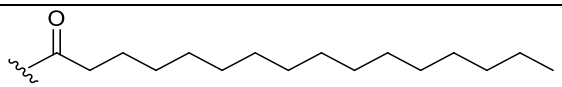
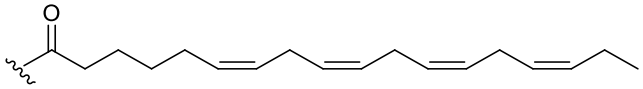
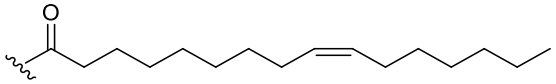
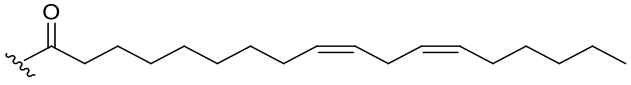
MGMG	R
1	
2	
3	
4	
11	
12	
13	

Table 1. Chemical structures of the MGMGs isolated from the biomass of the microalgae *M. pusillum* (1-4) and *I. galbana* (4, and 11-13).

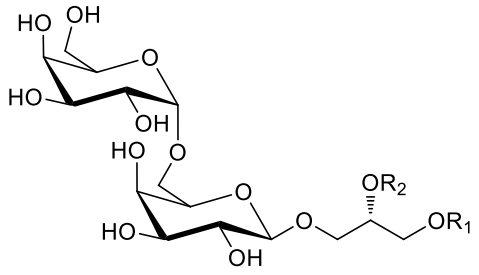
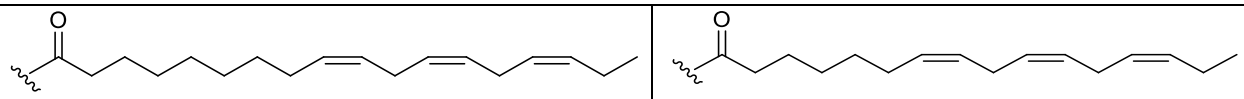
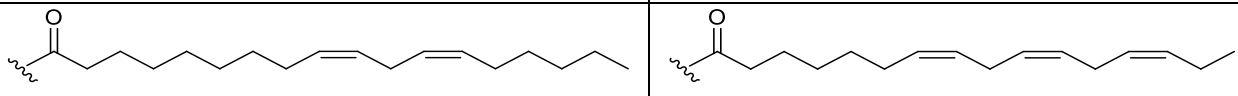
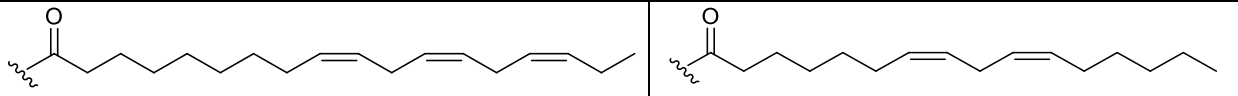
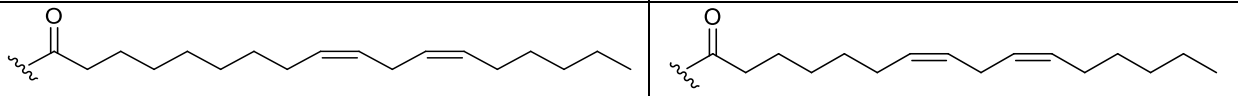
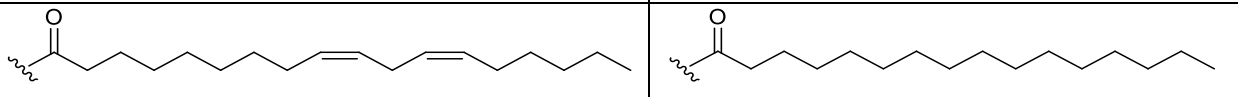
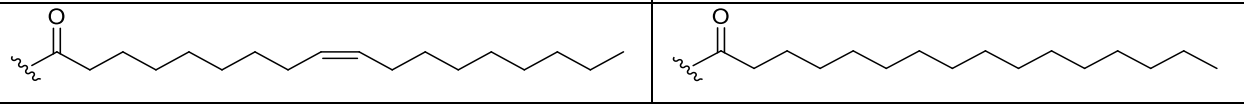
DGDG	
	R_1 R_2
5	
6	
7	
8	
9	
10	

Table 2. Chemical structures of the DGDGs 5-10 isolated from the biomass of *M. pusillum*.

Effect of glycolipids on cell viability

The effect of glycolipids on THP-1 macrophages and HaCaT cells viability was measured by using the SRB assay. Results from cytotoxicity study showed that none of the tested concentrations of the pure compounds (10 µM to 100 µM) and fractions (10 µg/mL to 100 µg/mL) affected cell viability. The inhibitory concentration 50 (IC_{50}) (half maximal inhibitory concentration) was above 100 µM (compounds) or 100 µg/mL (fractions) at 24, 48 and 72 h after treatment (data not shown).

Effect of glycolipids on TNF-α production

We have studied the activity of compounds 1-13 as inhibitors of the pro-inflammatory cytokine TNF-α Assays were performed on human THP-1 macrophages using LPS to stimulate the production of TNF-α. As shown in Fig. 1, LPS stimulation significantly increased TNF-α levels in THP-1 macrophages in comparison with unstimulated control cells ($P < 0.001$). In particular, pretreatment of cells with the MGMGs 2, 4, 11 and 12 at 50 µM significantly decreased LPS-induced TNF-α production by 40%, 47%, 57% and 45%, respectively, upon comparison with LPS-stimulated untreated THP-1 cells (Fig. 1A). Pure DGDGs 5, 6/7, 8, 9 and 10 were more active, causing at 50 µM inhibition of this cytokine levels by 60%, 55%, 59%, 63% and 59%, respectively ($P < 0.001$) (Fig. 1B). In addition, the fraction I-SQDG at 50 µg/mL reduced significantly the LPS-induced TNF-α production (Fig. 1C).

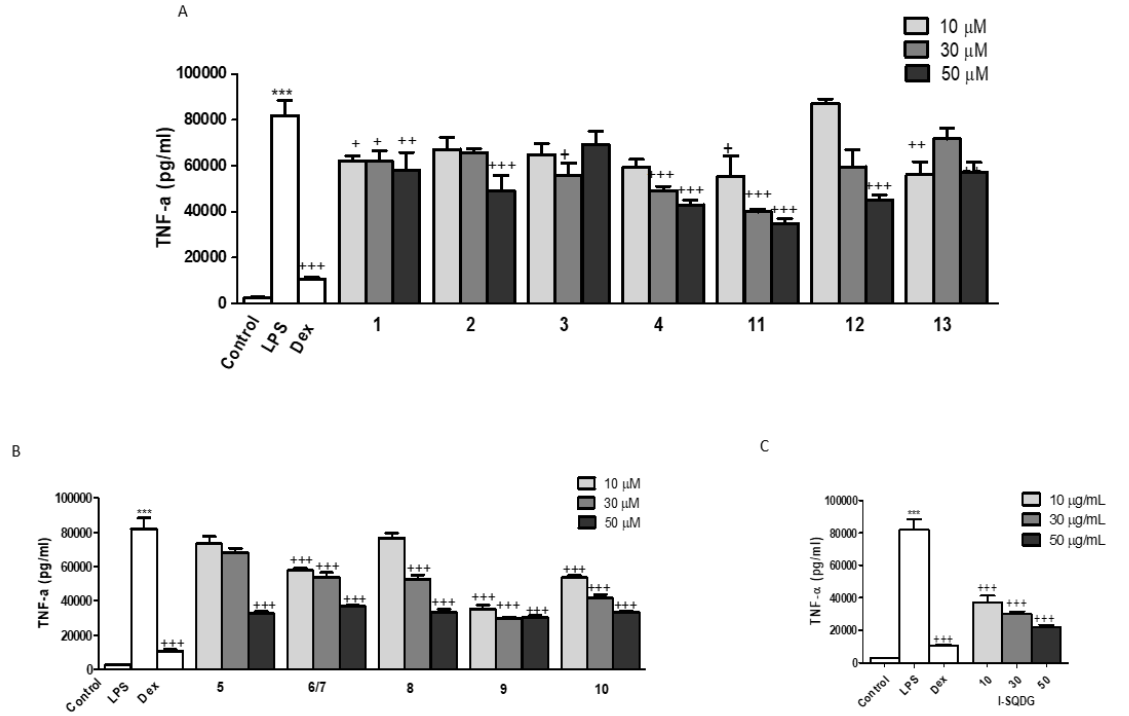


Figure 1. Effect of galactolipids 1-13 on TNF-α production in LPS-stimulated THP-1 macrophages. Cells were pretreated with monogalactosylmonoacylglycerols (MGMGs) 1-4 and 11-13 and digalactosyldiacylglycerols (DGDG) 5-10 at 10, 30 and 50 µM for 1 h, and then stimulated with LPS (1□g/mL). After incubation for 24 h, TNF-α levels were measured using ELISA assay. Dexamethasone (Dex) was used as positive reference compound at 1 µM. Data are means ± SEM from four independent experiments. ***$P < 0.001$ *vs.* untreated cells; + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$ *vs.* LPS-stimulated control.

Effect of glycolipids on intracellular LDH activity

The effect of glycolipids on intracellular enzymatic activity was examined by measuring the LDH enzyme activity in fresh cell lysates and supernatants 24 h after exposure of the cell cultures to UVB radiation. As expected, UVB irradiation to 50 mJ/cm^2 significantly increased LDH activity in HaCaT keratinocytes compared to unirradiated control ($P < 0.001$) (Fig. 2A). Pretreatment of cells with MGMGs 1-4 and 11-13 1 h prior to UVB exposure significantly decreased UVB-induced mortality, preserving cell membrane integrity, at all concentrations tested (10, 30 and 50 µM, $P < 0.001$) (Fig. 2A). Similar results were exhibited with keratinocytes pretreated with DGDGs 5-10 for 1 h (Fig. 2B). The study with fractions containing MGDGs, DGDGs, and SQDGs also showed a significant reduction on LDH enzyme activity ($P < 0.001$) (Fig. 2C).

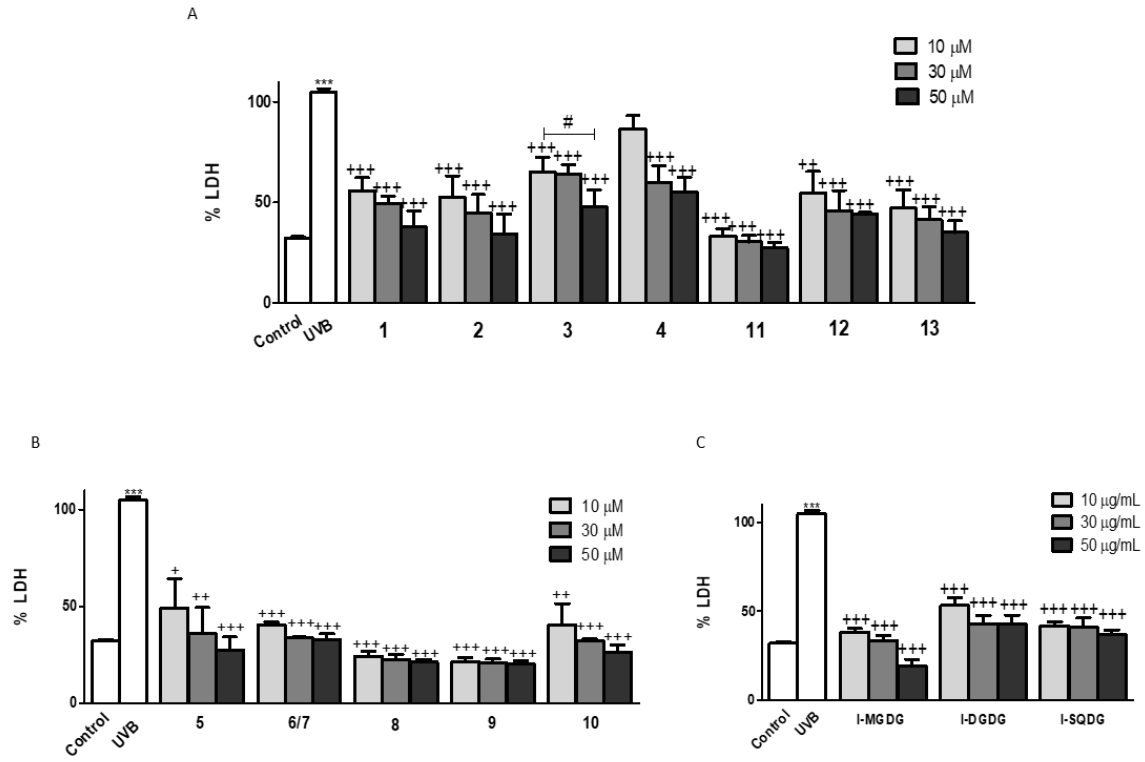


Figure 2. Effect of glycolipids from microalgae on UVB-induced LDH leakage in human HaCaT keratinocytes. Cells were pretreated with monogalactosylmonoacylglycerols (MGMGs) 1-4, and 11-13 (10, 30 and 50 µM) (A), digalactosyldiacylglycerols (DGDGs) 5-10 (10, 30 and 50 µM) (B) and glycolipid-containing fractions I-MGDG, I-DGDG, and I-SQDG (12.5, 25 and 50 µg/mL) (C) for 1 h, and then exposed to UVB light (50 mJ/cm2). After incubation for 24 h, cell viability was assessed by using LDH assay. Results are expressed as percentage respect to untreated control cells and bars represents mean ± SEM of four independent experiments (n = 4). *** $P < 0.001$ *vs.* untreated cells; + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$ *vs.* UVB-irradiated cells.

Effect of glycolipids on UVB-induced ROS production

We cultured HaCaT cells with the compounds for 1 h followed by UVB exposure and evaluated intracellular ROS levels based on the DCF-DA assay (Fig. 3). A significant increase in ROS content was observed in UVB-irradiated cells in relation to untreated control cells (without UVB irradiation) ($P < 0.001$). The MGMGs 1, 2 and 4 markedly reduced ROS levels by 42%, 34% and 37%, respectively, at the highest concentration tested ($P < 0.001$) (Fig. 3A). At the same concentration, compounds 3 and 11 were less active, causing 19% and 22%, respectively, of decrease of ROS production. As regards DGDGs, pre-treatment with 5, 6/7 and 9 at the concentration of 50 µM led to 25%, 22% and 18% of inhibition of ROS accumulation, respectively, in UVB-irradiated cells ($P < 0.001$, $P < 0.01$, $P < 0.05$, respectively) (Fig. 3B). However, only the fraction I-MGDG was able to reduce intracellular ROS production by 26% ($P < 0.05$) (Fig. 3C).

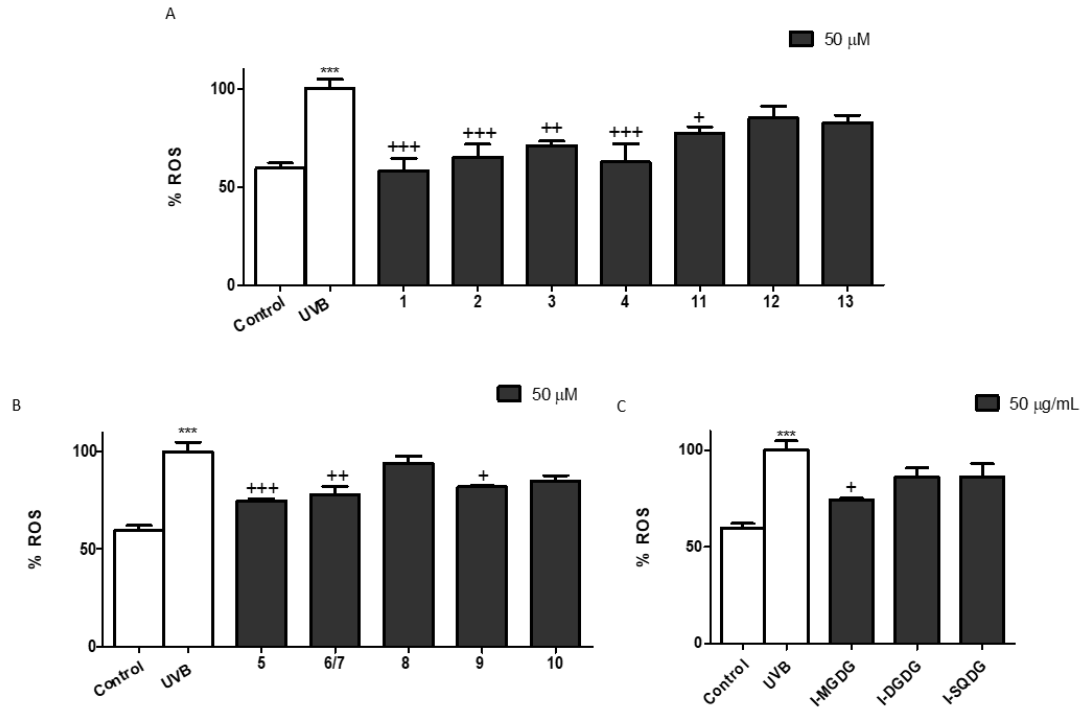


Figure 3. Effect of glycolipids from microalgae on UVB-induced ROS production in human HaCaT keratinocytes. HaCaT cells were pretreated with monogalactosylmonoacylglycerol (MGMGs) 1-4 and 11-13 (50 µM) (A), digalactosyldiacylglycerola (DGDG) 5-10 (50 µM) (B) and glycolipid-containing fractions I-MGDG, I-DGDG, and I-SQDG (50 µg/mL) (C) for 1 h, and then irradiated with UVB (50 mJ/cm2). ROS levels were estimated indirectly by measuring the fluorescence emitted by dichlorofluoresceine formed in proportion to the intracellular ROS, 30 min after UVB irradiation. Results are expressed as percentage respect to untreated control cells and bars represents mean ± SEM of four independent experiments (n = 4). ** $P < 0.01$ *vs.* untreated cells; + $P < 0.05$ and ++ $P < 0.01$ *vs.* UVB-irradiated cells.

Effect of glycolipids on IL-6 production

UVB exposure induces abnormally increased cytokine release from keratinocytes leading to inflammatory skin disorders. To evaluate the effects of the isolated glycolipids on the UVB-induced production of the pro-inflammatory cytokine IL-6, HaCaT cells were pre-treated with pure compounds and fractions for 1 h, and then were exposed to UVB (50 mJ/cm^2). UVB-irradiated HaCaT cells manifested high IL-6 levels in comparison with unirradiated control ($P < 0.001$) (Fig. 4A). Pre-treatment with the MGMGs 11, 12 and 13 substantially inhibited this cytokine levels by 86%, 85% and 66% at 50 µM, respectively. At this concentration, the compounds 1 and 2 exhibited less activity, causing 61% and 54% of inhibition, respectively (Fig. 4A). In relation to DGDGs, IL-6 production was only reduced after treatment with 8, 9 and 10 at all the concentrations tested. It is worth mentioning that compound 10 was the most active, reducing this cytokine levels by 66%, 88% and 88%, at 10, 30 and 50 µM, respectively (Fig. 4B). As regards fractions, I-MGDG caused the greatest inhibition of IL-6 production, which was decreased by 70%, 90%, and 99%, at 10, 30 and 50 µg/mL, respectively. Fraction I-DGDG also showed significant activity at the three concentrations tested, reducing IL-6 levels by 55%, 56% and 58%, respectively (Fig. 4C).

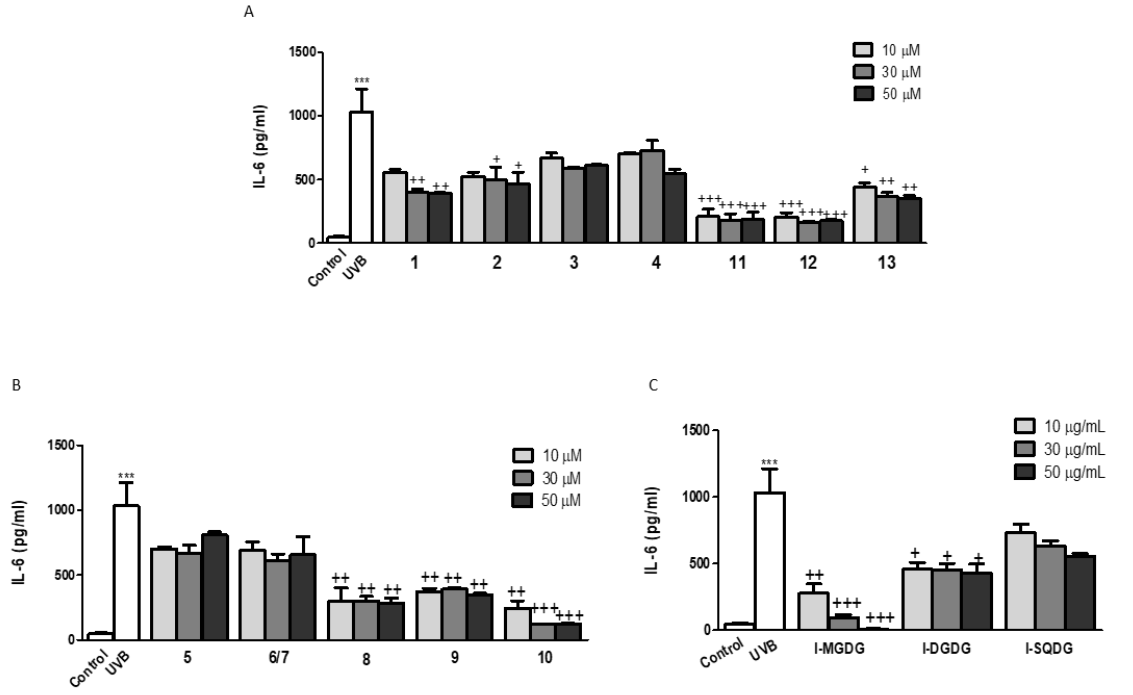


Figure 4. Effects of glycolipids on UVB-induced production of IL-6 in human HaCaT keratinocytes. HaCaT cells were pretreated with monogalactosylmonoacylglycerols (MGMGs) 1-4, and 11-13 (10, 30 and 50 µM) (A), digalactosyldiacylglycerols (DGDGs) 5-10 (10, 30 and 50 µM) (B) and glycolipid-containing fractions I-MGDG, I-DGDG, and I-SQDG (10, 30 and 50 µg/mL) (C) for 1 h, and then irradiated with UVB (50 mJ/cm^2). After incubation for 24 h, IL-6 levels were measured by ELISA kit. Data are means ± SEM from four independent

experiments (n = 4). *** P < 0.001 vs. untreated cells; + P < 0.05, ++ P < 0.01 and +++ P < 0.001 vs. UVB-irradiated cells.

Discussion

The skin is the main organ that participates in temperature maintenance of the body and offers protection from chemicals, microorganisms and UV radiation. Solar radiation has positive effects as mediator of vitamin D in the skin, improvement of endorphins and reduction of blood pressure (Pérez-Sánchez et al., 2016). However, an excessive UV exposition can lead to several skin pathological disorders, including erythema, sunburn, hyperpigmentation, immunosuppression, edema, premature aging and non-melanoma skin cancer (Li et al., 2016). Previous papers have reported the photoprotective properties of different molecules from natural origin through antioxidant and anti-inflammatory mechanisms (El-mahdy et al., 2009; Radice et al., 2016),. Therefore, treatment of the skin with oral or topical formulations containing these natural compounds may be a useful strategy for preventing UV-induced skin damage and ultimately skin cancer (Hu et al., 2016). Moreover, the use of natural products is advantageous since may be an alternative for reducing the pollution of marine waters (Sánchez-Quiles and Tovar-Sánchez, 2015).

Microalgae have been shown to be a rich source of bioactive molecules, including polar lipids such as the galactolipids. Lipid extracts and pure galactolipids from microalgae have previously demonstrated anti-inflammatory (Banskota et al., 2014; Robertson et al., 2015), and antitumor promoting (Morimoto et al., 1995) properties, which make them suitable candidates for further investigation. In our study, we have demonstrated that a variety of glycolipids of the MGMG, MGDG and DGDG classes can protect HaCaT keratinocytes against acute UVB radiation due to the inhibition of ROS generation and the decrease of the production of the pro-inflammatory cytokine IL-6, suggesting that these molecules could play an important role in protecting the skin exposed to UVB.

In a first experiment, we tried to confirm the anti-inflammatory potential of the compounds under study in the *in vitro* model of LPS-stimulated human THP-1 macrophages and TNF-α production. TNF-α is a potent pro-inflammatory cytokine whose dysregulation is associated with a variety of chronic inflammatory and autoimmune diseases, such as psoriatic arthritis, actinic keratosis, atopic dermatitis and skin cancer (Udommethaporn et al., 2016). We have recently demonstrated that

fractions containing MGDGs or DGDGs and pure DGDGs significantly inhibited TNF-α production in the LPS/THP-1 model (De los Reyes et al., 2016). Consistent with those results, in the present study we have confirmed the anti-inflammatory potential of the MGMGs 2, 4, 11 and 12 and of the DGDGs 5-10, which caused a marked reduction of TNF-α levels in LPS-stimulated THP-1 cells. Our results are also in line with a previous paper that showed that the MGDG containing two linolenoyl acyl chains isolated from Rose hip (*Rosa canina*) downregulated the expression of pro-inflammatory cytokines in macrophages and peripheral blood leukocytes and inhibited the inflammatory process in primary chondrocytes by reducing matrix metalloproteinases levels (Schwager et al., 2011). Moreover, in recent years several microalgal galactolipids, including MGMGs, MGDGs, and DGDGS with C14, C16, C18, and C20 acyl chains, have been shown to decrease the production of the inflammatory mediator NO in RAW264.7 macrophages (Banskota et al., 2014). In relation to *in vivo* studies, Bruno et al. (2015) demonstrated that fractions containing MGDGs, DGDGs, or SQDGs obtained from a blue green alga reduced the inflammation in croton-oil-induced ear edema and carrageenan-induced paw edema models.

Next, we aimed to study the possible protective effects of glycolipids in HaCaT keratinocytes, a well-established *in vitro* model for investigations on UV radiation-induced cell damage. UVB exposure promotes cell cytotoxicity by loss of cellular membrane integrity, which leads to liberation of LDH enzyme from cytosol to the culture medium. The cell membrane damage upon UVB exposure is correlated with increased ROS production. It has been reported that excessive generation of ROS results in oxidative stress in skin cells and plays an essential role in the initiation, promotion and progression of skin aging, carcinogenesis and many inflammatory disorders (Fehér et al., 2016). In the present study, pre-treatment of HaCaT keratinocytes with pure MGMGs and DGDGs and with fractions containing MGDGs or DGDGs significantly attenuated UVB-induced skin damage by increasing cell viability, as demonstrated by reduced LDH release, and inhibiting ROS levels. These results are in line with a previous report that showed that glycolipid extracts from spinach, with a high content in MGDGs and DGDGs, attenuated the PMA-induced ROS production in Caco-2 cells, the inhibitory effect of DGDG being much potent than that of MGDG (Shiota et al., 2010). In a similar way, two bacterial MGDGs exhibited protective activity against oxidative stress induced by alkyl peroxyl radicals (Matsufuji et al., 2000b), exogenous H_2O_2 or heat (Matsufuji et al., 2000a). In addition, a crude sulfoglycolipidic fraction from the microalga *Porphyridium*

cruentum, rich in long-chain PUFAs, inhibited superoxide anion generation in primed neutrophils (Bergé et al., 2002).

UVB irradiation of the skin also induces a robust inflammatory response, characterized by the production of numerous inflammatory mediators, such as chemokines and cytokines in epidermis, which in turn further elevate ROS production and increase oxidative stress (Decean et al., 2013). Excessive production of these factors leads to hyperpigmentation, erythema, photo-aging as well as chronic inflammation, which in turn could raise the risk of skin carcinogenesis (Aoki-Yoshida et al., 2013). It has been previously reported an up-regulation of the pro-inflammatory cytokine IL-6 in human and rat skin (Dawes et al., 2014), and human HaCaT keratinocytes after UVB-induced inflammation (Borland et al., 2011). Our results showed that glycolipids presented in this study inhibited IL-6 production in UVB-irradiated HaCaT cells. In a similar way, treatment of human articular chondrocytes with the MGDG fraction of the microalga *Phormidium sp*. ETS-05 suppressed this cytokine expression induced by IL-1α and TNF-α (Ulivi et al., 2011).

In summary, our findings confirm the anti-inflammatory properties of a variety of glycolipids of the MGMG and DGDG classes in macrophages and report for the first time the protective activity of these compounds against UVB-caused damage to HaCaT keratinocytes. These actions seem to be mediated by their antioxidant and anti-inflammatory properties. Future studies are needed to expand the vision of the effects and mechanisms by which these glycolipids exert their photoprotective activity. These investigations will support the potential use of these products as novel agents for the prevention of sun exposure-induced skin damage including cancer development.

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CAPÍTULO II

TOPICAL PRE-TREATMENT EFFECTIVENESS WITH FORMULATED GLYCOLIPIDS ISOLATED FROM THE MICROALGA *ISOCHRYSIS GALBANA* ON TPA-INDUCED EPIDERMAL HYPERPLASIA MURINE MODEL.

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Abstract

Chronic inflammatory skin diseases as psoriasis have a significant impact on society. The major topical treatments have many side effects making difficult their continued use for patients. Microalgae have emerged as a source of bio-active molecules, including glycolipids, with potent anti-inflammatory properties. In the present study, the effects of a glycolipid (MGMG-A) and a glycolipid-containing fraction (MGDG) obtained from the microalga *Isochrysis galbana* have been evaluated on a TPA-induced hyperplasia murine model. We examined the preventive effects of MGMG-A and MGDG dissolved in acetone on TPA-induced epidermal hyperplasia murine model as preliminary study. In a second step, we performed an *in vivo* permeability study, by using rhodamine-containing cream, ointment or gel, to ensure stability and enhanced permeation properties to the sample. Finally, MGDG-containing cream was assessed on the hyperplasia murine model. Pre-treatment with acetone-dissolved glycolipids reduced skin edema, epidermal thickness and pro-inflammatory cytokines production (TNF-α, IL-1β, IL-6, IL-17) in epidermal tissue. The *in vivo* and *ex vivo* permeation studies showed that the cream formulation had the best permeability profile and the MGDG-cream formulation permeated better than acetone-dissolved preparations. MGDG-

cream application attenuated TPA-induced skin edema, improved histopathological features and showed a reduction of the inflammatory cell infiltrate. In addition, this formulation inhibited epidermal expression of COX-2 on a similar way than dexamethasone. Our results suggest that a MGDG-containing cream could be an emerging therapeutic strategy for the treatment of inflammatory skin pathologies as psoriasis.

Abbreviations: CLSM, Confocal Laser Scanning Microscopy; Dex, Dexamethasone; IL, Interleukin; *I. galbana, Isochrysis galbana*; MGDG, monogalactosyldiacylglycerol; MPO, myeloperoxidase; RMS, root mean square; *SC, stratum corneum;* TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Keywords: glycolipids, MGDG, skin, inflammation, epidermal hyperplasia, microalgae, *Isochrysis galbana*.

Introduction

Inflammatory skin diseases have a significant impact on the quality-of-life of patients; one of them is psoriasis, considered as a common immune-mediated inflammatory skin disorder. It is estimated that 2-4 % of the population suffers psoriasis (An et al., 2016). Although the exact mechanism of this pathology is not completely understood, it is known that both genetic predisposition and environmental factors as stress, infection, trauma and drugs have an important role in its etiology (Arasa et al., 2014). This disease is associated with several comorbidities as cardiovascular diseases, metabolic syndrome, and psychiatric disorders.

Accumulating evidence has demonstrated that exposure of skin to the protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces a pleiotrophic tissue response and promotes macroscopic lesions as peeling and erythema, mimicking an apparent psoriasis phenotype. Furthermore, it has been observed an increase of thickness of epidermis resulting from hyperproliferation and aberrant differentiation of keratinocytes as well as infiltration of inflammatory leukocytes into the epidermis and dermis (Liu et al., 2015). The activated leukocytes cause uncontrolled production of reactive oxygen species (ROS), leading to peroxidative damage to skin membranes and contributing to the exacerbation of lesions. Moreover, these immune cells release growth factors, chemokines and pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-6, IL-1β and IL-17, which interact as a network in the pathogenesis of psoriasis (Lowes et al., 2007). The inducible enzyme cyclooxygenase-2 (COX-2) has also been

demonstrated to play a central role in skin proliferative disorders through overproduction of pro-inflammatory prostaglandins as PGE_2 (Zulfakar et al., 2016). Currently, treatment of psoriasis include topical agents (corticoids, vitamin D derivatives, retinoids and calcineurin inhibitors), photo-chemo-therapy, as well as systemic treatments (immunosuppressants and biological drugs) (Guerra et al., 2016). However, many patients, especially those with moderate-to-severe generalized psoriasis, are not adequately treated with effective, long-term therapies, and most of them have various degrees of side effects. Thus, the development of well-tolerated immune-modulatory agents can offer an alternative option for the treatment of psoriatic patients.

Microalgae have emerged as a source of bioactive compounds, including lipids, proteins, polysaccharides and carotenoids, which have attracted the interest of the pharmaceutical industry based on their anti-oxidant, anti-inflammatory or anti-carcinogenic activities in different skin inflammatory models (reviewed in Talero et al., 2015) (Talero et al., 2015). The topical application requires a suitable incorporation of these bio-compounds into a carrier that offers stability, permeation enhancer properties, and remaining time onto the skin. The formulation of these substances involves the selection of appropriate combinations of ingredients in the formula with the aim to exert the desirable local o systemic effect. Recently, microalgae derivatives are being used as cosmeceuticals, through their incorporation in many face and skin care products (e.g., anti-aging cream, regenerating care products, emollient and anti-irritant in peelers), sun protectors and hair care products (Martins et al., 2014). We have previously reported that galactosylglycerides isolated from the marine microalga *Isochrysis galbana* (*I. galbana*) inhibit the production of the pro-inflammatory cytokine TNF-α in lipopolysaccharide-stimulated human THP-1 macrophages (De los Reyes et al., 2016). Given the interesting anti-inflammatory properties elicited by these compounds, the aim of the present study was to evaluate the preventive effects of galactosylglycerides from *I. galbana* on a murine model of TPA-induced epidermal hyperplasia in female Swiss mice. To test this possibility, we have analysed the effects of topical application of acetone-dissolved glycolipids. However, it is known that acetone-dissolved glycolipids for further application onto the mice skin exhibit several drawbacks, such as amount of this organic solvent remaining in contact to the skin, spreading of the formulation and loss of sample, heterogeneity of the dose contacting with the skin and difficulty for applying the sample. The use of pharmaceutical carriers able to load the active molecule may

solve the above limitations of glycolipid solutions. Among them, topical formulations of different nature are used, including ointment, cream, or hydrophilic gel, in which the active compound is suspended or dissolved.

Materials and Methods

Glycolipids

The monogalactosylmonoacylglyceride (2*S*)-1-*O*-[(6*Z*,9*Z*,12*Z*,15*Z*)-octadeca-6,9,12,15-tetraenoyl]-3-*O*-β-D-galactopyranosylglycerol (MGMG-A) was isolated from the microalga *I. galbana*, as previously described in Rodríguez-Luna et al., (submitted). The fraction of monogalactosyldiacylglycerides (MGDG) was obtained from the microalga *I. galbana*, as previously described in (De los Reyes et al., 2016).

Cell culture

HaCaT human keratinocytes were obtained from the American Type Culture Collection and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in an atmosphere of 5 % CO_2 at 37 °C.

Cell viability assay

Viability of HaCaT cells upon exposure to glycolipid compounds was determined by the sulforhodamine B (SRB) assay (Skehan et al., 1990). Briefly, the cells were seeded into 96-well plates at $1x10^4$ cells/well. After 24 h, cells were incubated with compounds at the final concentrations range of 10-100 µM or 10-100 µg/mL (100 µL/well) that were prepared by dilution of stocks solutions (10 mM) in DMSO in fresh medium. After 24, 48, and 72 h, cells were fixed with 50 µL of trichloroacetic acid (TCA 50 % v/v), at 4 °C for 1 h and processed as described in the literature.

Determination of IL-6 and IL-8 production

HaCaT cells were seeded in 6-well plates (2 mL/well) at $5x10^5$ cells/well. After 24 h, the cells were treated with different concentrations of MGMG-A (10, 30 and 50 µM) and MGDG (10, 30 and 50 µg/mL) and dexamethasone (Dex) (1 µM) for 1 h, and then were stimulated with TNF-α (10 ng/mL) for 24 h. Then, supernatant fluids were collected and stored at -80 °C until IL-6 and IL-8 measurements. Controls contained medium with equivalent amounts of solvent compared to treatments, and

were incubated with and without TNF-α. Commercial enzyme-linked immunosorbent assay (ELISA) kits (Diaclone GEN-PROBE, France) were used to quantify cytokines according to the manufacturer's protocol. The absorbance at 450 nm was read by a microplate reader.

Skin topical formulations

- *Hydrophilic gel*. Carbopol® 934P was selected as a gelling agent at 1 % (w/v) due to its widespread use in pharmaceutical formulations and fast dispersion in water. Rhodamine 6G solution in ethanol absolute (10 µg/µL) was gradually added to the polymer dispersion under magnetic stirring. The dispersion was neutralized with triethanolamine to obtain an adequate consistency suitable for topical application.

- *Cream*. This O/W emulsion was prepared by gradually adding rhodamine (10 µg/µL), MGDG (10 mg/mL) or Dex (10 µg/µL) solution in ethanol absolute to a cold mix excipient composed of Caprylic/Capric Triglycerides, Glycol Stearate, PEG-3 Glyceryl Cocoate and Steareth-7 previously heated to improve the drug interposition. The final formulations contained 0.2 % (w/w) MGDG.

- *Lipophilic ointment*. This formulation was obtained using melt emulsification combined with stirring. Briefly, Brij® 72 (5 % w/w) and white soft paraffin (77.3 % w/w) were blended under gentle stirring in a water bath at 70 °C to form the lipid phase. Successively, liquid paraffin (5 % w/w) and α-tocopherol (0.002 % w/w) were added into the lipid phase until complete interposition at 60 °C. Then, rhodamine dissolved in propylene glycol (10 µg/µL) was added to the mixture. Meanwhile, two solutions of EDTA (0.0065 % w/w) and disodium phosphate dihydrate (0.026 % w/w) were prepared with distilled water at 60 °C and added dropwise to the lipid phase, with moderate magnetic stirring at 150 rpm for 20 min. Finally, rhodamine-loaded ointment was maintained at room temperature for further use.

Animals

For the present study, 8-week-old female Swiss CD-1 mice (25–30 g) were supplied by Janvier-Labs (Le Genest St Isle, France). Mice were maintained in our Animal Laboratory under standard conditions (temperature of 24–25 °C, humidity of 70–75 % and 12 h light–12 h dark cycle). Mice were allowed free access to a standard diet (Panlab) and water *ad libitum*. Dorsal region of the mice was shaved by using an electric clipper and treated with depilatory cream skin (Deliplus, Barcelona, Spain) without scratching or damaging the surface in order

to maintain the integrity of the skin. All studies were performed in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 2010/63/EU). The experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville.

In vivo skin depth permeation by Confocal Laser Scanning Microscopy (CLSM)

CLSM studies were performed in order to investigate the skin penetration ability of the different formulations through the skin layers (Álvarez-Roman et al., 2004). Towards this aim, Carbopol® hydrogel, cream and ointment were prepared by adding a hydrophobic fluorescent probe, i.e. rhodamine 6G, in the lipid or in the water-ethanol phase, according to the composition of formulations. Rhodamine 6G was selected as an equivalent marker for MGDG or Dex due to their comparable lipophilic properties. Appropriate samples of these formulations were taken and placed on stratum corneum (*SC*) of mice and were maintained in contact with the skin for 24 h. At the end of the experiment, the remaining preparation was carefully washed with purified water from the skin surface. Then, dorsal skin was excised and rinsed with pH 7.4 phosphate buffer solution, rapidly frozen by liquid nitrogen and then stored at -80 °C. Sections of skin (50 μm thickness) were then perpendicularly cut with a cryomicrotome and examined to investigate the fluorescent marker distribution in the different skin layers.

Analysis was carried out using a Leica TCS SP II Confocal Laser Scanning Imaging System (Leica, Heidelberg, Germany) equipped with a Kr–Ar–He–Ne ion laser and a Leica DM IRE 2 microscope endowed with HCPL Fluotar Leica X10 and X20 dry objectives and HCXPLAN APO Leica X40 multi-immersion objective (numeric aperture 0.85). For excitation of the fluorescent label the 488nm wavelength was used and the fluorescence emission was detected at 520 nm.
From the confocal images, a mathematical treatment was carried out in order to evaluate the intensity of fluorescence in the samples, as appeared in the histogram curve provided from Leica software. Statistical calculations were as follows:

- Arithmetical mean value: $\mu(I) = \frac{1}{N_{pixel}} \cdot \sum I_i$
- Average image energy: $I^2_{mean} = \frac{1}{N_{pixel}} \cdot \sum_{pixel} I_i^2$
- Root mean square value: $I^2_{mean} = \sqrt{\frac{1}{N_{pixel}} \sum_{pixel} I_i^2}$

- Skewness of the distribution: $\mathrm{Skew(I)} = \frac{1}{N}\sum_i \left[\frac{I_i - \mu(I)}{\sqrt{VAR(I)}}\right]^3$

Where *I* is the energy intensity, N_{pixel} is the number of pixels of the image and *VAR* is the variance.

***Ex-vivo* permeation studies**

Ex-vivo diffusion studies were carried out using a Franz diffusion cell apparatus (SES-Gmgh Analyses system, Germany) with an effective diffusional area of 3.14 cm^2. Excised mice skin was used as membrane. Animals were sacrificed and full thickness dorsal skin was excised. A specific portion of the skin was cut and used for the permeation study after washing it with distilled water. Study was carried out following the methodology previously reported (López-Pinto et al., 2005). Animal skin was inserted between the donor and receiving compartments and adjusted by means of a pinch clamp. The receiving chamber was filled with 14.5 mL of degassed ethanol absolute and was thermostated by means of a water bath circulator and a jacket surrounding the cell, maintaining 32 °C in the skin surface. The receiving medium was continuously stirred to avoid diffusion layer effect.

Once selected the cream carrier from the previous study, MGDG-cream (0.2 % w/w), MGDG control solution (0.2 % w/w) and Dex-cream (0.2 % w/w) were accurately measured and placed on *SC* in the donor compartment and sealed with parafilm. Aliquots of 0.5 mL were withdrawn from the receiving medium at predetermined time intervals (0, 0.5, 1, 2, 3, 4, 5, 6 and 24 h) according to international guidelines and the same volume was replaced with fresh ethanol absolute at the same temperature. These samples were quantified by HPLC (Hitachi Elite LaChrom) for MGDG quantification and spectrophotometry UV-visible (Agilent 8453) was used for Dex quantification.

The HPLC system is equipped with an L-2130 isocratic pump, a diode array detector L-2455 and L-2200 autosampler. The chromatographic separation was performed on a reverse phase LichroCART® C18 column (5 µm, 4.6 mm ID × 150 mm) using as mobile phase acetonitrile (ACN, solution A): formic acid solution (0.1 % v/v), solution B) adjusted to pH 2.67 in the following gradient (v/v): 0–6 min, 90 % A, 10 % B, flux 1 mL/min; 6–15 min, 100 % A. The injection volume was 30 µL.
Cumulative amount of drug in receptor chamber for the three formulations (MGDG-cream, MGDG control solution and Dex control solution) was plotted as a function of time (*t*, h). The cumulative amount (%) of drug permeated

through the skin (P %) was determined as per the following equation (Li Z, Liu M, Wang H, 2016):

$$P\% = \frac{C_n \cdot V + \sum_{i=1}^{n-1} C_i \cdot V_i}{M} \cdot 100$$

Where C_n is the drug concentration of the n^{th} sampling point (mg/mL), C_i is the drug concentration of the i^{th} sample point (mg/mL), V is the total volume (14.5 mL) of liquid in receiving pool, V_i is the volume (0.5 mL) of the i^{th} sampling points and M is the mass of drug (MGDG or Dex).

TPA-induced epidermal hyperplasia model and treatments

We evaluated the effect of glycolipids from *I. galbana* on TPA-induced hyperplasia in murine skin by using two experimental protocols. Briefly, dorsal skin of female Swiss mice was shaved and 24 h later, animals that displayed no evidence of hair regrowth or injury were assigned to the different groups.

In the first protocol, MGMG-A and MGDG dissolved in acetone (10 µg/µL, 200 µg per site), the reference agent Dex dissolved in acetone (10 µg/µL, 200 µg per site), or vehicle (acetone) were topically administered to the skin surface of the backs in an area of 1 cm^2 by using a micropipette (total volume of 20 µL). After 30 min, TPA (2 nmol per site, dissolved in acetone) was topically applied to the same areas (day 1). A reference control group was included for comparison with the TPA-induced group: sham group received acetone, instead TPA solution, in a comparable volume. This procedure was repeated during two consecutive days (Arasa et al., 2014).

In the second protocol, MGDG-cream formulation (100 mg per site, containing 200 µg of MGDG dissolved in ethanol at 10 µg/µL), Dex (100mg per site, equivalent at 200 µg of compound dissolved in ethanol) or vehicle (cream with a comparable volume of ethanol) were applied on the dorsal skin in an area of 1 cm^2 by using a syringe, and starting 2 days before the hyperplasia induction. Twenty-four hours after the second cream administration, TPA (2 nmol per site, dissolved in ethanol) was topically applied to the same areas (day 1). After 1 h, MGDG-cream, Dex or vehicle were administered following the mentioned protocol. This procedure was repeated during two consecutive days.

For the two experimental protocols, 3 days after the first TPA application (day 4), mice were sacrificed by cervical dislocation and punch biopsies were collected from the treated dorsal skin and weighed to evaluate edema. One tissue piece per animal was fixed in 4% buffered paraformaldehyde. Other pieces were frozen in liquid nitrogen for measurement of biochemical parameters.

Histological study

Tissue samples from the dorsal skin of four animals were fixed in 42 % buffered paraformaldehyde, dehydrated by increasing concentrations of ethanol and embedded in paraffin. Tissue sections were cut at 7 µm on a rotary microtome (Leica Microsystems, Germany), were mounted on slides, deparaffinized with xylene, rehydrated through graded alcohols and stained with haematoxylin and eosin, according to standard protocols. All tissue sections were examined under an Olympus BH-2 microscope (GMI Inc., MIN, USA) for determination of histopathological changes. Epidermal thickness was measured using Scientific Imaging Systems (Biophotonics ImageJ Analysis Software; National Institutes of Health, USA).

MPO activity

Myeloperoxidase (MPO) activity was assayed as a marker of neutrophil infiltration according to the method of Grisham et al. (Grisham et al., 1990). The tissue was thawed, weighed and homogenised in ten volumes of 50 mM-PBS (pH 7.4). The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C. The pellet was again homogenized in ten volumes of 50 mM-PBS (pH 6) containing hexadecyl trimethylammonium bromide (0.5%) and 10 mM-EDTA. This homogenate was subjected to one cycle of freezing/thawing and a brief period of sonication. A sample of the homogenate (50 µL) was added to a 96-well microplate and incubated at 37 °C for 3 min with a mixture containing *o*-dianisidine dihydrochloride (0.067 %), hexadecyl trimethyl-ammonium bromide (0.5 %) and 0.3 mM-H_2O_2. Changes in absorbance at 450 nm were measured with a microplate reader (Labysystem Multiskan EX, Thermo Scientific, NY, USA). One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0u nit/min at 37 °C in the final reaction volume containing the acetate. Results are expressed as units/mg tissue.

Measurement of cytokines in skin homogenates

Frozen skin biopsies were homogenised in three volumes of ice-cold tissue lysis buffer containing PBS (pH 7.2) with 0.1 M of EDTA, 1 mg/mL of leupeptin, 1 mg/mL of pepstatin, 1 mg/mL of aprotinin and 1 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 12,000 *g* for 10 min at 4 °C. Supernatants fluids were stored at -80 °C until measurements. Levels of TNF-α, IL-1β, IL-6, IL-17 and IL-10 were measured by quantitative Enzyme-Linked ImmunoSorbent Assay kits (ELISA) (Peprotech, Germany), according to the manufacturer's instructions. Data are reported as pg/mg tissue.

Immunohistochemical analysis

Staining of COX-2 was performed using a streptavidin-biotin-peroxidase method (Talero et al., 2011). Paraffin-embedded dorsal skin sections (7 µm) were mounted on slides, deparaffinized with xylene and rehydrated through graded alcohols. These sections were boiled (10 mM citrate buffer, pH 6.0 for 3 min) for antigen retrieval, followed by cooling at room temperature for 20 min. Endogenous peroxidase was quenched with 0.3 % (v/v) hydrogen peroxide for 20 min. Sections were rinsed with PBS for 10 min. Nonspecific adsorption was minimized by incubating sections in normal horse serum (Vectastain Kit; Vector Laboratories, Burlingame, CA) for 20 min. Subsequently, slides were incubated with rabbit polyclonal anti-COX-2 antibody (Cayman Chemical, MICH, USA) (1:300) overnight at 4 °C. Then, slides were treated with anti-mouse IgG antibody for 30 min and incubated with the streptavidin-peroxidase complex for 30 min, at room temperature (Vectastain Kit; Vector Laboratories, CA, USA). The enzymatic activities were developed with 3,3´-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin. Negative control sections were treated in the same way, omitting the primary antibody (Talero et al., 2012). COX-2 immunoreactivity was examined on all sections using a microscope Olympus BX61 (Olympus Optical Co. Ltd. Tokyo, Japan) The quantification of immunohistochemical data was done by counting the number of immuno-stained cells as percent of total epidermal cells from ten microscopic fields of immunostained tissues per animal.

Statistical Analysis

All values in the figures and text are expressed as arithmetic means ± SEM. Data were evaluated with GraphPad Prism version 5.00 software (GraphPad Software, Inc., San Diego, CA, USA). In all cases, the Shapiro-Wilk test was used to verify the normality of the data. The Mann-Whitney U-test was chosen for non-parametric values. The parametric

values groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. P values < 0.05 were considered statistically significant. In the histological experiment, results shown are representative of at least four independent experiments performed on different days.

Results

Effects of glycolipids on IL-6 and IL-8 production in TNF-α-stimulated HaCaT human keratinocytes

To investigate the effect of the glycolipid MGMG-A and the fraction MGDG on the production of the pro-inflammatory cytokines IL-6 and IL-8, HaCaT cells were pretreated with non-cytotoxic concentrations of glycolipids (10, 30, and 50 µM for pure compound and µg/mL for fraction) for 1 h, and then, were stimulated with TNF-α (10 ng/mL) for 24 h. TNF-α-stimulated HaCaT cells manifested high IL-6 and IL-8 levels in comparison with unstimulated control cells (P < 0.001) (Table 1). Pre-treatment with the compound and fraction significantly inhibited IL-6 and IL-8 production at the different tested concentrations.

				MGMG-A (µM)			MGDG (µg/mL)		
	Control	**TNF-α**	**Dex**	**10**	**30**	**50**	**10**	**30**	**50**
IL-6 (pg/ml)	**8.738 ± 2.5**	**123.2 ± 10.8 *****	**27.9 ± 10.7 ++**	**36.6 ± 16.2 +**	**34.6 ± 14.8 +**	**32.5 ± 13.9 +**	**44.5 ± 13.5 +**	**45.3 ± 8.8 +**	**45.9 ± 8.9 +**
IL-8 (pg/ml)	**5473 ± 971.1**	**55505 ± 7148 *****	**20848 ± 2848 +++**	**28822 ± 3938 ++**	**26727 ± 4170 ++**	**25205 ± 3610 ++**	**31838 ± 5851 +**	**30601 ± 6962 +**	**27979 ± 6116 ++**

Table 1. Effects of glycolipids from *I. galbana* on IL-6 and IL-8 production in TNF-α-stimulated HaCaT human keratinocytes. IL-6 levels and IL-8 levels in TNF-α-stimulated HaCaT human keratinocytes. Cells were pre-incubated with the glycolipid MGMG-A (10, 30, 50 µM) and the fraction MGDG (10, 30, 50 µg/mL), and then stimulated with TNF-α (10 ng/mL) for 24 h. Dexamethasone (Dex) was used as positive reference compound at 1 µM. After 24 h, the production of cytokines in the supernatants was measured by ELISA assay. Results are representative of six independent experiments (n=6). Values are means with standard errors represented by vertical bars. Mean value was significantly different compared with the control group (*** P < 0.001; Student *t* test). Mean value was significantly

different compared with the TNF-α group (+P < 0.05, ++P < 0.01, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

Topical application of acetone-dissolved glycolipids inhibits skin inflammation and hyperplasia in the murine TPA-induced model

We studied the effect of MGMG-A and MGDG on the murine TPA-induced epidermal hyperplasia model, which reproduces certain biochemical and histopathological parameters typical of human psoriasis (Andrés et al., 2013). TPA administration to mouse skin resulted in the development of macroscopic lesions (Fig. 1a) and skin edema, confirmed by a higher weight of the 1 cm^2 punch biopsies compared with the sham group (P < 0.001) (Fig. 1b). Topical treatment with MGMG-A and MGDG (200 µM per site in acetone) 30 min prior to TPA application (2 nmol per site for three consecutive days) inhibited macroscopic damage and the skin punch weight (P < 0.001 and P < 0.05, respectively), suggesting an inhibition of skin edema (Fig. 1b). We next examined haematoxylin and eosin-stained sections of mouse skin (Fig. 1c). Consistent with macroscopic changes, TPA-treated animals exhibited a clear evidence of edema, epidermal hyperplasia and a massive neutrophilic infiltration compared with sham animals (Fig. 1c). Moreover, a marked increase in epidermal thickness was evident in the TPA group (P < 0.001) (Fig. 1d). These results correlated with increased MPO activity, an established marker for inflammatory cell infiltration into the skin (Fig. 1e). Treatment with compound and glycolipid fraction markedly prevented epidermal hyperplasia (P < 0.01 and P < 0.001, respectively) (Fig. 1c and d), which was associated with a reduction of MPO activity being significant for compound (P < 0.01) (Fig. 1e).

To support the beneficial effects of glycolipids on skin inflammation, we analyzed the production of several pro-inflammatory cytokines that are highly involved in psoriasis as well as the anti-inflammatory cytokine IL-10. Immune cell infiltration detected in the histological examination of the skin from TPA-treated mice correlated with increased levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17, in comparison with sham group (P < 0.05, P < 0.01, P < 0.01, and P < 0.001, respectively) (Fig. 2). In accordance with the reduction of the skin edema, the production of TNF-α, IL-6 and IL-17 were significantly reduced in animals treated with the glycolipid MGMG-A (P < 0.05, P < 0.001, P < 0.05, respectively) (Figs. 2a-d). Regarding the fraction MGDG, its application resulted in a strong significant

suppression, comparable with Dex treatment, of TNF-α and IL-6 levels ($P < 0.01$, and $P < 0.001$) (Figs. 2a, c). IL-10 production analysis revealed increased levels in the TPA group in comparison with sham animals ($P < 0.05$). Pre-treatments with these compounds tended to reduce this cytokine levels; however, these changes were not statistically significant (Fig. 2e).

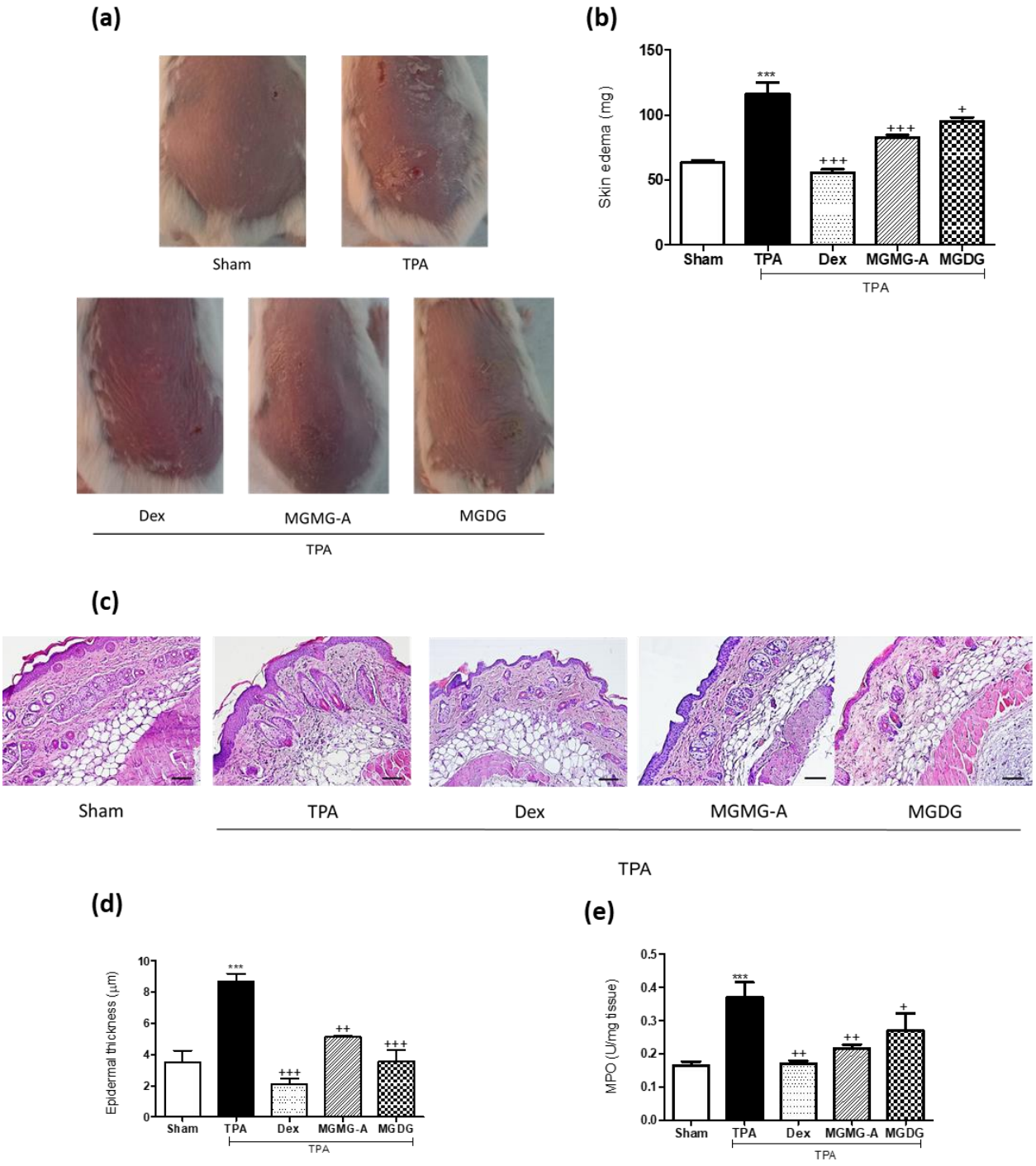


Figure 1. Topical application of acetone-dissolved glycolipids from *I. galbana* inhibits skin inflammation and hyperplasia on the murine 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced model. The glycolipid MGMG-A (200 µM per site) or the fraction MGDG (200 µg/mL per site) were topically administered 30 min before TPA application (2 nmol per zone) during three consecutive days. Dexamethasone (Dex) was used as positive reference compound (200 µg per site). (a) Representative images of macroscopic appearance of the dorsal skin. (b) Skin edema as punch biopsy; weight of edema was employed as marker of inflammatory skin process. (c) Histological appearance of mouse dorsal skin after haematoxylin/eosin (H&E)-staining; Bar = 100 µm. Original magnification 100X (d) Epidermal thickness assessment in H&E-stained skin slides. (e) Myeloperoxidase (MPO) activity in dorsal skin. Values are means with standard errors represented by vertical bars. Data are means ± SEM (n=6-8 mice/group). Mean value was significantly different compared with the sham group (***P < 0.001; Student *t* test). Mean value was significantly different compared with TPA group (+P < 0.05, ++P < 0.01, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

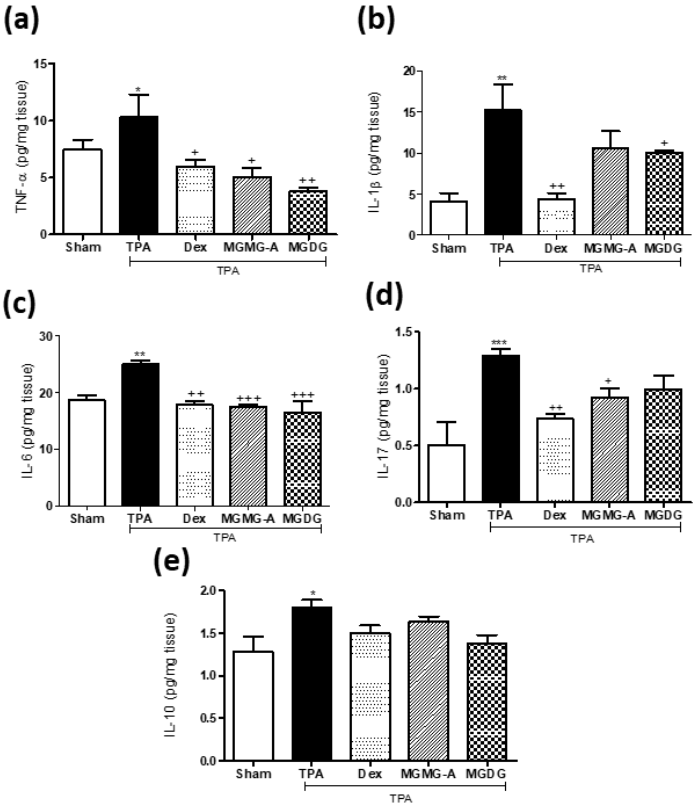


Figure 2. Effect of the glycolipid MGMG-A and the fraction MGDG from *I. galbana* on the production of cytokines in skin homogenates in the murine 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced hyperplasia model. (a) TNF-α

(pg/mg tissue), (b) IL-1β (pg/mg tissue), (c) IL-6 (pg/mg tissue), (d) IL-17 (pg/mg tissue) and (e) IL-10 (pg/mg tissue). Values are means with standard errors represented by vertical bars. Data are means ± SEM (n=6). Mean value was significantly different compared with the sham group (*P < 0.05, **P < 0.01***P < 0.001; Student *t* test). Mean value was significantly different compared with TPA group (+P < 0.05, ++P < 0.01, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

Effect of the formulation

The development of topical formulations implies the selection of excipients leading to improvement in the drug skin delivery. In order to evaluate the skin accumulation and penetration properties of the examined formulations, sections of the mice skin were analysed by CLSM at the end of permeation experiments. For these studies, rhodamine 6G, a fluorescent hydrophobic probe, was added as a model drug (Mura et al., 2007). The penetration depth of the fluorescent probe and the relative intensity of fluorescence in the skin layers were compared in three types of semisolid formulations. CLSM images revealed that all the examined formulations penetrated deeply into the *SC* and diffused into the whole skin thickness excepting the ointment (Fig. 3a). Cream showed the higher probe permeation in 24 h, following the control formulation containing only ethanol and incorporated into Carbopol® gels. However, the rhodamine 6G incorporated into the lipid ointment was observed to show a low penetration capacity. In addition to the effect of the carrier nature, deeper skin layers were more easily visualized when ethanol was present in the composition, as occurred in all the formulations excepting the ointment where the labeling probe was dissolved in propylene glycol.

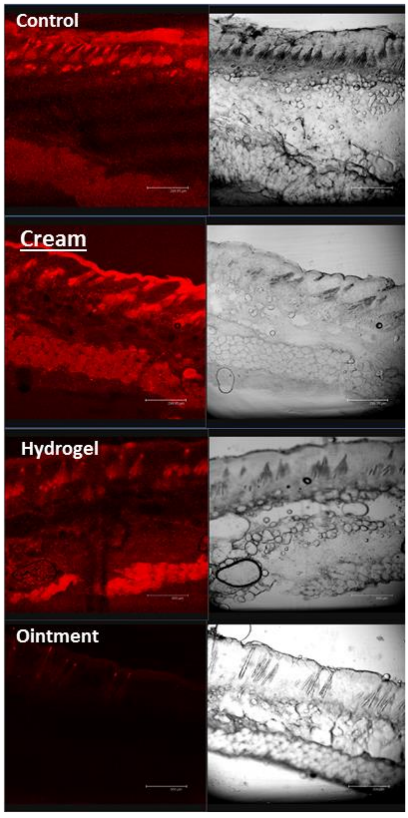
The quantitative parameters of histogram distribution revealed a higher fluorescent intensity and accumulation of rhodamine 6G in the presence of ethanol (Fig. 3b). Among all the samples, the cream system offered the higher fluorescence intensity and adequate symmetry of the normal distribution of histogram.

***Ex vivo* permeation studies**

Permeation profiles of MGDG from the ethanol solution and cream through mice skin membranes were obtained from the equation previously described in experimental section. Dex-loaded cream was used as control formulation. Results showed that permeation of MGDG from cream (100 % ± 1.9 of the applied dose) was considerably greater than that observed from ethanolic control solution (49.3 % ± 3.5), about 2-fold higher. On the other hand, the permeated amount of Dex from cream was lower (15 %

± 3.1) in comparison with the other preparations (Fig. 3c). This value can be attributed to lower partition coefficient of this molecule (logP 1.83) compared to MGDG, which lipophilicity could be resembled to a reference diacylglycerol in terms of lipophilic acyl groups (logP 3.85) (Maréchal et al., 1994). It is well-known that the partition coefficient has been widely used as a measurement for defining the lipophilicity of a drug and the diffusion efficiency across the membranes (Zhongqiu, L., Stephen, Wang., 2009).

(a)



(b)

Formulation	Mean	Mean Energy	RMS	Skewness
Control solution	76,64	9260,70	96,23	0,98
Cream	89,48	11112,34	105,42	1,14
Hydrogel	71,39	7945,02	89,13	1,24
Ointment	12,89	325,48	18,04	3,38

(c)

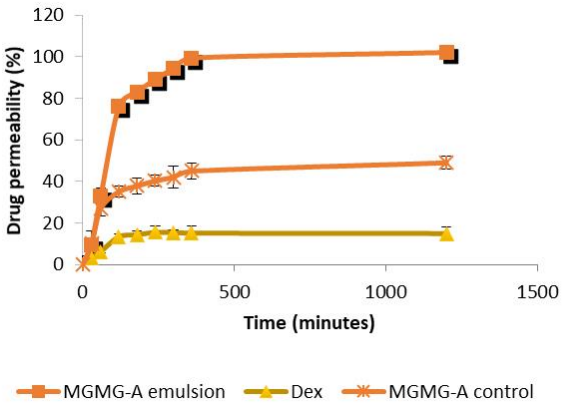


Figure 3. Effect of the vehicle composition and physicochemical properties of the drug on the permeation characteristics. (a) Confocal micrographs of mice skin cross-sectioning corresponding to rhodamine-loaded ethanolic control solution, cream, hydrogel and ointment. Bar = 200 µm. Original magnification 100X. (b) Numerical data corresponding to the intensity histogram for each sample; Mean: arithmetical mean value; Mean energy: average image energy; RMS: root mean square value; Skewness: skewness of the distribution. (c) *Ex vivo* permeability percentages of MGDG formulations in 24 h (ethanolic control solution, cream and ointment).

Topical pre-treatment with cream containing the glycolipid fraction decreases skin inflammation and hyperplasia on the murine TPA-induced model

We evaluated the effect of MGDG-cream formulation on the murine TPA-induced epidermal hyperplasia model; this cream formulation demonstrated enabling lipid preservation and high permeation in comparison with the acetone vehicle. After treatment with TPA for 3 consecutive days, the mice exhibited the expected psoriasis phenotype, including peeling, erythema and thickening of the back skin, accompanied by a marked increase of dorsal skin thickness and weight and substantial inflammatory cell infiltration in the dermis ($P < 0.001$) (Fig. 4). Pre-treatment with MGDG-cream (100 mg per site containing 200 µg of MGDG) attenuated the macroscopic lesions formation (Fig. 4a) and significantly reduced skin edema ($P < 0.001$) 24 h after the last application of TPA, compared with cream-TPA group (Fig. 4b). Histological analysis of H&E-stained skin lesions revealed an improvement in the microscopic features of hyperplasia in mice treated with MGDG-cream, evidenced by a reduction of epidermal thickness ($P < 0.05$) in relation to cream-TPA group (Fig. 4c and d). These results were accompanied by a clear inhibition of MPO activity following MGDG-cream administration ($P < 0.001$); interestingly, glycolipid formulation was as effective as the reference topical treatment with Dex, reaching similar levels to those in the healthy group (Fig. 4e). It is known that COX-2 plays an important role in skin pathologies. Immunohistochemical analysis of this enzyme showed that stimulation with TPA significantly increased COX-2-positive cell numbers ($P < 0.001$), predominantly localized in epidermal layer (Fig. 5a), when compared with sham group. As shown in the Fig. 5b, skin from MGDG-cream-treated mice revealed a significant downregulation in the number of epidermal COX-2-positive stained cells in comparison with cream-TPA group ($P < 0.001$).

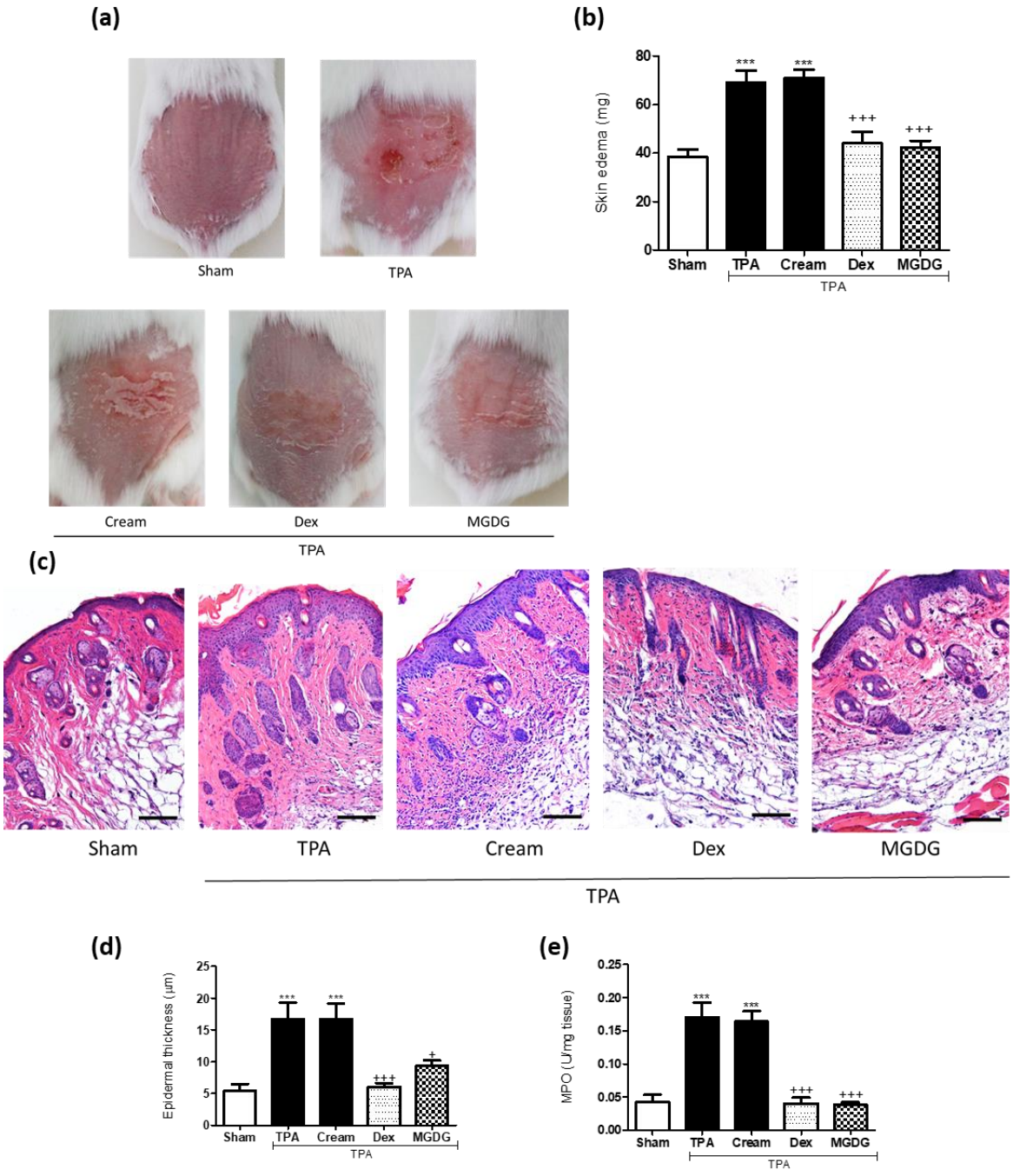


Figure 4. Topical pre-treatment with cream containing the glycolipid fraction MGDG from *I. galbana* decreases skin inflammation and hyperplasia on the murine 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced model. Glycolipid cream formulation (100 mg per site containing 200 µg of MGDG), Dexamethasone (Dex) (100 mg per site, equivalent at 200 µg of compound) or vehicle (cream with a comparable volume of ethanol) were topically administered from 2 days before hyperplasia induction and 30 min after each TPA application (2 nmol per zone for three consecutive days). Dex was used as positive reference compound. (a) Representative images of macroscopic appearance of the dorsal skin. (b) Determination of skin edema as punch biopsy weight. (c) Histological appearance of mouse dorsal skin after haematoxylin/eosin (H&E)-staining; Bar = 100 µm.

Original magnification 100X. (d) Epidermal thickness assessment in H&E-stained skin slides. (e) Myeloperoxidase (MPO) activity. Values are means with standard errors represented by vertical bars. Data are means ± SEM (n=6-8 mice/group). Mean value was significantly different compared with the sham group (***P < 0.001; Student *t* test). Mean value was significantly different compared with cream-TPA group (+P < 0.05, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

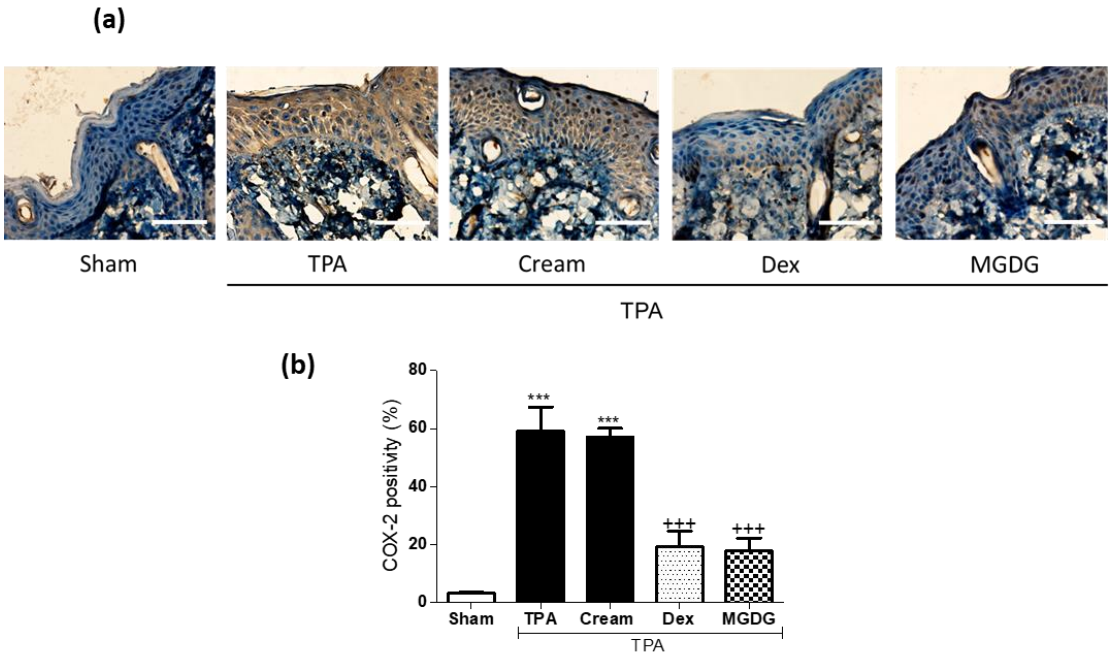


Figure 5. Topical pre-treatment with cream containing the glycolipid fraction MGDG from *I. galbana* attenuates 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in mouse skin. (a) Representative photographs of epidermal COX-2 distribution by immunohistochemical detection; Bar = 200 µm. Original magnification 200X. (b) Positive COX-2 epidermal layer was assessed by counting the COX-2 positive cells *versus* total cells in different immunostainned dorsal skin sections per animal; photographs are representative from one out of three mice investigated. Values are means with standard errors represented by vertical bars. Data are means ± SEM (n=6). Mean value was significantly different compared with the sham group (***P < 0.001; Student *t* test). Mean value was significantly different compared with cream-TPA group (+++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

Discussion

Inflammatory skin diseases have a significant impact in society, being atopic dermatitis, acne, sunburn and psoriasis the most common manifestations. Psoriasis is a chronic, autoimmune and multisystem

inflammatory disease that affects at 2-4 % of population (Anand et al., 2017). Currently, conventional treatments of this disease are based on the degree of severity and include from topical therapy and systemic agents through to phototherapy or combinations of those. However, many of these therapies are not recommended for a vast majority of patients afflicted of mild forms of psoriasis because of their potential risk (Malatestinic et al., 2017). Therefore, other approaches for mild psoriasis treatment that require topical therapy only are still needed. In this regards, natural products provides some options for increasing safety and efficacy in the management of this pathology (Herman et al., 2016). Microalgae species are a promising source of a variety of bioactive molecules, including polar lipids such as glycolipids. Lipid enriched extracts or pure glycolipids have previously demonstrated their *in vitro* anti-inflammatory (Banskota et al., 2014; Robertson et al., 2015), and antitumor promoting properties (Hossain et al., 2005), which make them suitable candidates for further investigation. However, the use of galactosylglycerides to prevent skin pathologies such as psoriasis has not been previously evidenced. In this sense, we have recently observed that this kind of metabolites, protect human keratinocytes HaCaT against UVB radiation through inhibition of ROS generation and decrease of the production of the pro-inflammatory cytokine IL-6 (Rodríguez-Luna, et al., submitted). These findings suggest that this type of molecules could play a main role not only in protecting the skin against UVB exposition but also in preventing the skin inflammatory process. In this context, we aimed to evaluate the anti-inflammatory effects of the glycolipid MGMG-A and, the fraction MGDG in an experimental TPA-induced hyperplasia model in mice. Moreover, we used different semisolid formulations in which the glycolipid was loaded in order to facilitate its topical application and to enhance the permeation mechanism compared to conventional liquid preparations.

Firstly, we tried to demonstrate the anti-inflammatory potential of the compounds under study in the *in vitro* model of TNF-α-stimulated HaCaT keratinocytes. This cytokine plays a crucial role in the pathogenesis of skin inflammatory diseases as psoriasis (Paya et al., 2007). We proved that pre-treatment with the compound MGMG-A or the fraction MGDG significantly reduced the production of the pro-inflammatory cytokines IL-6 and IL-8 in stimulated HaCaT keratinocytes. These results led us to evaluate the possible preventive effects of these products on TPA-induced hyperplasia in murine skin by using two experimental approaches. In the first *in vivo* experiment, glycolipids,

dissolved in acetone, were topically administered to dorsal skin 30 min before TPA administration. Our data showed that TPA clearly caused peeling, erythema, and a strong inflammatory reaction, produced by a marked influx of mononuclear and polymorphonuclear leukocytes in epidermis and dermis. Treatment of mice with the compound MGMG-A or the fraction MGDG from *I. galbana* reduced the hyperplasia manifestations and the inflammation grade, presumably due to an inhibition of inflammatory cells infiltration, as revealed MPO study. These findings are interesting since the cellular infiltrate has pathogenic roles in psoriasis and thus, its control is extremely important for the attenuation of this disease (Yazici et al., 2016). Microscopic analysis of the dorsal skin was in accordance with macroscopic study reflecting the attenuation of the keratinocytes hyperproliferation in the epidermis after the treatment with the acetone-dissolved glycolipids. Our results are in line with a previous report that showed that topical pre-treatment with the glycoglycerolipids MGDG, DGDG, or SQDG, obtained from a blue green alga, reduced the inflammation in croton-oil-induced ear edema, and in carrageenan-induced paw edema models (Bruno et al., 2005).

The role for TNF-α, IL-17, and IL-1β in psoriasis pathogenesis has been well documented (Mahil et al., 2016), as strategies to block these cytokines showed to be particularly effective in the treatment of this disease (Campa et al., 2016). In the present study, dorsal skin samples from TPA group showed increased levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17, which were decreased in skin samples from mice treated with MGMG-A or MGDG fraction. On the other hand, IL-10 is considered an anti-inflammatory cytokine because it inhibits the T cells and macrophages pro-inflammatory cytokine production (Geginat et al., 2016). Our data showed no lower significant levels of IL-10 in mice treated with the acetone-dissolved glycolipids in comparison with that in TPA group, suggesting a lower role of this cytokine in the immune response by MGMG-A or MGDG.

After examining the preventive effects of the compound MGMG-A and fraction MGDG obtained from *I. galbana*, we aimed to optimize the permeation behaviour of these substances with respect to previously acetone-dissolved preparations. In the skin diseases, such as psoriasis, local topical delivery can be improved by following two main approaches. Firstly, the adequate choice of formulation can optimize the local targeting. Secondly, the physicochemical parameters of the drug itself, such as lipophilicity, can affect the degree of delivery as well.

Concerning to the adequate choice of carrier composition, different formulations, including hydrophilic gel, cream and ointment were prepared and analyzed. For these studies, a fluorescent hydrophobic marker such as rhodamine 6G, with an oil/water partition coefficient of 2.62 was added, as a model drug (Wang et al., 2012). The *in vivo* CLSM study defined the cream as the best vehicle to dig deeper in the skin preserving the entity of different tissue layers. This may be assigned to the fact that the surfactant-like properties of this heterogeneous disperse system could enhance the amount permeated of rhodamine 6G. On the other hand, control solution using ethanol as solvent and hydroalcoholic Carbopol® gels, exhibited also high drug permeation. This could be also attributed to the presence of ethanol in the formulation, which was used as solubilizer and permeation enhancer. On the contrary, the deposition of the fluorophore from lipid ointment onto the skin was very low. This would be ascribed to the hydrophobic nature of the vehicle, being insoluble, which did not penetrate through the skin and remained onto the skin surface, as visualized in figure 3.
Usually, the permeation pathway across the *SC* resides in the intercellular lipid domains. Conversely, at the viable epidermis level, the fluorophore is not restricted to the cell membranes only, but also accumulates in the cytosol. In this study, a relationship between lipophilicity of the applied formulation on the accumulation of the fluorophore in *SC* and viable epidermis was observed. Although the fluorescent label was applied onto the *SC* of mice, only low relative accumulation in the deeper layers of the skin was observed for the most lipophilic vehicle, the ointment. This can be explained since the lipid lamellae constitute only a small region of the *SC* compared to the corneocytes. However, the adjacent viable epidermis is much more brightly stained than *SC* in samples from the rhodamine-loaded cream and hydrogel formulations, because in this layer the label can distribute throughout the entire epidermis, which results in a brighter appearance (Aggarwal et al., 2012).

Once selected the cream for further studies, an *ex vivo* permeation process was planned for comparison MGDG-loaded cream with MGDG reference solution and Dex cream. This corticoid was selected for being the most used drug in the psoriasis treatment. In this study, the permeation through mice excised skin was determined using Franz diffusion apparatus and degassed absolute ethanol. Since the goal of this study was to find the experimental conditions that allowed a fast and accurate method to evaluate the permeability properties of different formulations across the skin, we decided to use ethanol as solvent forming the receiving

compartment because the assayed molecules (MGDG and Dex) are freely soluble in this medium. This solvent has been used for analyzing the skin diffusion kinetic of other molecules, such as α-tocopherol acetate (Mahamongkol et al., 2005). However, the use of this solvent has the potential to extract lipids from the *SC* and to artificially increase the skin permeability. Since all the formulations tested in these experiments had ethanol as penetration enhancer interacting with skin constituents to increase drug flux, this would not affect the relative results.
MGDG and Dex from the three formulations were detected in the receiver medium in a time-dependent and cumulative amount permeated curves were plotted. Concerning MGDG cream respect to MGDG ethanolic solution, the difference in the permeability percentage may be attributed to the surfactant-like properties of emulsion components and their affinity to the phospholipids of the skin membrane (Williams and Barry, 2012). A common mechanism of action of surfactants as penetration enhancers involves firstly a "push" effect to increase the drug solubility and hence to create a high concentration gradient. Secondly, a "pull" effect is related to the flux of the permeation enhancer through the skin, which can induce skin structural transformations (Puglia and Bonina, 2008).

On the other hand, findings from our study indicate that the cream exerted the best effect in increasing skin permeation of MGDG compared to Dex, in which the permeated amount was lower (15 % ± 3.1). In accordance to another studies (Wang et al., 2004), this behaviour can be attributed to lower partition coefficient of this molecule (logP 1.83) with respect to MGDG (Maréchal et al., 1994). The interaction of the drug between water and oil phase can determine the extent of lowering of the thermodynamic activity in external phase, which is in contact with the skin. Partitioning of the drug into internal oily phase (higher logP) is due to hydrophobic characteristic of the drug. Being both molecules highly lipophilic substances, the extremely slow permeation of Dex from the emulsion through the mice skin can be explained by the fact that diffusion of drug through oily phase is the limiting step for drug permeation (Grams et al., 2003).

An important consideration in psoriasis is the skin condition. Topical formulations may be applied either to opened lesions that have lost *SC* barrier properties or to thickened lesions that represent an additional barrier to absorption (Garnier T, 2002). Thus, our findings suggest that a hydrophilic vehicle (cream) could be an interesting alternative to improved topical delivery of MGDG in both conditions.

Based on the results above, we selected the fraction MGDG from *I. galbana*, and elaborated a cream with MGDG to evaluate its effects on a second TPA-induced hyperplasia model in mice. In this experimental model, TPA was applied 1 h before the administration of MGDG-containing cream to ensure the correct absorption of the product as well as increase its permeation and protect the lipid integrity. Our data showed that dorsal application of TPA caused similar manifestations to those detected in the previous model. Consistently with the anti-inflammatory activity of glycolipids, pre-treatment with MGDG-cream ameliorated macroscopic cutaneous lesions induced by repetitive application of TPA, which was correlated with the histological normalization of epidermal. Interestingly, the reduction of skin edema and MPO activity were higher with MGDG-cream than those detected in the compound dissolved in acetone, reaching similar levels to those of the reference corticosteroid Dex.

It has been shown that topical application of TPA activates intracellular transduction signals, enhancing aberrant expression of COX-2 in mouse skin (Kundu et al., 2009; Passos et al., 2013). To further elucidate the mechanisms for the anti-inflammatory role of MGDG-cream in damaged skin, we detected this protein expression by immunohistochemistry. The results evidenced that pre-treatment with MGDG-cream markedly attenuated COX-2 expression in TPA-stimulated mouse skin, reaching similar levels to Dex. These findings, at least partly, suggest that suppression of COX-2 expression may be involved in the preventive effect of this glycolipid fraction on TPA-induced epidermal hyperplasia.

In conclusion, our study demonstrates for the first time the preventive effects of topical administration of the glycolipid MGMG-A or a fraction MGDG from *I. galbana*, in the inflammatory model of TPA-induced skin hyperplasia. These actions may be associated with a reduction of edema, leucocyte infiltration, proinflammatory cytokines production and COX-2 expression in skin mouse. Topical application of MGDG-cream enhanced the sample permeability and consequently significantly increased the preventive effects of this product. Future studies are needed to expand the vision of the mechanisms by which these lipid products improve skin inflammation and will support their potential use in the development of effective therapeutic strategies for skin pathologies as psoriasis.

Conflicts of interest

The authors have no conflict of interest to declare.

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The authors' contributions are follows: MC. T., E.T., V. M. and J. A-R designed the study protocol; C. DLR., performed chemical characterization of glycolipids from *I. galbana*; A. R-L., J. A-R. and E. T., conducted the *in vivo* and histological experiments and analysed the data; A. R-L. and ML. G-R executed technologic experiments; A. R-L., E. T., J. A-R., V. M. and ML. G-R wrote the draft of the manuscript. All the authors critically reviewed and approved the final version of the manuscript. None of the authors has any conflicts of interest to declare.

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CAPÍTULO III

A CREAM CONTAINING FUCOXANTHIN PREVENTS EPIDERMAL HYPERPLASIA AND UVB-INDUCED SKIN ERYTHEMA IN MICE

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ABSTRACT

Nowadays, chemoprevention with natural products is a potent strategy to reduce the development of skin inflammatory pathologies. In this study, anti-inflammatory, anti-oxidative and photoprotective effects of the carotenoid Fucoxanthin (FX) isolated from the microalgae *Isochrysis galbana* were investigated in *in vitro* and *in vivo* models. Firstly, the anti-inflammatory activity was evaluated in LPS-stimulated THP-1 macrophages and TNF-α-stimulated HaCaT keratinocytes, and the anti-oxidant activity in UVB-irradiated HaCaT cells (50 mJ/cm^2). Permeation studies were developed to determinate the most suitable formulation for topical application of FX. Then, we evaluated the preventive effects of FX-containing cream on TPA-induced epidermal hyperplasia model in Swiss CD-1 mice, as well as in UVB-induced acute erythema model in hairless mice (360 mJ/cm^2). Our results confirmed the *in vitro* reduction of anti-inflammatory mediators as TNF-α and IL-6 and photoprotective effects of FX, reducing ROS and LDH production. The permeation results showed that cream was the most favorable vehicle for FX, consequently FX-cream was elaborated for topical application. In addition, the pre-treatment with the FX-cream effectively ameliorated TPA-induced hyperplasia in mice, by reducing skin edema, epidermal thickness, MPO activity and COX-2 expression levels. Moreover, we showed that FX reduced UVB-induced erythema in mice thorough down-regulation of COX-2 and iNOS protein expressions as well as up-regulation of the anti-oxidant HO-1 protein levels through Nrf-2 signalling pathway. These findings suggest that FX, administered in a topical formulation, could

be a novel approach for preventing psoriatic exacerbations and protecting skin against UV radiation.

KEYWORDS: Fucoxanthin, skin, inflammation, epidermal hyperplasia, UVB, microalgae, *Isochrysis galbana*.

ABBREVIATIONS: AK, actinic keratosis; DCF, 2',7'-dichlorofluorescein; DCF-DA, dichlorofluorescein diacetate; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; Dex, Dexamethasone; FX, Fucoxanthin; HO-1, heme oxygenase 1; IL, Interleukin; *I. galbana, Isochrysis galbana*; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NMSC, non-melanoma skin cancer; Nrf2, nuclear factor E2-related factor 2; ROS, reactive oxygen species; SRB, Sulforhodamine B; TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

INTRODUCTION

Skin is the organ that acts as principal defence against external environment factors, protecting the organism from harmful substances, mechanical damage, pathologic invasion and radiations. In addition, skin regulates body homeostasis by modulation of water loss and body temperature. It is well-known that skin is an indispensable piece of the immune system. The disturbance of skin structure and its functionality could be caused by external factors such as toxic compounds or ultraviolet (UV) radiation, or by internal factors including genetic predisposition, immune and hormone disorders, or stress. The result of these perturbations could trigger the inflammatory process, a lower immune response or an oxidative stress, among others (Fernández-García, 2014).

Inflammatory skin diseases such as psoriasis, atopic dermatitis or rosacea have a significant impact on the quality-of-life of patients. These pathologies have clinical implications because of their chronic curse along the life of patients, which experiment comorbidities that difficult their treatment (Nijsten, 2017). Atopic dermatitis is the most common chronic inflammatory skin disease and its prevalence is increasing in the last years with 15-20 % of children and 1-3 % of adults affected (Yoshihisa et al., 2016). In the same line, psoriasis is an incurable skin disease whose incidence can vary depending to age and geographic region affecting up to 8 % of population. Moreover, it has been related with some comorbidities such as cardiovascular diseases, metabolic

syndrome or Crohn's disease (Griffiths et al., 2017). Other skin alterations are initiated by sun exposure, particularly UVB light (280-315 nm). UVB radiation promotes a strong acute inflammatory response characterized by activation and recruitment of innate immune cells such as neutrophils and macrophages to the epidermis and dermis. In addition, UVB radiation is the main source of reactive oxygen species (ROS) production and it enlarges the inflammatory response, causing DNA oxidative damage in keratinocytes (Duncan et al., 2009). These conditions promote lesions as actinic keratosis (AK), one of the premalignant stages of non-melanoma skin cancer (NMSC), which is the most frequently diagnosed cancer in caucasian people worldwide (Zink et al., 2016).

Currently, natural compounds are taking importance in skin care products due to their potent anti-inflammatory and antioxidant actions as well as the safety and low risk in the administration. Microalgae are a vast source of bio-active molecules with potential activity in skin inflammation, reviewed in Talero et al. 2015. In this sense, carotenoids have shown anti-oxidant, anti-inflammatory or anti-carcinogenic properties in different skin inflammatory models. Fucoxanthin (FX) is an orange carotenoid present in some brown seaweeds but also it can be isolated from microalgae. Specifically, in this work we used FX from the microalga *Isocrhysis galbana (I. galbana)*. This carotenoid has demonstrated antioxidant properties in previous studies (D'Orazio et al., 2012). Particularly, FX has shown to enhance AKT/ nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/glutathione (GSH)-dependent antioxidant response in keratinocytes (Zheng et al., 2014), as well as reduce wrinkles formation and epidermal hypertrophy in mice (Urikura et al. 2011) and supress melanogenesis and prostaglandin synthesis (Shimoda et al., 2010). In addition, FX is proposed as a photoprotective compound by stimulating restoration of the skin barrier in UVA-induced sunburn (Matsui et al., 2016). However, anti-inflammatory/protective effects of a topical formulation containing FX on skin inflammatory pathologies have not yet been described. The possibility to administer FX topically has subjected to several drawbacks because of its molecular weight and lipophilicity. As it is known, the drug must diffuse across stratum corneum and tight junction to achieve effective permeation. In skin disease treatment, topical dosage forms such as creams, ointments, lotions and gels are commonly used for enhancing the cutaneous penetration. Their composition will affect the drug permeation. Concerning FX, several preparations have been studied with this aim (Dai , 2013; Dai and Kim, 2016). In all of these, the evaluation of cutaneous penetration is a useful approach to predict the safety and efficacy of

formulations. To date, we have developed topical experiments in mice with ethanol or acetone-dissolved compounds (Urikura et al. 2011; Arasa et al., 2014). With the aim to solve the irritant effect of this solvent for use in human, in the present work, FX will be dissolved in ethanol absolute to further be incorporated into several common topical formulations. The aim of this study was to evaluate the pharmacological properties of a FX-loaded galenic formulation in the 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced hyperplasia model in mice, which mimics psoriatic parameters in dorsal murine skin, and in the UVB-induced erythema model in hairless mice, which reproduces the consequences expected in humans receiving acute UVB radiation. Herein, we reported the *in vitro* anti-inflammatory and photoprotective effects of FX. In addition, the pre-treatment with the FX-loaded galenic preparation effectively ameliorated TPA-induced hyperplasia by decreasing skin edema, epidermal thickness, MPO activity and COX-2 expression levels. Moreover, we showed that FX reduced UVB-induced erythema in mice thorough down-regulation of COX-2 and iNOS protein expressions as well as up-regulation of the anti-oxidant heme oxygenase 1 (HO-1) protein levels through nuclear factor E2-related factor 2 (Nrf-2) signalling pathway.

MATERIALS AND METHODS

Materials

Dried biomass derived from culture of the microalgae *I. galbana* was provided by the Department of Biotechnology of the Instituto Tecnológico de Canarias (35119 Santa Lucía, Gran Canaria, Canary Islands, Spain). The extraction of the dried biomass of *I. galbana* and structural determination of FX was developed by the Department of Organic Chemistry of the Cadiz University.

Cell culture

The THP-1 human monocytic leukemia cell line and HaCaT human keratinocytes were obtained from the American Type Culture Collection (ATCC, USA). THP-1 cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. HaCaT human keratinocytes were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) with the same proportion of compounds (Aoki-

Yoshida et al., 2013). Both cell lines were grown in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cell viability assay

Sulforhodamine B (SRB) assay was used for determining the viability of THP-1 macrophages and HaCaT cells upon exposure to FX (Skehan et al., 1990). Firstly, for differentiation into macrophages, THP-1 cells (10^4 cells/well) were incubated with phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO) (0.2 µM) for 72 h in 96-well plates and HaCaT cells were seeded into 96-well plates in the growth medium (10^4 cells/well) for 24 h to ensure the adherence. After that, cells were treated with FX at final concentrations range of 10-100 µM in DMSO 0.1% (v/v) and the cytotoxicity was measured after 24, 48 and 72 h of incubation. The absorbance was determined at 492 nm in a microplate spectrophotometer (Sinergy HT, Biotek®, Bad Friedrichshall, Germany).

Determination of TNF-α production

THP-1 monocytes were differentiated into macrophages in 96-well plates (10^4 cells/well). Then, cells were incubated for 1 h with FX (10, 30 and 50 µM). The positive reference compound used was dexamethasone (Dex) (Sigma-Aldrich, St. Louis, MO) (1 µM). Inflammatory response was induced by addition of lipopolysaccharide (LPS, 1 µg/mL) except for control group. After 24 h, commercial enzyme-linked immunosorbent assay (ELISA) kit (Diaclone GEN-PROBE, France) was used to quantify TNF-α according to the manufacturer's protocol.

Determination of IL-6 and IL-8 production

HaCaT cells were seeded in 6-well plates ($5x10^5$ cells/well). After 24 h, cells were washed twice (PBS, 4 °C) and medium was added containing FX (10, 30 and 50 µM) or Dex (1 µM) as reference compound, for 1 h. Then, cells were stimulated with TNF-α (10 ng/mL) except for control group. After 24 h, commercial enzyme-linked immunosorbent assay (ELISA) kit (Diaclone GEN-PROBE, France) was used to quantify IL-6 and IL-8 according to the manufacturer's protocol.

UVB irradiation of HaCaT Keratinocytes

Human keratinocytes were exposed to UVB radiation as previously described (Huang et al., 2010). Briefly, the cells were grown to confluence and were treated with different concentrations of FX (10, 30 and 50 µM) for 1 h. Then, medium was removed and a thin layer of PBS was

added. Cells were exposed to a single dose of UVB radiation (50 mJ/cm^2) for 1 min. The UVB source was a CL-1000M UV Crosslinker (UVP, Upland, CA, USA), which was used to deliver an energy spectrum of UVB light (280-315 nm; peak intensity, 302 nm). After UVB irradiation, the cells were supplied with fresh complete medium and incubated for 24 h.

Analysis of intracellular LDH activity

The measure of cytosolic enzyme lactate dehydrogenase (LDH) is one of the commonly used methods for assessing loss of cellular membrane integrity. Assay is based on the conversion of lactate to pyruvate in the presence of LDH with the consequence oxidation of NADH as previously described (Verhulst et al., 1998). HaCaT cells were seeded in 6-well plates (5x10^5 cells/well). After 24 h, cells were treated with FX (10, 30 and 50 µM) for 1 h and then, were irradiated with UVB. After 24 h, free-cells supernatants and cell lysates were mixed in a 96-well plate and the absorbance was read by using a microplate reader system (Sinergy HT, Biotek®, Bad Friedrichshall, Germany). LDH leakage was estimated calculating the LDH activity in the cell-free medium and LDH activity in lysates ratio. Results were represented as the percentage (%) of change in activity compared with the control cells.

Intracellular ROS scavenging activity

For quantification of ROS in HaCaT cells, the dichlorofluorescein diacetate (DCF-DA) assay was employed (Wang and Joseph, 1999). HaCaT cells were seeded in 96-well plates (10^4 cells/well). Non-irradiated cells were used as negative control. After 24 h, cells were treated with FX (10, 30 and 50 µM) for 1h and then, were exposed to UVB. Post irradiation, cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) solution (5 mg/mL) in PBS for 30 min. The fluorescence of the 2',7'-dichlorofluorescein (DCF) product was determined using a fluorescence plate reader (Sinergy HT, Biotek®, Bad Friedrichshall, Germany) at 485 nm for excitation and 535 nm for emission.

Determination of IL-6 production in UVB-exposed HaCaT keratinocytes

HaCaT cells (5x10^5 cells/well) were seeded in 6-well plates and allowed to attach . After 24 h, cells were treated with FX (10, 20 and 50 µM) for 1 h and then, were exposed to UVB. After 24 h, supernatant fluids were collected and ELISA kit (Diaclone GEN-PROBE, France) was employed to quantify IL-6 according to the manufacturer's protocol. The

absorbance at 450 nm was read by a microplate reader (Labysystem Multiskan EX, Thermo Scientific, NY, USA).

Preparation of topical formulations

Topical creams containing 3 mg/g of drug (beta-carotene (β-Carot), FX or Dex) were prepared using a very simple preparation method of Oil/Water (O/W) emulsion. β-Carot was employed as reference compound because of its structural similarity with FX. The drug was dissolved in absolute ethanol (10 mg/mL) and then was added to an excipient cold mixture composed of Caprylic/Capric Triglycerides, Glycol Stearate, Polyethylene glycol (PEG)-3 Glyceryl Cocoate and Steareth-7, which was previously heated up to 50°C to improve the drug interposition. The preparation was then mixed and stirred until to obtain a homogeneous semisolid formulation.

Lipophilic ointment was prepared using a traditional preparation method of semisolid formulations combining both the melt emulsification and stirring. In brief, a first portion of the oily phase, consisting of Brij® 72 (5 % w/w) and white soft paraffin (77.3 % w/w) were blended under gentle stirring in a water bath at 70 °C following the addition of liquid paraffin (5 % w/w) and α-tocopherol (0.002 % w/w) until complete interposition at 60 °C. Then, β-Carot dissolved in ethanol (10 mg/mL) was added to the mixture. The aqueous phase was prepared by adding two solutions of EDTA (0.0065 % w/w) and disodium phosphate dihydrate (0.026 % w/w) prepared with distilled water at 60 °C dropwise to the lipid phase, with moderate magnetic stirring at 150 rpm for 20 minutes until a homogeneous semisolid formulation was obtained.
For preparing the hydrophilic gel, Carbopol 934P (1% w/v) was soaked in water for 2 h and then dispersed by stirring at 500 rpm (IKA) to obtain a smooth dispersion. After the dispersion was allowed to stand, β-Carot solution in ethanol absolute (10 mg/mL) was gradually added to the polymer dispersion under magnetic stirring. At this stage, triethanolamine was added to the preparation with slow-rate stirring. The presence of ethanol in the preparation makes that no additional preservative is required. The formulations were kept in vacuum overnight in order to escape any entrapped air in the gel base.

***In vitro* permeation studies from topical formulations**

In vitro drug permeation studies from the topical formulations were performed using a Franz diffusion cell apparatus (SES-Gmgh Analyses system, Germany), with 14,5 mL receptor volume and 3,14 cm^2 diffusion area.

An appropriately conditioned regenerated cellulose nitrate membrane (Tuffryn®; Pall Corporation, Port Washington, NY, USA) impregnated with lauryl alcohol (membrane weight increase 90–110%) was used as artificial lipophilic membrane simulating the epidermal barrier (Mura et al., 1993; Maestrelli et al., 2005). The membrane was placed in the diffusion cell sandwiching the donor and receptor compartments. The receiver solution contained absolute ethanol and the donor chamber was filled with 1 g of formulation (cream, ointment or hydrogel). The whole diffusion cell was thermostated maintaining the temperature at 32°C. The amount of all the formulations was equivalent to 3 g of β-Carot, FX or Dex. Aliquots of 0,5 mL were withdrawn from the receptor chamber after 0.5, 1, 1.5, 2 and 3 h and replaced by fresh ethanol. The concentrations of β-Carot and Dex were spectrometrically assayed at 454 nm and 254 nm (UV/vis 1601 Shimadzu), respectively. The FX content in the ethanol solutions was analyzed by using HPLC method. The correction for the cumulative dilution was calculated. FX analysis was performed on a Hitachi Elite Lachron HPLC system equipped with a L-2130 isocratic pump, a diode array detector L-2455 and a L-2200 autosampler. Separation was carried out within a chromatographic C18 column (Merck, RP-18 LichroCART® 150 mm × 4 mm, 5 µm). For drug analysis, the injection volume was 10 mL, and the flow rate and column temperature was set at 1.0 mL/min and 25 °C, respectively. The mobile phase consisted of A (formic acid 0.1%) and B (acetonitrile). The elution program was: 6 min isocratic at 90% B, followed by a gradient to 100% B at 10 min. Afterwards, the column was re-equilibrated during 5 min at 90% B.

The cumulative amounts of permeated drug per unit area in the receiver chamber (µg/cm^2) were plotted as a function of time (h). The slope of the linear portion of the plot was presented as steady state flux (Jss, µg/cm^2/h). From the permeation profiles, the AUC (area under the drug permeation curve) was calculated using the trapezoidal rule as a quantitative parameter for studying the permeation magnitude (Cirri et al., 2015).

Animals

Female Swiss CD-1 mice (25-30 g) were purchased from Janvier-Labs (Le Genest St Isle, France) and female SKH-1 hairless mice (18-20 g) from Charles River Laboratories (France). Mice were maintained on a standard conditions (temperature of 24–25°C, humidity of 70-75% and 12 h light-12 h dark cycle) and were allowed free access to a standard diet (Panlab) and water *ad libitum*. All studies were performed in accordance

with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 2010/63/EU). The experiments followed protocols approved by the Animal Ethics Committee of the University of Seville.

TPA-induced epidermal hyperplasia model and treatments

Female Swiss CD-1 mice (*n* = 10 per group) were used to study the effect of FX from *I. galbana* on TPA-induced hyperplasia in dorsal mice skin (Andrés et al., 2013). Briefly, the dorsal hair of animals was removed with an electric clipper and treated with depilatory cream (Deliplus, Barcelona, Spain) and 24 h later, the animals were assigned to the different groups: Sham (100 mg of cream with 0.2% of ethanol), TPA (20 µl of ethanol containing 2 nmol of TPA per site), TPA-Cream (100 mg of cream and 2 nmol of TPA, dissolved in 20 µl of ethanol), Dex (100 mg per site, equivalent at 200 µg of compound dissolved in ethanol) and FX-cream formulation (100 mg per site, containing 200 µg of FX dissolved in ethanol at 10 µg/µl) were applied on the dorsal skin in an area of 1 cm^2 by using a syringe, during 2 days before the hyperplasia induction. Day 4, TPA was topically applied to the same areas. After 1 h, FX-cream, Dex or vehicle were administered following the mentioned protocol. This procedure was repeated during two consecutive days (Fig. 1).

After 24 h of the last TPA dorsal application (day 7), mice were sacrified by cervical dislocation. Edema was assessed by punch biopsies from the treated dorsal skin. Skin pieces were fixed in 4 % buffered paraformaldehyde for histological studies. Other pieces were frozen in liquid nitrogen for measurement of biochemical parameters.

EXPERIMENTAL GROUPS	
1.Sham	Cream vehicle 100mg
2.TPA control	TPA (20 µl of ethanol containing 2 nmol of TPA)
3. TPA-cream	Cream vehicle 100mg + TPA
3. Dex-cream	100 mg/zone (200 µg of Dex dissolved in EtOH) +TPA
4.FX-cream	100 mg/zone (200 µg of FX dissolved in EtOH)

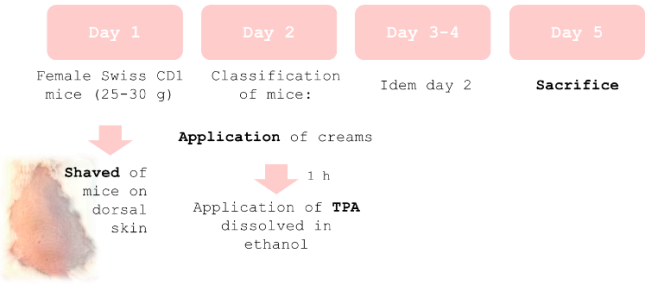

Figure 1: Experimental setting for the TPA-induced epidermal hyperplasia model in mice and treatment groups. TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Dex, dexamethasone; FX, Fucoxanthin; EtOH, ethanol.

UVB-induced erythema in hairless mice

Female SKH-1 hairless mice (*n* = 8 per group) were used to study the effect of FX from *I. galbana* on UVB-induced erythema. Animals were pre-treated with β-carotene-cream (100 mg per site, containing 200 µg of β-carotene dissolved in ethanol at 10 µg/µl) as reference control and FX-cream formulation (100 mg per site, containing 200 µg of FX dissolved in ethanol at 10 µg/µl) or vehicle (cream with a comparable volume of ethanol) (Fig. 2). Sham control mice were treated topically with cream vehicle. The formulations were applied on the dorsal skin (1 cm^2/area) by using a syringe, and starting 2 days before irradiation. On day 3, 30 min after application of the substances, mice were exposed to an acute UVB dose (360 mJ/cm^2) as previously described (Sirerol et al., 2015). 48 h after UVB exposure, mice were macroscopically evaluated and sacrificed. At this end-point time, a pronounced UVB-induced cutaneous inflammation was shown (J Duncan, 2009).

EXPERIMENTAL GROUPS	
1.Sham	Cream vehicle 100mg
2.UVB	Cream vehicle 100mg
3.β-carot cream	100 mg/zone (200 µg of β-Carot dissolved in EtOH)
4.FX-cream	100 mg/zone (200 µg of FX dissolved in EtOH)

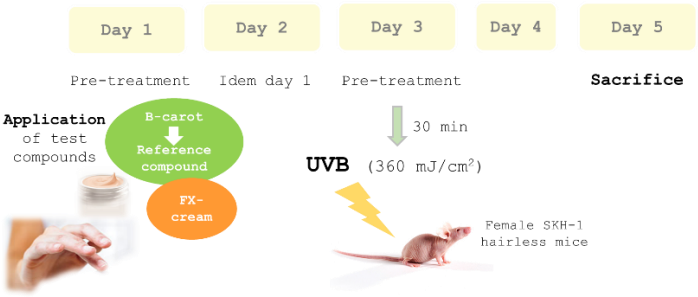

Figure 2: Experimental setting for the of UVB-induced acute erythema model in hairless mice and treatment groups. β-carot, βeta-carotene; FX, Fucoxanthin; EtOH, ethanol; UVB, ultraviolet B.

Dermatoscope measures

Dorsal skin was macroscopically examined every day by using a multi-dermatoscope (Dermatoscope MDS 800, Microcaya, Bilbao, Spain). Corneometer® was used to determinate the hydration in the stratum corneum through electrical capacitance of the skin surface (Haruta-Ono et al., 2012). Cutometer® probe was used as suction method to analyse the real elasticity of skin (Nishimori Y., Edwards C., Pearse A., Matsumoto K., Kawai M., 2001). In the last step, Mexameter® was employed to analyse the redness skin by melanin index (Wang et al., 2001). Every day, dorsal skin mice was assessed twice and finally the results were expressed in arbitrary units (a. u.) or by a melanin index scale (1-36).

Histological study

The samples were fixed in paraformaldehyde, dehydrated by increasing concentrations of ethanol and embedded in paraffin. For haematoxylin and eosin stains, tissue sections were cut at 7 µm on a rotary microtome (Leica Microsystems, Germany), mounted on slides, deparaffinized with xylene, rehydrated through graded alcohols and stained according to standard protocols. All tissue sections were examined under an Olympus BH-2 microscope (GMI Inc., MIN, USA) for determination of histopathological changes. Epidermal thickness was measured by using Scientific Imaging Systems (Biophotonics ImageJ Analysis Software; National Institutes of Health, USA).

MPO activity

For acute inflammatory models, the measurement of myeloperoxidase (MPO) activity was used as a marker of neutrophil infiltration (Grisham et al., 1990). The tissue was thawed, weighed and homogenised in ten volumes of 50 mM-PBS (pH 7.4). Then, the homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C and the pellet was again homogenized in ten volumes of 50 mM-PBS (pH 6) containing hexadecyl trimethylammonium bromide (0.5 %) and 10 mM-EDTA. This homogenate was subjected to one cycle of freezing/thawing and sonicated. The homogenate samples (50 µl) were added to 96-well microplate and incubated at 37 °C for 3 min with a measurement mix (*o*-dianisidine dihydrochloride (0.067 %), hexadecyl trimethyl-ammonium bromide (0.5 %) and 0.3 mM-H_2O_2). The changes in absorbance were monitorized at 450 nm with a microplate reader (Labysystem Multiskan EX, Thermo Scientific, NY, USA). MPO activity unit was defined as the amount of enzyme present that produced a change in absorbance of 1.0 unit/min at 37 °C in the final reaction volume containing acetate. Results are expressed as units/mg tissue.

Immunohistochemical analysis

COX-2 was measured by immunohistochemical analysis by using a streptavidin-biotin-peroxidase method (Talero et al., 2011). Paraffin-embedded dorsal skin sections (7 µm) were mounted on slides, deparaffinized with xylene and rehydrated through graded alcohols. These sections were boiled (10 mM citrate buffer (pH 6.0) for 3 min) for antigen retrieval, followed by cooling at room temperature for 20 min. Endogenous peroxidase was quenched with 0.3 % (v/v) hydrogen peroxide for 20 min and the sections were washed (PBS, 10 min). To minimize the non-specific adsorption the sections were incubated in normal horse serum (Vectastain Kit; Vector Laboratories, Burlingame, CA) for 20 min.

Afterwards, slides were incubated with rabbit polyclonal anti-COX-2 antibody (Cayman Chemical, MICH, USA) (1:300) overnight at 4 °C. Then, the samples were treated with anti-mouse IgG antibody. After 30 min the cells were incubated with the streptavidin–peroxidase complex for 30 min, at room temperature (Vectastain Kit; Vector Laboratories, CA, USA). The enzymatic activities were developed with 3,3´-diaminobenzidine (DAB), and the sections were counterstained with hematoxylin. Negative control sections were treated in the same way, omitting the primary antibody. To examine COX-2 immunoreactivity, the microscope Olympus BX61 was used (Olympus Optical Co. Ltd. Tokyo, Japan). The quantification of immunohistochemical data was done by counting the number of immunostained cells as percent of total epidermal cells from ten microscopic fields of immunostained tissues per animal.

Western Blot Assay

Frozen dorsal skin tissues from UVB-induced erythema model were randomly selected (6 per group), weighed and homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N',N'-tetra acetic acid, 0.5 mM EDTA, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 250 mM NaCl). The homogenates were centrifuged (12,000 g, 15 min, 4°C), and the supernatants were collected and stored at -80°C. To determinate the protein concentration of the homogenates was used the Bradford colorimetric method (Bradford, 1976). Samples of the supernatants with equal amounts of protein (25 µg) were separated on 10 % acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-iNOS (1:1000; Stressgen-Enzo Life Sciences, Farmingdale, USA); rabbit anti-COX-2 (1:3000; Cayman Chemical, Michigan, USA); rabbit anti-Nrf2, rabbit anti-HO-1 (1:1000Cell Signaling, Danvers, MA, USA) overnight at 4°C. After rising, the membranes were incubated with the horseradish peroxidase-linked (HRP) secondary antibody anti-rabbit (Cayman Chemical®, Ann Arbor, MI, USA) (1:1000) or anti-mouse (Dako®, Atlanta, GA, USA) (1:1000) containing blocking solution for 1 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma Aldrich®, MO, USA). Immunodetection was performed using an enhanced chemiluminescence light-detecting kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, IL, USA). Then, the immunosignals were monitorized by using LAS-3000 Imaging System (Fujifilm Image Reader,

USA) and densitometric data were studied after normalization to the control (housekeeping gene). The signals were analyzed and quantified with a Scientific Imaging Systems (Biophotonics ImageJ Analysis Software, National Institute of Mental Health, Bethesda, MD, USA) and expressed as percentage respect to sham control group.

Statistical Analysis

All values in the figures and text are expressed as arithmetic means ± SEM. Data were evaluated with GraphPad Prism version 5.00 software (GraphPad Software, Inc., San Diego, CA, USA). In all cases, the Shapiro-Wilk test was used to verify the normality of the data. The Mann-Whitney U-test was chosen for non-parametric values. The parametric values groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. P values < 0.05 were considered statistically significant.

RESULTS

Effects of FX on TNF-α production in LPS-stimulated THP-1 macrophages and IL-6 and IL-8 production in TNF-α-stimulated HaCaT human keratinocytes.

The anti-inflammatory effect of FX was assayed in both THP-1 macrophages and human keratinocytes. As shown in figure 1, all concentrations tested significantly reduced the production of inflammatory cytokines in both cell models. Interestingly, the pre-treatment with the carotenoid at 50 µM showed a significant reduction of the TNF-α production in LPS-stimulated THP-1 macrophages, reaching values similar to Dex ($P < 0.001$) (Fig. 3a). In relation to IL-6 and IL-8 production in HaCaT keratinocytes, similar results were exhibited in cells pretreated with the highest dose of FX ($P < 0.001$) (Fig. 3b-c).

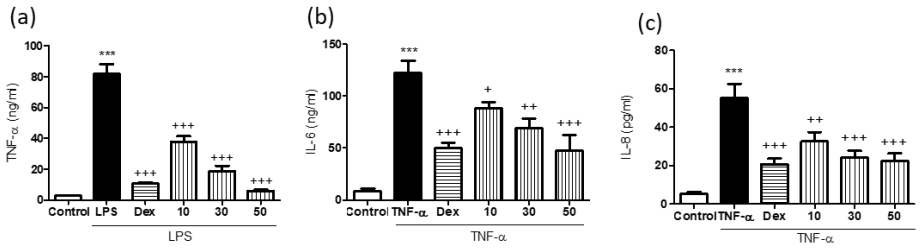

Figure 3. Effects of Fucoxanthin (FX) from *I. galbana* on pro-inflammatory cytokines production in THP-1 macrophages and HaCaT keratinocytes. (a) Tumor necrosis factor alpha (TNF-α) in LPS-stimulated THP-1 macrophages. (b)

Interleukin (IL)-6 and (c) IL-8 in TNF-α-stimulated HaCaT keratinocytes. Cells were treated with FX (10, 30 and 50 µM) for 1 h, and then stimulated with LPS (1µg/mL) in THP-1 macrophages or TNF-α (10 ng/mL) in HaCaT cells for 24 h. Dexamethasone (Dex) was used as positive reference compound at 1 µM. Production of cytokines in supernatant was measured by ELISA assay. Results are representative of six independent experiments. Values are means with standard errors represented by vertical bars. Mean value was significantly different compared with the control group (*** $P < 0.001$; Student *t* test). Mean value was significantly different compared with the LPS or TNF-α group (+$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

***In vitro* permeation studies of β-Carot from different topical formulations**

The objective of this study was to evaluate the *in vitro* permeation through artificial membranes of β-Carot from various topical formulations. β-Carot was selected because of its structural similarity with FX. The formulations tested were kept simple and ranged from ethanolic solution as control to gel, cream and ointment formulations.

The experimental setup used in this study, Franz diffusion cells, consists in β-Carot that leaves an unstirred donor, crosses through a membrane mimicking the skin barrier, crosses an area and accumulates in a stirred receiver for which sink conditions are maintained. Therefore, the transport across the membrane is the rate-controlling step. Moreover, we decided to use absolute ethanol in the receiver chamber because β-Carot is freely soluble in this medium. Finally, β-Carot was detected in the receiver medium in a time-dependent way, and cumulative amount permeated curves have been built (Figure 4).

The permeation profiles showed the cream as the most favorable vehicle as penetration enhancer. When compared with the ethanolic control solution, a high percentage of β-Carot permeated was obtained at a flux very similar to the control solution, as reported in Table 1. However, the ointment and hydrogel preparations made difficult the pass through the membrane, probably due to their lipophilic and hydrophilic nature, respectively. This was visualized in terms of lower permeation percentages and flux rates.

Hydrophilic cream provided the highest release of β-Carot in comparison with lipophilic ointment or hydrogels, as was obtained for flavonoids (Bernatoniene et al., 2011). Therefore, the hydrophilic cream (O/W emulsion) was chosen for incorporation of FX and Dex for further studies. As these emulsions have hydrophilic external phase, they are

miscible with water and skin secretions and thus they are not occlusive and are easily removed from skin.

Formulation	% perm (180 min)	Jss (µg/cm².min)	r^2
Cream	68.38	0.1159	0.9795
Ointment	47.31	0.0548	0.9704
Hydrogel	25	0.0383	0.9403
Control	100	0.1350	0.9862

Table 1: Total percentage permeated across artificial lipophilic membrane of β-Carot from different topical formulations, and flux rate (µg/cm2.min) calculated from the slope of amount permeated per area versus time. % perm, % permeation.

In vitro permeation studies of FX-containing cream

Once the cream was selected as vehicle for drug formulation, FX-loaded cream was prepared following the same methodology than with β-Carot. In addition, Dex-loaded cream was formulated as positive control. The permeation profiles of FX contained in the hydrophilic cream were evaluated with the aim to allow an enhancement in the skin penetration compared to a control solution and Dex in cream.

Results obtained showed a clear enhancement of FX permeated with time (Figure 4). As evidenced from the AUC calculated (48610, 19914 and 8671 % .min for FX-cream, FX control and Dex-cream, respectively), the cream vehicle offered a higher amount of FX permeated with time, whereas the control solution of this molecule showed lower percentages. Although the ethanol acted as permeation enhancer, the cream composition, rich in surfactants, made the formulation to improve the amount of FX to cross the membrane. Surprisingly, the permeation profile of Dex showed a 15% approx. of drug permeated. This lack of diffusion across the membrane could be attributed to a lower partition coefficient than FX (1.83 versus 14.76). Dex has a lower molecular weight than FX, but the lipophilic nature of FX makes it a better candidate to interact with the surfactant components of the cream, which aids the solubilized molecule to reach the receiver medium.

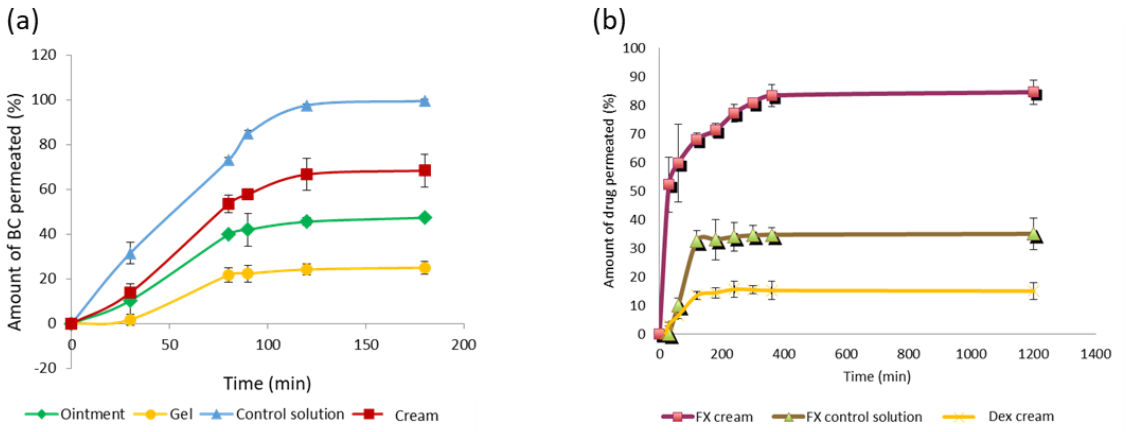

Figure 4. Permeation profiles. a) *In vitro* permeation profile of β-Carotene (BC) from different topical formulations: cream, ointment and gel. β-Carotene dissolved in absolute ethanol was used as control solution. Artificial membranes impregnated with N-dodecanol mimicked the skin barrier. Absolute ethanol was used as release medium. b) *In vitro* permeation profiles of fucoxanthin (FX) and dexamethasone (Dex) from cream formulation. FX dissolved in absolute ethanol was used as control solution. Dex-loaded cream was used as positive control. The experimental procedure was similar than for β-Carot. Data are represented as the mean ± SD (n=2).

Topical application of FX-containing cream decreases skin inflammation and hyperplasia on the murine TPA-induced model

To analyse whether topical pre-treatment with FX could reduce the inflammatory effects *in vivo*, we studied this compound on the murine TPA-induced epidermal hyperplasia model. Topical pre-treatment with FX-cream (100 mg per site containing 200 µg of FX) and Dex (100 mg per site containing 200 µg of Dex) were administered from 2 days before TPA-induced hyperplasia and 1 h after each TPA application (2 nmol per zone for three consecutive days). 24 hours after the last application of TPA, mice were sacrificed and skin biopsies were removed and weighted (Fig. 1). Mice treated with TPA or TPA-cream revealed an increment of weight of dorsal skin, in comparison with sham group ($P < 0.001$), with no significant differences between both groups (Fig. 5a). This comparison led us to confirm that cream did not interfere in TPA action in our experiment. As expected, pre-treatment with the reference compound Dex significantly reduced the skin punch weight ($P < 0.001$). Similarly, mice treated with FX-cream showed a significant attenuation of skin edema when compared with TPA-cream group ($P < 0.001$) (Fig. 5a).

We next analysed the skin homogenates to evaluate MPO activity as a neutrophil infiltration parameter with important relevance in hyperplasia model (Fig. 5b). Our results confirm that this marker significantly increased after TPA application in comparison with sham group ($P < 0.01$). Dex application markedly diminished MPO activity in relation to TPA-cream group ($P < 0.01$). In a similar way, this parameter was reduced by FX-cream ($P < 0.05$) (Fig. 5b). These results were confirmed with histological analysis of H&E-stained skin lesions in mice (Fig. 5c), which showed that TPA administration produced a massive neutrophilic infiltration and an epidermal hyperplasia, because of uncontrolled and abnormal keratinocyte production. This effect was evidenced by a marked increase of epidermal thickness in this group ($P < 0.001$). Animals pre-treated with Dex and FX-cream evidenced a marked improvement in epidermal hyperplasia in relation to TPA-cream group ($P < 0.001$, and $P < 0.01$, respectively) (Fig. 5c-d).

To support the beneficial effects of FX on skin inflammation and explore its possible mechanism of action, we examined the COX-2 expression in skin samples (Fig. 5e and f). This enzyme has been shown to have an important role in pathogenesis of skin diseases. Immunohistochemical analysis of this enzyme showed that TPA-induced hyperplasia significantly increased COX-2-positive cell numbers ($P < 0.001$), predominantly localized in epidermal layer, when compared with sham (Fig. 5e). As shown in Figure 5e and 3f, the results of mice pre-treated with Dex confirmed the anti-inflammatory effect of this corticoid ($P < 0.001$). Interestingly, pre-treatment with FX-cream resulted in a significant downregulation in the number of epidermal COX-2-positive stained cells in comparison with TPA-cream group, reaching expression levels lower than those in Dex group ($P < 0.001$).

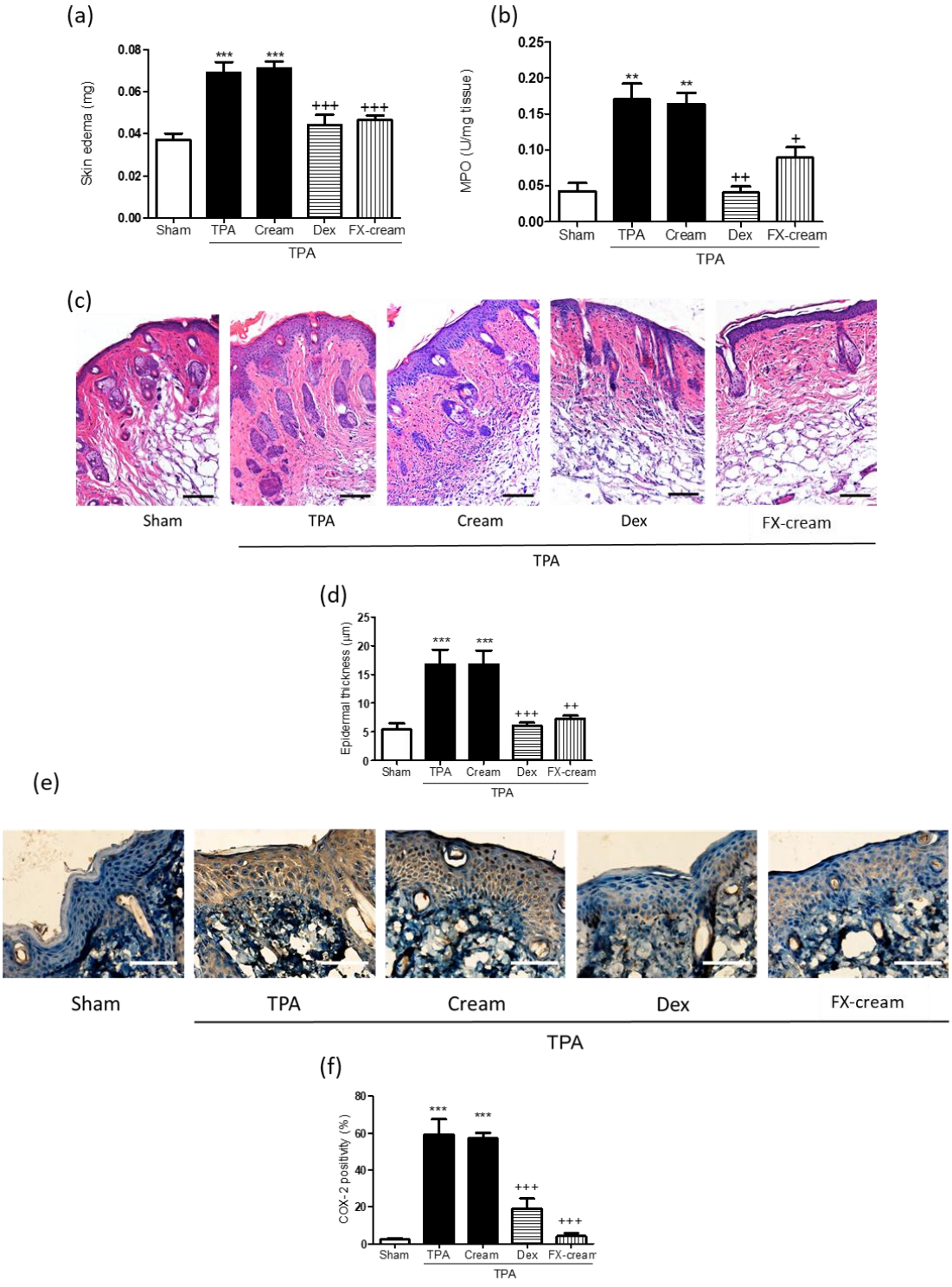

Figure 5. Fucoxanthin (FX) ameliorates skin hyperplasia and inflammation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mice (n = 8 mice/group). FX-cream formulation (100 mg per site at 200 µg), Dexamethasone (Dex, 100 mg per site at 200 µg) or vehicle (cream with 0.2 % ethanol) were topically administered as described in Material and methods. (a) Skin edema as punch biopsy. (b) Myeloperoxidase (MPO) activity. (c) Histological appearance of mouse dorsal skin after haematoxylin/eosin (H&E)-staining; Bar = 10 mm. Original magnification

100x (d) Epidermal thickness assessment in H&E-stained skin slides. (e) Representative photographs of epidermal COX-2 distribution by immunohistochemical detection; Bar = 12.7 mm. Original magnification 200x. (f) Percent of COX-2 positivity in epidermal layer was assessed by counting the number of COX-2 positive cells *versus* total cells from 10 equal sections of immunostained dorsal skin per animal (n=3) Values are means with standard errors represented by vertical bars. Mean value was significantly different compared with the sham group (**P < 0.01, ***P < 0.001; Student *t* test). Mean value was significantly different compared with TPA-cream group (+P < 0.05, ++P < 0.01, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

FX protects human HaCaT keratinocytes against UVB-caused damage.

To examine the protective effect of FX in irradiated HaCaT cells, we evaluated cell viability by LDH enzyme activity, ROS levels and IL-6 production after exposure to UVB. As expected, UVB irradiation significantly increased LDH activity, ROS content and IL-6 production in HaCaT keratinocytes compared to unirradiated control (P < 0.001) (Fig. 6a-c). Pre-treatment of cells with FX 1 h prior to UVB exposure significantly decreased UVB-induced mortality, preserving cell membrane integrity in a dose-dependent manner at all concentrations used (10, 30 and 50 µM, P < 0.05, P < 0.01, P < 0.001, respectively) (Fig. 6a). We next evaluated intracellular ROS levels in UVB-irradiated cells based on the DCF-DA assay (Fig. 6b). FX markedly reduced ROS levels by 23, 31 and 32 % at the concentration of 10, 30 and 50 µM, respectively (P < 0.01, P < 0.001, P < 0.001) (Fig. 6b).

It is known that UVB exposure induces abnormally augmented cytokine production from keratinocytes, leading to inflammatory skin disorders. To evaluate the effect of FX on IL-6 production, HaCaT cells were pre-treated for 1 h with this carotenoid, and then were exposed to UVB. Pre-treatment with FX at the concentration tested of 10, 30 and 50 µM substantially inhibited this cytokine levels by 23, 31 and 59 %, respectively (P < 0.001) (Fig. 6c).

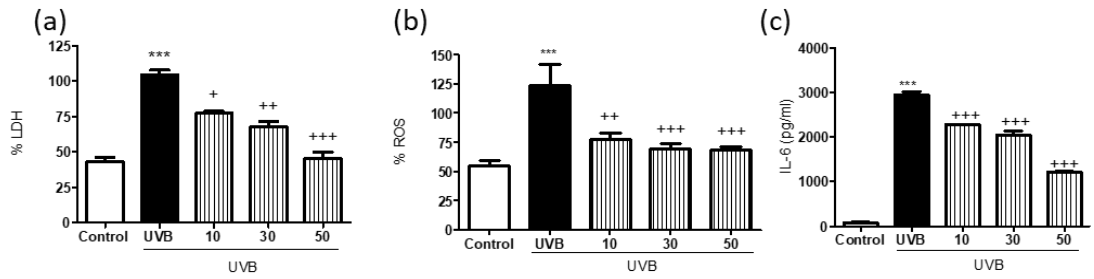

Figure 6. Protective effect of Fucoxanthin (FX) on UVB-induced damage in HaCaT cells. Keratinocytes were preincubated with FX (10, 30 and 50 µM) for 1 h prior to UVB (50 mJ/cm^2) exposure. (a) After 24 h, cell viability was assessed by using Lactate deshydrogenase (LDH) assay. (b) Intracellular ROS generation was measured 30 min after UVB irradiation by relative fluorescence intensity using DCF-DA assay. (c) IL-6 production was evaluated by ELISA assay, 24 after UVB exposure. Cell viability and ROS production are expressed as percentage respect to UVB-irradiated cells and IL-6 levels as pg/mL. Results are representative of four independent experiments. Values are means with standard errors represented by vertical bars. Mean value was significantly different compared with the control group (***P < 0.001; Student *t* test). Mean value was significantly different compared with UVB group (+P < 0.05, ++P < 0.01, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

Topical application of FX-containing cream protects against UVB-induced skin erythema in SKH-1 hairless mice

The possible photoprotective effect of the topical pre-treatment with FX in hairless mice was assayed using an acute proinflammatory UVB dose (360 mJ/cm^2). β-carotene was employed as reference compound due to its antioxidant and photoprotective activity (Stahl and Sies, 2012). Animals were examined with a dermatoscope for five days, evaluating UVB-induced skin alterations (Stahl and Sies, 2012). As shown in Figure 7, a single UVB exposition accelerated skin damage, showing typical symptoms as loss of moisture, reduction of elasticity and increase of melanin production in comparison with sham controls (Fig 7a-c). The progressive evaluation of mice showed that pre-treatment with FX increased the skin moisture (Fig. 7a) and elasticity (Fig. 7b) and decreased the production of melanin (Fig. 7c), with similar values to β-carotene. In order to confirm the protective profile of FX, 48 h after UVB irradiation, the mice were sacrificed and dorsal skin was examined. Similarly to hyperplasia model, skin edema, MPO activity and epithermal thickness were assessed. Our data reflected significant increase of these levels in UVB-irradiated group when compared with sham (P < 0.001). The reference group, β-carot-cream, showed pronounced lower levels of these parameters when compared to UVB-irradiated group (P < 0.001, P < 0.05, P < 0.001, respectively) (Fig. 7d-f). In a similar way, mice pre-treated with FX-cream evidenced a reduction of skin edema, MPO activity and epidermal thickness in a significant manner (Fig. 7d-f). These results were confirmed by histological study by using H&E staining. Histologically, we observed a significant increase of superficial skin layer thickness due to unregulated keratinocytes proliferation in UVB-

irradiated animals group in comparison to sham ($P < 0.001$) (Fig. 7f-g). FX-cream treatment was as effective as topical application of β-carotene; our data revealed a decrease in keratinocytes proliferation when compared to UVB-irradiated group, evidenced by a significant reduction of epidermal thickness ($P < 0.001$) (Fig. 7f-g). To further explore the possible mechanisms of action of FX, we examined the expression levels of different inflammatory and anti-oxidant proteins in dorsal skin samples. Exposure of skin to UVB induced a significant increase in the pro-inflammatory enzymes COX-2 ($P < 0.01$) and iNOS ($P < 0.01$) expression (Fig. 8b-c). Neither of these proteins changed significantly in animals treated with the reference compound β-carotene. However, pre-treatment with FX resulted in a significant downregulation of COX-2 expression levels in irradiated mice ($P < 0.01$) in comparison with not treated mice. As regards iNOS protein, although its expression tended to decrease in FX-cream-treated animals, no significant differences were observed in relation to UVB-treated mice.

UVB exposure significantly down-regulated the expression of Nrf-2 protein ($P < 0.01$) and its target gene HO-1 ($P < 0.05$) (Fig. 8d-e). β-carot-cream application induced a marked increase in Nrf2 levels ($P < 0.001$) accompanied with a rise of HO-1 ($P < 0.05$). In a similar way, FX-cream significantly increased these anti-oxidant proteins ($P < 0.05$) in UVB-exposed skin.

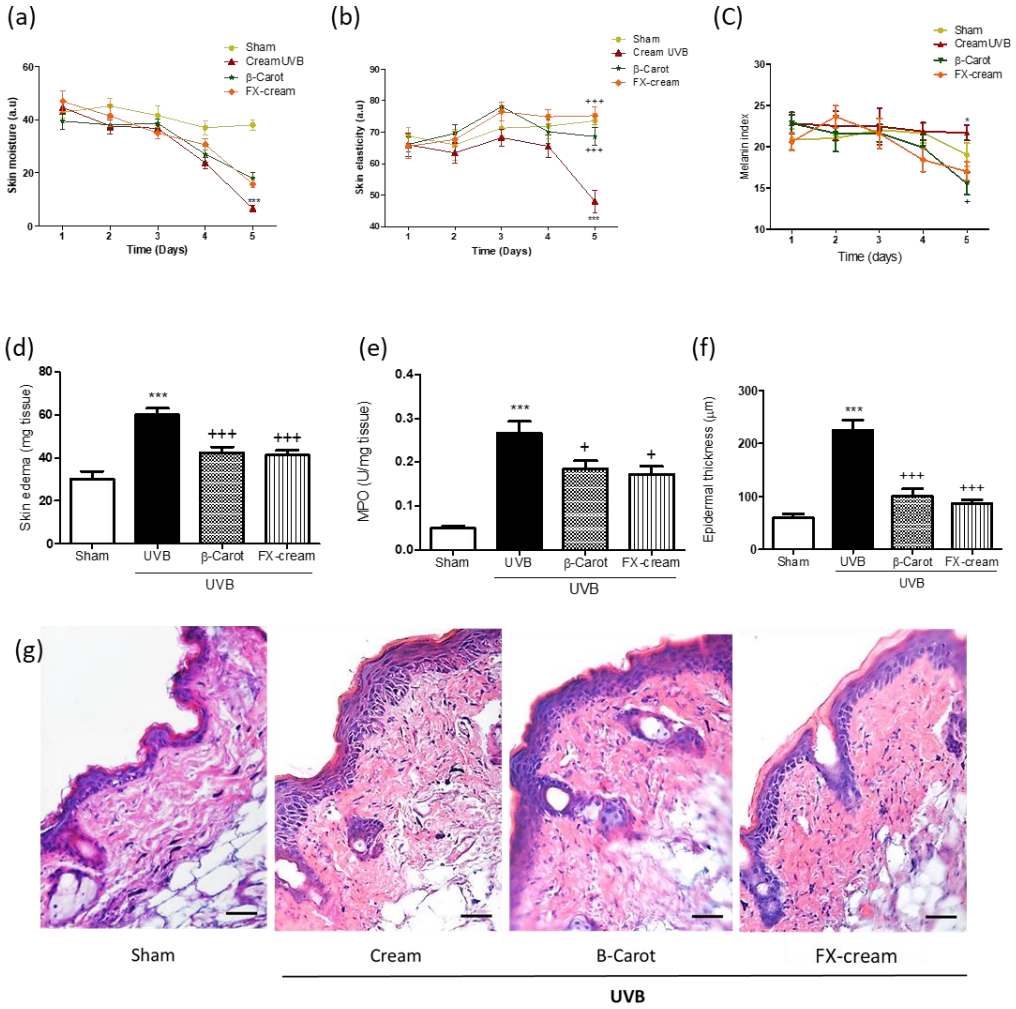

Figure 7. Fucoxanthin (FX) has photoprotective effects in UVB-induced erythema model in hairless mice (n = 8 mice/group). Mice received a single UVB radiation dose of 360 mJ/cm^2. FX-cream formulation (FX-cream, 100 mg per site at 200 µg), β-carotene-cream (β-carot, 100 mg per site at 200 µg) or vehicle (cream with 0.2 % ethanol) were topically administered as described in Material and methods. (a) Skin moisture, (b) skin elasticity and (c) melanin index were evaluated during all experiment. (a.u), arbitrary units. (d) Determination of skin edema as punch biopsy weight. (e) Mieloperoxidase (MPO) activity. (f) Measurement of the epidermal thickness in haematoxylin/eosin (H&E)-stained skin slides. (g) Photomicrographs of mouse dorsal skin after (H&E)-staining; Bar = 10 mm. Original magnification 100x. Values are means with standard errors represented by vertical bars. Mean value was significantly different compared with the sham group (***P < 0.001; Student *t* test). Mean value was significantly different compared with UVB-exposed group (+P < 0.05, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

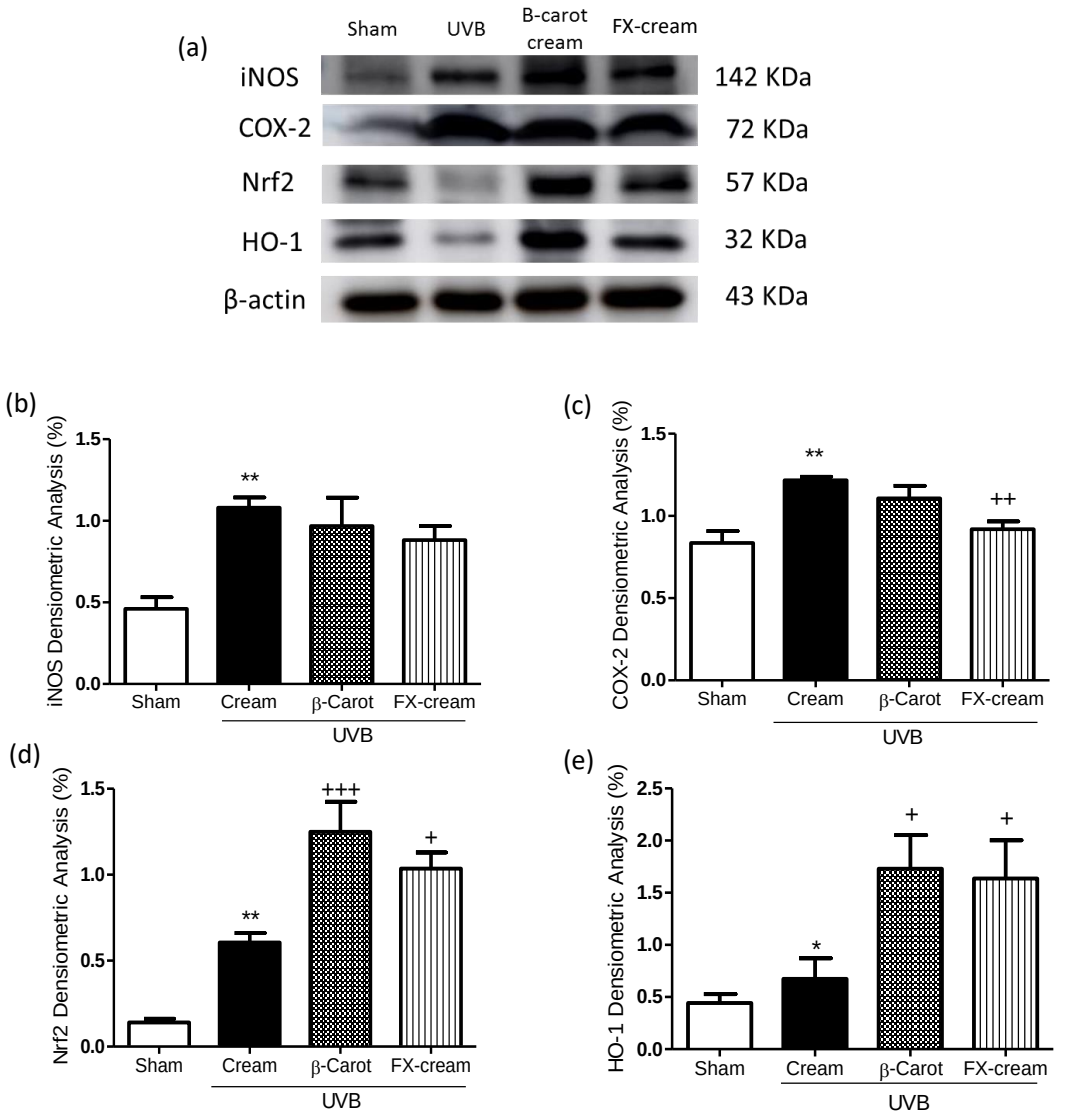

Figure 8. Anti-inflammatory and antioxidant effects of Fucoxanthin (FX) in UVB-induced erythema model in hairless mice (n = 8 mice/group). Mice received a single UVB radiation dose of 360 mJ/cm^2. FX-cream formulation (100 mg per site at 200 µg), β-carotene-cream (β-carot, 100 mg per site at 200 µg) or vehicle (cream with 0.2 % ethanol) were topically administered as described in Material and methods. (a) Representative Western blot images of different skin proteins. Densitometric analysis of (b) inducible nitric oxide synthase (iNOS), (c) cyclooxygenase-2 (COX-2), (d) nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and (e) heme oxygenase (HO-1) proteins. Data were studied following normalization to the control (housekeeping gene, β-actin). Values are means with standard errors represented by vertical bars. Mean value was significantly different compared with the sham group (*P < 0.05, **P < 0.01; Student *t* test). Mean value was significantly different compared with UVB-exposed group (+P < 0.05, ++P < 0.01, +++P < 0.001 one-way ANOVA followed by Bonferroni's Multiple Comparison test).

Discussion

Microalgae are a rich source of bioactive compounds, including carotenoids as FX. This carotenoid have formerly demonstrated *in vitro* and *in vivo* anti-inflammatory and anti-oxidant activities, (Kim et al. 2010; Zheng et al. 2014b ; Matsui et al. 2016). However, the use of topical formulations containing FX, as cream/emulsion, to prevent skin inflammatory pathologies has not been previously evidenced. Treatment of the skin with these formulations is a rational approach since avoids gastrointestinal degradation and preserves bioavailability.

Firstly, in the present study, we confirmed the *in vitro* anti-inflammatory activity of FX through decrease in TNF-α production in LPS-stimulated macrophages and reduction of the production of the pro-inflammatory cytokines IL-6 and IL-8 in TNF-α-stimulated HaCaT keratinocytes. These results led us to evaluate the possible preventive effects of this carotenoid on two *in vivo* acute skin inflammatory models: a hyperplasia model induced by multiple applications of TPA and an erythema model induced by a single UVB challenge.

Drug delivery across the skin remains as an important challenge in the development of drug delivery systems. This is mainly attributed to the highly-organized of intercellular lipids and poor permeability of stratum corneum. It is also well-known that lipophilic molecules move across the barrier by a transcellular mechanism whereas hydrophilic agents are likely to follow a paracellular pathway to cross the skin.

FX is water-insoluble and has a high partition coefficient (log P 6.83-7.54). To date, some studies with FX have been realized by dissolving FX in ethanol for applying the drug dissolved onto the skin (Urikura et al., 2011). To improve its topical application, various hydrophilic and lipid-based formulations have been developed, by using β-Carot as model drug because of its structural similarity to FX (Mahamongkol et al., 2005). In this sense, is necessary some options to avoid other formulations less comfortable as vaseline (Shimoda et al., 2010). In order to have a prediction of the *in vivo* permeation behaviour of the drug and to avoid the expensive cost of the use of animals or human skin, alternative artificial skin diffusion apparatus was used for conducting *in vitro* permeation studies. Among the formulations tested (cream, ointment and hydrogel), it was found that lipid-based formulations improved most efficiently the diffusion of β-Carot through the permeation membrane in a Franz diffusion cell. In contrast, water-based formulations, such as Carbopol gel, exhibited poor penetration.

The composition of cream, which is highly charged in surfactants, makes it to act as a permeation enhancer that changes the environment of the lipids to promote the diffusion of lipid molecules (β-Carot or FX) and/or influence their solubility (Williams and Barry, 2012). The blend of agents with polar and non-polar properties, which probably mimic the complex lipid/polar nature of the stratum corneum, makes the cream thermodynamically similar to the stratum corneum, facilitating the permeation of β-Carot through the skin. Other authors incorporated surfactant-like molecules (D-a-tocopheryl polyethylene glycol 1000 succinate) into the formulations for synergistically act with ethanol as solubilizing agents of griseofulvin (Aggarwal et al. 2012). This is the reason because formulations too occlusive and lipophilic (ointment) make difficult the permeation process of poorly water-soluble compounds. On the contrary, concerning to highly hydrophilic formulations (gel), it is known that water can generate a resistant boundary at the donor-skin interface (impregnated in our artificial membranes with dodecanol) and may prolong or delay the permeation of poorly water-soluble molecules such as β-Carot.

Once selected the cream as vehicle for our studies, we proceeded to dilucidate the FX behaviour in terms of permeation kinetics through a comparative study with control solution and Dex cream. This carotenoid has been previously formulated in oil-in-water formulations by other authors (Dai J, 2013). Our results show that, under the experimental conditions used, the FX cream has a higher permeability though artificial membrane than the other formulations (control solution FX and Dex cream). Other authors reported that the differences in penetration might be associated with the variation in the lipophilicity of the tested compounds (Taofiq et al., 2017), as was obtained in this case. Although Dex has a lower molecular weight than FX, which might favor the diffusion across the membrane, its low lipophilia (log P 1.83) compared to FX, makes more difficult this passage, showing permeation profiles more reduced (Xie et al., 2016).

The efficacy of FX-loaded O/W emulsions has been demonstrated as anti-obesity formulations being prepared using medium chain triglyceride as an oil phase and l-α-phosphatidylcholine as an emulsifier. This heterogeneous disperse system has been also used for incorporating other antioxidant molecules such as green tea polyphenols with the aim of preventing UVB-induced oxidation of lipids in mouse skin (Bernatoniene et al., 2011). Thus, hydrophilic cream may serve as an optimal delivery system for FX use in animal models.

TPA is an activator of protein kinase C isoenzymes, and well-known inducer of inflammatory response in murine skin rising expression of the inflammation mediators (Laihia et al., 2012). TPA-induced hyperplasia model in dorsal murine skin reproduces typical manifestations of inflammatory skin pathologies as psoriasis. In this regards, TPA administration causes macroscopic lesions such as erythema, as well as increment of epidermis thickness by hyperproliferation and aberrant differentiation of keratinocytes or leukocytes infiltration into the dermis and consequent cytokines and chemokines production (Perera et al., 2012). Our study has shown the pre-treatment with FX-cream significantly reduced skin edema and MPO activity. These results were related with the improvement of histological damage by reducing epidermal thickness and neutrophil infiltration, getting similar levels to those of the reference corticosteroid dexamethasone.

The relation between epidermal differentiation and COX-2 expression has been strongly reported, as well as their connection with the pathogenesis of psoriasis (Ikai, 1999). In this sense, repeated applications of TPA in dorsal murine skin causes inflammation symptoms that are accompanied by COX-2 epidermal overexpression in relation with healthy group (Andrés et al., 2013). Our data evidenced a reduced COX-2 expression in mice treated with FX in comparison with that in TPA group. These findings, at least partly, suggest that suppression of COX-2 expression may be involved in the preventive effect of this carotenoid on TPA-induced epidermal hyperplasia. Our data are in agreement with a previous *in vitro* study that showed the capacity of FX to modulate inflammatory response through inhibition of COX-2 and iNOS expression and the consequent decrease of NO and PGE_2 levels (Kim et al., 2010). More recently, FX has shown to downregulate COX-2 levels in a murine model of high-fat-diet-induced obesity (Tan and Hou, 2014).

This experimental model has revealed for the first time the anti-inflammatory activity of a topical formulation containing FX from *I. galbana* in skin dorsal hyperplasia induced by TPA, suggesting its use in inflammatory pathologies such as psoriasis or dermatitis. Additionally, it is worth to highlight that since these conditions benefit from therapeutic sun exposition, it is very important to assure the photoprotection of exposed skin. Sunscreens are used to prevent the deleterious effects of UVB; however, its limited efficacy supports the need of further therapeutic strategies targeting UVB inflammation and oxidative stress. Thus, our next objective was to examine the photoprotective effects of FX in an UVB-induced cell damage model in

HaCaT keratinocytes, as well as on a UVB-induced erythema model in hairless mice.

UVB irradiation has been reported to induce cell cytotoxicity through loss of cellular membrane integrity, which leads to liberation of LDH enzyme from cytosol to the culture medium (Lakatos et al., 2013). Moreover, UVB radiation causes increased ROS production and DNA damage, as well as a strong inflammatory response, characterized by the production of inflammatory cytokines, such as IL-6, which leads to premature skin aging and carcinomas (Leerach et al., 2017). Recently, it has been demonstrated that the anti-oxidative role of FX in HaCaT keratinocytes is related with DNA protection against oxidative stress and the prevention of apoptosis (Heo and Jeon 2009; Zheng et al. 2013). According with these results, we have shown that pre-treatment of HaCaT keratinocytes with FX significantly attenuated UVB-induced damage by increasing cell viability and inhibiting ROS and IL-6 levels.

Acute UVB radiation promotes erythema, edema and loss of skin moisture and elasticity, as well as increases melanin production, which is involved in melanogenesis. Recently, several studies have revealed the photoprotective effects of FX in UVB-irradiated mice by reducing melanogenesis parameters (Shimoda et al., 2010) and in UVA-induced sunburn by promotion of skin barrier formation (Matsui et al., 2016). In addition, this carotenoid prevented skin photoaging in hairless mice through its antiangiogenic and antioxidant effects (Urikura et al. 2011). Herein, we report that the topical pre-treatment with FX-cream formulation ameliorated erythema induced by UVB as well as edema and MPO activity, which are important inflammation and neutrophil recruitment parameters. Overexpression of COX-2 is highly related with some carcinomas including skin cancer (Kanekura et al. 2000). On this regard, and in accordance to the above results from TPA model, we also reported the reduction of COX-2 expression by FX-cream pre-treatment on UVB-exposed skin.

It has been reported that excessive generation of ROS results in oxidative stress in skin cells and plays an essential role in the initiation, promotion and progression of skin aging, carcinogenesis and many inflammatory disorders (Fehér et al., 2016). Nrf2 is a transcription factor that perceives variation in cellular oxidative stress and promotes the transcription of antioxidant genes and detoxification enzymes such as Heme-oxygenase 1 (HO-1) to protect against UVB-induced oxidative damage (Zhong and Li 2016; Furue et al. 2017). Our data show that the pre-treatment with FX increased the expression of Nrf2 and its downstream

target HO-1. In line with our *in vivo* results, an *in vitro* study has elicited the anti-oxidant activity of FX by enhancing the Akt/Nrf2/GSH pathways in human keratinocytes (Zheng et al., 2014). Our findings are in accordance with recent studies that suggest the UVB oxidative damage could be counterated by antioxidant supplements, acting as a valuable strategy for reducing UVB-induced oxidative stress and skin damage (Sun et al., 2016).

In conclusion, topical application of FX-containing cream, isolated from the microalga *I. galbana* has shown to have anti-inflammatory effects in hyperplasic skin, reducing cell infiltrate and epidermal COX-2 expression, which has a main role in the progression of hyperplasia and the consequent skin damage. In addition, this carotenoid protects mice against superficial skin damage induced by UVB through inhibition of inflammatory mediators and promotion of antioxidant responses. For these reasons, this natural carotenoid, administered in a topical formulation, which improvement its permeation, could be a novel approach for preventing psoriatic exacerbations as well as protecting skin against UV radiation.

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CAPÍTULO IV

EFFECT OF ROSMARINIC ACID IN NANOTRANSETHOSOMES TOPICALLY APPLIED ON IMIQUIMOD-INDUCED PSORIASIS ANIMAL MODEL

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ABSTRACT

Nowadays, is necessary the search for new alternatives for psoriasis management, because the majority of patients remain untreated or undertreated, which lead to poor adherence and dissatisfaction with the current therapies. In this line, an interesting option is the use of natural products as polyphenols, concretely rosmarinic acid (RA). Nevertheless, its topical use by incorporation in dermocosmetic is limited due to the poor water solubility, discolouration and chemical instability. In this regard, anionic nanotransethosomes represent a type of phospholipid vesicles developed due to well-known skin permeation enhancing effect of ethanol, offering enhancer properties to the vesicles. The aim of this study was encapsulate two compounds, RA and dexamethasone (Dex), in this type of nanoliposomes (NL) and to evaluate their anti-inflammatory activity on Imiquimod-induced psoriasis-like skin inflammation mice model. Our *in vivo* study did not yield the expected results: RA-NL had not a significant pharmacological effect whereas Dex-NL gave statistical significance in all the parameters evaluated. A possible explanation of these results could be related to the different ionization characteristics of both molecules. In this sense, we can determinate that the ionization constant (pKa) could become the limiting factor for drug release rate, permeation and therapeutic effect.

KEYWORDS: nanotransethosomes, nanoliposomes, rosmarinic acid, inflammation, psoriasis, skin.

ABBREVIATIONS: Ch, cholesterol; Dex, Dexamethasone; EPC, L-α phosphatidylcholine from egg-yolk; IL, Interleukin; IMQ, Imiquimod; MPO, myeloperoxidase; NL, nanoliposomes; PdI, polidispersity index;

pKa, ionization constant; RA, Rosmarinic acid; SDC, sodium deoxycholate; TNF-α, tumor necrosis factor alpha.

INTRODUCTION

Psoriasis is a common immune-mediated inflammatory skin disease that affects at 2-4 % of the population (An et al., 2016). Nowadays, treatment of this pathology comprises a wide range of possibilities, including topical agents (corticoids, vitamin D derivatives, and retinoids) and phototherapy for mild cases as well as systemic treatment (immunosuppressants and biological drugs) for severe cases. However, some of these treatments can cause severe side effects. Moreover, the majority of patients remain untreated or undertreated, which lead to poor adherence and dissatisfaction with the therapies (Feldman et al., 2016). For this reason, it is necessary the search for new alternatives for the management of psoriasis. In this line, an interesting option is the use of natural products as polyphenols. Rosmarinic acid (RA) is a phenolic ester widely studied and with remarkable biological and pharmacological activities (Amoah et al., 2016). Nevertheless, its topical use by incorporation in dermocosmetic is limited due to the poor water solubility, discolouration and chemical instability (Kim et al., 2010).

Nowadays, the development of delivery systems to administer certain compounds is a key to improve the treatment of psoriasis (Vogt et al., 2016) (Doppalapudi et al., 2017a). Among them, the use of lipid vesicles has been widely developed in this research field. Concerning the permeation mechanism, there is a controversy, because some studies have illustrated that the *stratum corneum* serves as a short-term reservoir for storing nanoparticles in contrast to the hair follicles because of depletion of the dead *stratum corneum* layer through desquamation and replacement with new cells. In contrast, other authors defended the passage of certain lipophilic substances through the skin by utilizing the intercellular lipid matrix and diffusing further down into the epidermis whilst few others permeate directly across the corneocytes into the deeper skin layers.
Liposomes are phospholipid vesicles composed of one or more phospholipid bilayers surrounding inner aqueous compartments. These biodegradable and biocompatible nanocarriers have the advantage of possessing a lipid composition similar to the intercellular lamellae and keratinocytes, which increase the affinity of them for the skin surface. In addition, they act as penetration enhancers, altering the intercellular lipid matrix. The versatility of these vesicles to

entrap hydrophilic molecules in the aqueous phase and also lipophilic and amphiphatic substances into the lipid bilayer makes liposomes very interesting systems for skin drug delivery (Bozzuto and Molinari, 2015). However, other vesicles have been developed in order to enhance and optimize the penetration process of drugs (Ascenso et al., 2014)(Hua, 2015). Among them, transfersomes, also named elastic or ultradeformable vesicles represent a relatively novel type of phospholipid vesicles designed to penetrate through the skin since they present an edge activator (single chain surfactant), which destabilizes the phospholipid bilayers of vesicles by increasing their deformability (elasticity), squeezing through pores in *stratum corneum* (Romero and Morilla, 2013). Recently, Doppalapudi et al (2017c) have formulated ultradeformable liposomes co-loaded with psoralen and resveratrol for evaluation of PUVA and anti-oxidant combination in vitiligo treatment. They found that, compared to control, these nanocarriers stimulated significantly the melanin production and tyrosinase activity, also exhibiting potential free radical scavenging activity.

Therefore, the presence of ethanol in the formulation contributes to improve the permeating characteristics of vesicles, named in this case ethosomes, due to well-known skin permeation enhancing effect of ethanol (Touitou and Godin, 2007).

In some cases, both strategies above mentioned are present, resulting in new structures known as transethosomes, which are mainly used when the drug has limited water solubility and the addition of ethanol is needed. Important contributions have been reported by Song et al. (2012) and Ascenso et al. (2015), who studied the enhanced both *in vitro* and *in vivo* skin deposition of voriconazole and vitamin E, respectively, in the dermis/epidermis region compared to conventional vesicles and deformable liposomes.

In skin drug delivery, many studies have focused on the modification of permeation properties of the drug as a function of the type of vesicles developed, the lipid composition and the surface charge of vesicles (Kasetvatin et al., 2015). Very few of these results have been evaluated *in vivo*, thus, conclusions obtained cannot be extrapolated to the animal or human behavior. *In vitro* drug release and permeation studies have been used as the most important methods to assess the quality of the formulation. In this regards, the *in vitro* release testing provides detailed information about drug release mechanism and kinetics, allowing the establishment of an adequate *in vitro*/*in vivo* correlation (Balzus et al., 2016). Regarding the

permeation results, hair follicles have been recently suggested to play a critical role in the permeation of nanocarriers across the *stratum corneum* compared to the conventional transcellular and intercellular mechanisms. Although the authors suggested that the surface charge of nanocarriers plays an important role in the extent of permeation into the skin layers and onto the *stratum corneum* (Baroli, 2010)(Boakye et al., 2015), the studies are not definitive to conclude that nanocarriers permeate the skin mostly by the transfollicular pathway. Similar results have been obtained by (Liu et al., 2017).

On the basis of these approaches and taking into account the relevance of charge interaction vesicle-skin, one important parameter to be considered in these systems is the ionization constant (pKa) of the drug because the ionized fraction can be located into the lipid bilayer (in the case of lipophilic drugs), modifying the surface charge of vesicles. This parameter could become the limiting factor for drug release rate, permeation and therapeutic effect.

In this work, an interesting study has been carried out because two tasks were conjugated: firstly, the analysis of the effect of ionization constant of two substances used as antipsoriasic drugs (RA and dexamethasone (Dex)) on the release rate and secondly, the evaluation of *in vivo* efficacy of transethosomes in an imiquimod-based animal model.

MATERIALS AND METHODS

Materials

RA (98%) from *Rosemarinus officinalis L.*, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). RA was dissolved in ethanol at 10 µg/µL. L-α phosphatidylcholine from egg-yolk (EPC), cholesterol (Ch) and sodium deoxycholate (SDC) were purchased from Sigma-BioChemika (Steinhein, Germany). Trichloromethane, methanol, ethanol and 2 - [4 (2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Hepes) were obtained from Panreac Química (Barcelona, Spain). Polycarbonate membranes with 800, 200 and 100 nm pore-size were purchased from Millipore (County Cork, Ireland). All other chemicals were of analytical degree.

Quantification of RA by HPLC and Dex by UV-visible spectrophotometry

The amount of RA was evaluated by applying an HPLC method, slightly modified from (Wang et al., 2004). The method was developed

using the Hitachi Elite LaChrom system equipped with a L-2130 isocratic pump, a diode array detector L-2455 and a L-2200 autosampler. The separation was performed on a C18 (Merck, RP-18 LichroCART (150 mm x 4 mm, 5 µm) column thermostated to 30°C. The mobile phase consisted of solvents A (0.1 % v/v orthophosphoric acid in water) and B (0.1 % v/v orthophosphoric acid in methanol) using the following gradient elution: 40 % of B at time 0, 50 % of B at 10 min, 60 % of B at 15 min and then returned to the initial conditions after 5 minutes reequilibrating. The total run time was 20 minutes. The analysis was carried out at a flow rate of 1 mL/min, 10 µL injection volume, with the detection wavelength at 330 nm.
Stock standard solutions were prepared by accurately weighing 10 mg of RA into a 10-mL volumetric flask and dissolving in ethanol/Hepes buffer (50:50, v/v) with the aid of sonication. Working standard solutions, 10–600 µg/mL, were prepared by dilution from the stock standard solutions with ethanol/Hepes buffer (50:50, v/v).
Dex concentration was determined by UV-spectroscopy (Modelo del espectrofotometro). Absorption at 245 nm was measured.

Preparation of anionic nanotransethosomes

In this study, anionic nanotransethosomes were prepared. However, in order to simplify the nomenclature, we will refer them as nanoliposomes. Firstly, liposomes were prepared by using the thin film hydration method, also employed for entrapping other drugs (Mura et al., 2007); (Arroyo et al., 2017). The composition of these modified liposomes was carried out by previous formulations made from our research group. The proportion of EPC and Ch was 62.81 and 20.90 mol-percent, respectively. Dihexadecyl phosphate was added as negative charge donor, at a 10.41 mol-percent and sodium deoxycholate was selected from previous studies, as edge-activator for generating ultradeformable liposomes, at 5.87 mol-percent.

Briefly, RA-loaded liposomes were prepared by the film method as follows: EPC, Ch and DCP were dissolved in chloroform (4 mL) and sodium deoxycholate was dissolved in methanol (2 mL); both solutions were mixed and the solvent was evaporated under vacuum using rotary evaporator (Büchi R-210 with Heating Bath Buchi B-491, Switzerland) at 58°C for 30 min. Once removed the residual solvent, the lipid film was hydrated and vortexed (Velp Scientifica Zx3) with 6 mL of ethanol:Hepes buffer pH 7.4 mixture (20:80 v/v) containing 5 mg/mL of RA. The formulation was quickly sealed in glass containers and stored

in the darkness at 4 °C. Finally, unilamellar nanoliposomes (RA-NL) were obtained after extruding the above samples through 400 and 200 nm pore sized polycarbonate membrane filters equipped in the Lipex Thermobarrel extruder (Northern Lipids Inc., Canada) under high pressure of nitrogen (N_2). Dex-loaded nanoliposomes (Dex-NL) were produced by following the previous procedure. Dex concentration in liposome sample was 2 mg/mL. Control liposomal formulations without drug were obtained by following the same methodology.

Characterization of nanoliposomes

Particle size, PdI and zeta potential

The average size and polidispersity index (PdI) of vesicles were determined by dynamic light scattering technique by using a Zetasizer Nano-S equipment at room temperature (Malvern Instruments, Malvern, UK). PdI less than 0.2 indicate a homogeneous and monodisperse population whereas larger PdI (> 0.3) indicates heterogeneity.

Zeta potential was determined from electrophoretic mobility (μ) measurements. The mobility μ was converted to Z by the Smoluchowski equation:

$Z = \mu\eta/\varepsilon$

where η is the viscosity and ε is the permittivity of the solution.

For both measurements, 200 µL of samples were diluted with Hepes solution (1/20).

Entrapment efficiency

The percentage of drug entrapment was obtained after removing the unentrapped fraction after centrifugation in a cooling centrifuge (Eppendorf Centrifuge 5804 R) at 10000 rpm at 4 °C for 60 min. The whole supernatant was filtered and analyzed by HPLC for the RA content or spectrophotometry for Dex content. The residual solid was further extracted with 0.5% w/v sodium dodecylsulfate aqueous solution with sonication for 10 min in order to disrupt the vesicles, and centrifuged as above (Bhardwaj et al., 2010).
This entrapment parameter was calculated as follows:

$$PDE(\%) = \frac{Q_I}{F_t} \cdot 100$$

where PDE is the percentage of drug entrapped, Ft is the total amount of drug in the sample and Qi is the amount of drug retained into the vesicles.

Preparation of liposomal gels

Hydrogels were prepared as described earlier with slight modifications (Maestrelli et al., 2010). In brief, Carbopol hydrogels were prepared by blending of Carbopol 940 powder in Hepes buffer (1% w/w), stirring for 24 h at room temperature following the addition of triethanolamine up to pH 7.0 for gelation and swelling. The resulting gel was stored in capped glass containers, at 4 °C, in the dark. RA and Dex anionic liposomal gels (RA-NL-Gel and Dex-NL-Gel) were prepared after the swelling time. The liposomal dispersion (33.3% (w/w)) was added and stirred carefully until an even distribution within the hydrogel was achieved. Final concentration of 0.167 and 0.067 % w/w for RA and Dex, respectively, were obtained.

***In vitro* release studies**

In vitro release testing from nanoliposomes was determined using a dialysis bag method (Doppalapudi et al., 2017c), wherein 1 mL of RA-NL, Dex-NL and control solutions for both drugs (equivalent to 5 mg and 2 mg for RA and Dex, respectively), were inside a dialysis bag (Spectra®/Por 12-14 kD MWCO membranes), which was previously hydrated with Hepes buffer. The dialysis sacs containing the liposome suspensions were placed in glass beaker containing 50 mL Hepes buffer maintained at 37 °C under magnetic stirring at 100 rpm. At predetermined time points (0.5, 1, 2, 3, 4, 5, 6 and 24 h) 1 ml of the release medium was collected and replaced with 1 mL of the fresh release medium. Then, samples were analyzed by HPLC method as described above for RA and spectrophotometrically for Dex. The results were reported as mean ± SD (n = 3). *In vitro* drug release data was fitted to different kinetic models (first order, Higuchi, zero order) to elucidate the release pattern of RA and Dex from the developed formulations. The regression analysis was performed and best fit correlation was obtained.

Animals

For the present study, female BALB/c mice (8-weeks) were supplied by Janvier-Labs (Le Genest St Isle, France). Mice were maintained in our animal laboratory under standard conditions (temperature of 24–25 °C, humidity of 70–75 % and 12 h light–12 h dark

cycle). Mice were allowed free access to a standard diet (Panlab) and water *ad libitum*. Dorsal region of the mice was shaved by using an electric clipper and treated with depilatory cream skin (Deliplus, Barcelona, Spain) without scratching or damaging the surface in order to maintain the integrity of the skin. The study was performed in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 2010/63/EU). The experiments followed a protocol approved by the Animal Ethics Committee of the University of Seville.

Imiquimod-induced psoriasis-like inflammation in mice

A daily topical dose of 62.5 mg from commercially available IMQ cream (Aldara 5%; Meda AB, Solna, Sweden) was administered on the shaved dorsal selected areas of female BALB/c mice for 6 consecutive days (Andrés et al., 2013). Repeated topical application of IMQ promotes skin inflammation with structural features typical of psoriasis (van der Fits et al., 2009).

Mice were stratified randomly into 6 experimental groups:

1. Sham: mice were treated with non-loaded NL dispersed in gel (100 mg per site)
2. IMQ: mice were pre-treated with non-loaded NL dispersed in gel (100 mg per site) and IMQ cream
3. IMQ-gel: mice were pre-treated with non-loaded NL dispersed in gel (100 mg per site) and IMQ cream (Data no shown)
4. Dex-NL-Gel: Dex-loaded NL dispersed in gel (100 mg per site, containing 200 µg of Dex dissolved in ethanol at 10 µg/µL) (0.2 % w/w) and IMQ cream
5. RA-NL-Gel: RA-loaded NL dispersed in gel (100 mg per site, containing 200 µg of RA dissolved in ethanol at 10 µg/µL) (0.2 % w/w) and IMQ cream
6. RA-Gel: RA-Gel formulation (100 mg per site, containing 200 µg of RA dissolved in ethanol at 10 µg/µL) (0.2 % w/w) and IMQ cream

The pre-treatments of Dex-NL-Gel, RA-NL-Gel and RA-Gel were applied daily on the selected back area thirty minutes before the IMQ application (62.5 mg). One day after last application (day 7), mice were sacrificed by cervical dislocation and punch biopsies were collected from the treated dorsal skin and weighed to evaluate edema. All pieces were frozen in liquid nitrogen for measurement of biochemical parameters.

MPO activity

Myeloperoxidase (MPO) activity was assayed as a marker of neutrophil infiltration according to the method of Grisham et al. (Grisham et al., 1990). The tissue was processed as previously reported (Rodríguez-Luna et al., submitted). One unit of MPO activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 unit/min at 37 °C in the final reaction volume containing the acetate. Results are expressed as units/mg tissue.

Measurement of cytokines in skin homogenates

Frozen skin biopsies were homogenised, centrifuged and supernatants fluids were stored at -80°C until measurements as detailed in (Rodríguez-Luna et al., submitted). Levels of TNF-α, IL-1β, IL-6 and IL-17 were measured by quantitative Enzyme-Linked ImmunoSorbent Assay kits (ELISA) (Peprotech, Germany), according to the manufacturer's instructions. Data are reported as ng/mg tissue.

Statistical Analysis

All values in the figures and text are expressed as arithmetic means ± SEM. Data were evaluated with GraphPad Prism version 5.00 software (GraphPad Software, Inc., San Diego, CA, USA). In all cases, the Shapiro-Wilk test was used to verify the normality of the data. The Mann-Whitney U-test was chosen for non-parametric values. The parametric values groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. P values < 0.05 were considered statistically significant. In the histological experiment, results shown are representative of at least four independent experiments performed on different days.

RESULTS

Characterization of nanoliposomes

Anionic nanoliposomes of RA were prepared by thin layer evaporation technique, wherein they were composed of EPC, Ch, DCP and sodium deoxycholate. This composition was fixed based on our experience and our previous studies (González-Rodríguez and Rabasco, 2011; Villasmil et al., 2010; Arroyo et al., 2016).

Dex-NL were formulated as control formulations since this drug has been extensively used as model substance in psoriatic disease. Liposome formulations were selected because recent studies have been

regarded them as effective and promising delivery systems for treatment of skin diseases, such as atopic dermatitis, with corticosteroids (Eroğlu et al., 2016). These vesicular systems were also characterized. RA-NL and Dex-NL had different characteristics in terms of dimensions and surface charge. Before the extrusion process, multilamellar vesicles were obtained, with mean vesicle sizes being of 1325 and 604 nm, for RA-NL and Dex-NL respectively. As expected, increased volume of vesicles as a consequence of RA loading into the aqueous compartment gave rise to higher mean sizes compared to plain NL (758,77 nm). However, interesting results were obtained with Dex formulations, since reduced mean sizes were obtained compared to plain NL. Probably, a rearrangement of drug into lipid bilayer is the cause for these dimensions. Regarding the zeta potential values (Table 1), the great difference among the formulations can be attributed to the affinity and displacement of the drug (in the case of Dex) to the lipid bilayer as a consequence of the effect of lipophilicity and ionization characteristics of this drug into the aqueous compartment. However, RA remains into the liposomal core and the surface charge was maintained unaffected. This hypothesis was corroborated viewing the percentage of drug entrapped into the unilamellar vesicles wherein higher values were obtained for RA batches than for Dex formulations.

Batch	**Size (nm)**	**PdI**	**Zeta (mV)**	**PDE (%)**
Plain NL_b	758,77 ± 38,89	0,39 ± 0,08	-23,77 ± 1,22	-
Plain NL	160,43 ± 9,23	0,10 ± 0,03	-17,05 ± 0,98	-
RA-NL_b	1325 ± 128,75	0,27 ± 0,12	-23,6 ± 0,1	86,5 ± 4,6
RA-NL	315 ± 10,25	0,35 ± 0,07	-21,7 ± 0,25	71,9 ± 6,7
Dex-NL_b	604,07 ± 18,76	0,25 ± 0,03	-9,16 ± 0,65	52,6 ± 3,1
Dex-NL	150,1 ± 3,54	0,15 ± 0,01	-8,9 ± 0,05	42,8 ± 4,4

Table 1. Characterization parameters of the different batches. Plain NL_b: nanoliposomes without drug characterized before the extrusion step. Plain NL: nanoliposomes without drug. RA-NL_b: rosmarinic acid-loaded nanoliposomes before the extrusion step. RA-NL: rosmarinic acid-loaded nanoliposomes . Dex-NL_b: dexamethasone-loaded nanoliposomes before the extrusion step. Dex-NL: dexamethasone -loaded nanoliposomes.

In vitro release profiles evidenced the sustained release of drug from liposomes

The _in vitro_ release profiles of RA and Dex from NL dispersions were compared with profiles obtained from plain drug solutions. These anionic deformable liposomes exhibited a sustained release of the drug from the vesicles, which is evident by the percentage cumulative of drug release profile. Figure 1 showed that the release rate of RA from RA-NL was slower compared to the RA solution; on the contrary, Dex release was improved compared to Dex solution when the drug was formulated into lipid vesicles (Dex-NL). RA solution exhibited about 95% drug release in 24 h with about 70% in two hours, whereas the RA-NL was comparatively slower, with 32% of RA released at this time. In the case of Dex samples, drug release from solution was slower and the formulation of Dex-NL improved the release rate and the cumulative amount released in 24 h, being about 54 and 72% for solution and Dex-NL, respectively.

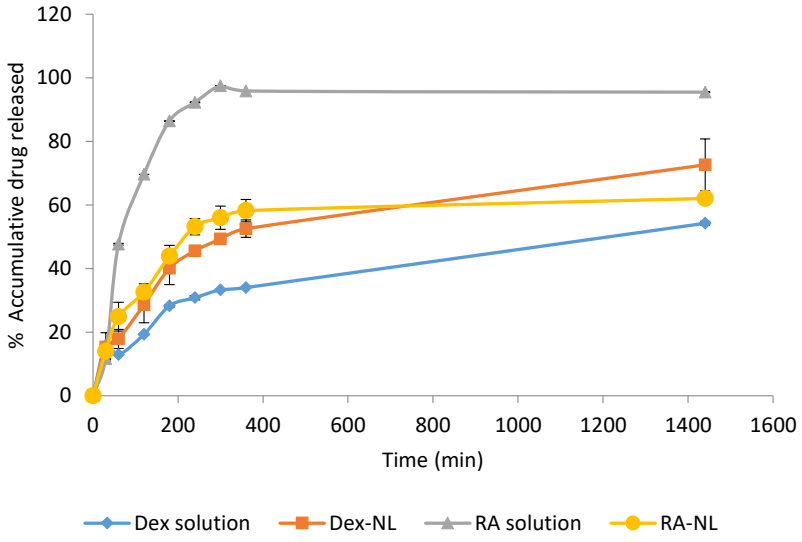

Figure 1. _In vitro_ release profiles of Rosmarinic acid (RA) in nanoliposomes (RA-NL), Dexamethasone (Dex) in nanoliposomes (Dex-NL) and free drug solution (RA solution and Dex solution). The study was carried out using dialysis bag method at 37 °C, 100 rpm and Hepes buffer solution (pH 7.4) was used as release medium. All data represent mean ± SD (n=3).

RA ameliorates IMQ-induced psoriasis-like skin inflammation

We studied the topical effect of gel with RA and RA-NL (RA-Gel and RA-NL-Gel, respectively)on the murine IMQ-induced psoriasis-like inflammation model, which reproduces certain biochemical and histopathological parameters typical of human psoriasis (van der Fits et al., 2009). IMQ-cream administration to dorsal skin resulted in the development of macroscopic lesions as peeling and skin edema, confirmed by a higher weight of the 1 cm^2 punch biopsies compared with the sham group ($P < 0.01$) (Fig. 2A). As expected, pre-treatment with the reference compound Dex-NL-Gel significantly reduced the skin punch weight ($P < 0.001$). Similarly, topical treatment with both RA-Gel and RA-NL-Gel 30 min prior to IMQ application (62.5 mg per site for 6 consecutive days) inhibited macroscopic damage and the skin punch weight in a similar way ($P < 0.001$), suggesting an reduction of skin edema (Fig. 2A). In accordance with macroscopic changes, IMQ-treated animals exhibited a massive neutrophilic infiltration compared with sham animals, evidenced by the increase of MPO activity, an established marker for inflammatory cell infiltration into the skin ($P < 0.001$) (Fig. 2B). Dex-NL application markedly diminished MPO activity in relation to IMQ-cream group ($P < 0.001$). In a similar way, this marker was reduced by treatment with RA-Gel ($P < 0.05$); however, no significant changes were detected after RA-NL-Gel administration (Fig. 2B).

Immune cell infiltration detected in the MPO measures of the skin from IMQ-treated mice were correlated with increased levels of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17, in comparison with sham group ($P < 0.05$, $P < 0.001$, $P < 0.001$, and $P < 0.05$, respectively). The production of TNF-α, IL-1β, IL-6 and IL-17 were significantly reduced in animals treated with RA-Gel in comparison with IMQ group ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.05$, respectively). As regards RA-NL-Gel, this formulation only was able to reduce TNF-α and IL-6 levels in a significant manner. These results suggest a better anti-inflammatory profile following RA application in comparison to RA-NL (Fig. 2C-F).

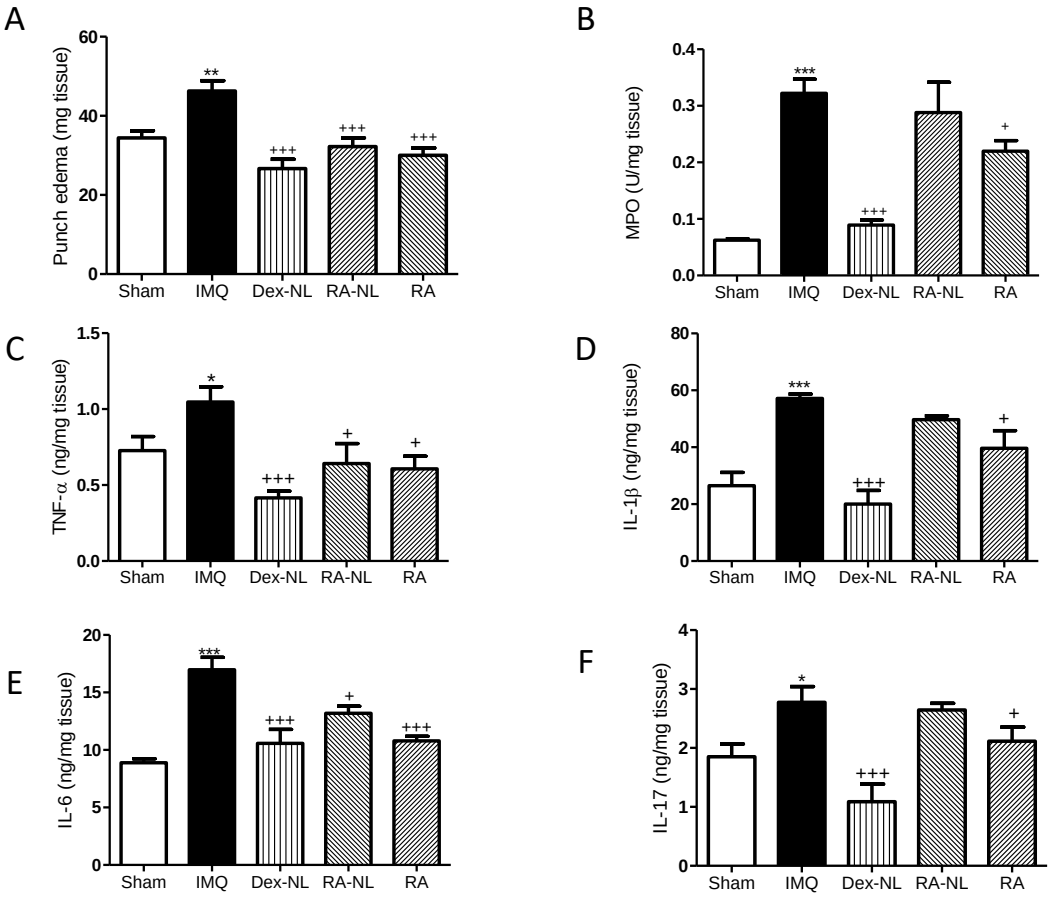

Figure 2. Rosmarinic acid (RA) ameliorates skin inflammation on the murine IMQ-induced psoriasis-like model. The formulations with RA-loaded nanoliposomes in gel (RA-NL) (100 mg per zone, 0.2 % w/w) and RA in gel (RA) (100 mg per zone, 0.2 % w/w) were topically administered 30 min before the IMQ application (62.5 mg per zone) during six consecutive days. Dexamethasone-loaded nanoliposomes in gel (Dex-NL) was employed as positive reference compound (100 mg per zone, 0.2 % w/w). (A) Skin edema as punch biopsy; weight of skin was used as marker of inflammatory skin process. (B) Myeloperoxidase (MPO) activity in dorsal skin. (C) TNF-α levels (ng/mg tissue). (D) IL-1β levels (ng/mg tissue). (E) IL-6 levels (ng/mg tissue). (F) IL-17 levels (ng/mg tissue). Values are means with standard errors represented by vertical bars. Data are means ± SEM (n=6). Mean value was significantly different compared with the sham group (*P < 0.05, **P < 0.01***P < 0.001; Student t test). Mean value was significantly different compared with IMQ group (+P < 0.05, +++P < 0.001; one-way ANOVA followed by Bonferroni's Multiple Comparison test).

DISCUSSION

In this study, additional information in the liposome field has been provided after analyzing the release behavior and *in vivo*

efficacy of two lipophilic compounds with different (opposite) ionization properties in aqueous medium at physiological pH (7,4), one negatively charged (RA) and one positively charged (Dex), when dissolved in hydrogels (control gels) or dispersed in the form of NL (liposomal gels).

Although similar studies have been previously performed with drugs of the same lipophilic nature, such as tretinoin (Ascenso et al., 2014), ellagic acid (Junyaprasert et al., 2012), psoralen, (Doppalapudi et al., 2017a), doxorubicin (Boakye et al., 2015), conclusions have been obtained *in vitro* or provided from different *in vivo* studies. However, *in vivo* parameters referred to a same pathology by using two different compounds with these characteristics have not been evaluated. This is the first time that the elucidation of the influence of ionization properties of a drug into a lipid vesicle is studied under identical conditions and based on a common *in vivo* model.

The use of NL in this work was mainly focused on the capability of these nanocarriers to potentially increase the stability of labile drugs such as RA and to offer a sustained release of the drug, which permits the dose optimization for psoriasis treatment. In this study, ultradeformable vesicles have been formulated with the aim to increase the deformability and elasticity when passing through the skin pores. Additionally, ethanol has been added to the composition as solubility enhancer of both molecules, RA and Dex, in order to be incorporated into the aqueous compartment of liposomes. Although Tsotas et al. (2007) have anticipated that multilamellar vesicles retain Dex for long periods of incubation in buffer, previous results have demonstrated that adding the drug into the aqueous space increase as the stability as the pharmacological effect (Mura et al., 2007). In this regard, our *in vitro release profiles showed that* the developed liposomes have the ability to provide a sustained release of the incorporated substance over a prolonged time. However, Dex and RA control solutions showed different release characteristics, proposing that the effect of molecule nature is crucial to improve or to retain the drug prior to permeation process.

Surprisingly, our *in vivo* study did not yield the expected results: RA-Gel showed better anti-inflammatory activity that RA-NL-Gel. In this sense, the production of pro-inflammatory cytokines as TNF-α, IL-1β, IL-6 and IL-17 were markedly inhibited in animals treated with RA-Gel in comparison with IMQ group. While, the pre-treatment with RA-NL-Gel had not a significant pharmacological effect,

because it was only able to reduce TNF-α and IL-6 levels in a significant manner. Nevertheless, at was expected, Dex-NL gave statistical significance in all the parameters evaluated, in accordance with its demonstrated anti-inflammatory activity in this model (Andrés et al., 2013). A possible explanation of these results is related to the different ionization characteristics of both molecules, which have similar physicochemical properties in terms of molecular weight (392,46 for Dex and 360,32 for RA), water solubility (very slightly soluble) and lipophilicity (log P 1,83 for Dex and 1,82 for RA). However, Dex is a weak base (pKa 12,14) that is positively ionized in the formulation (pH 7,4) and RA is negatively ionized at this pH (pKa 3,57). These findings have been recently reported by Chantasart et al. (2015) in a continuous effort to understand the skin permeation behavior of weak acids and bases, suggesting that the observed permeability–pH relationships could not be explained solely by possible pH differences between skin and donor solution but considering other properties such as the molecule ionization.

The effect of this difference in charge of both compounds on the liposomal characteristics has been elucidated from the characterization parameters. Nanoliposomes obtained in this work have resulted, after extrusion, in deformable unilamellar anionic vesicles with a size less than 200 nm, as exception of RA-NL, which had higher sizes. As postulated (Verma et al., 2003), vesicle size plays an important role in skin penetration of vesicular systems, being liposomes with size less than 300 nm able to deliver their contents to some extent into the deeper layers of the skin. Relative higher sizes obtained in RA-NL can be explained because the drug remains negatively charged into the aqueous space of liposomes as a consequence of electrostatic repulsion caused by ionic bilayer. From zeta potential values, we can conclude that no interaction with the lipid bilayer exists (no difference in zeta values between plain liposomes and RA-NL). This repulsion effect gives rise to increase the PDE parameter, which was about 72% in RA-NL and favors the maintenance of RA into the aqueous compartment. These results are in line with the slow drug release profile and consequently with the low anti-inflammatory activity observed by the pre-treatment with RA-NL-Gel. In the case of Dex, this molecule tends to migrate to the lipid bilayer as a consequence of an electrostatic attraction and lipid character, producing a displacement of structures in the bilayer, such as cholesterol, obtaining zeta potential values less negative caused by charge neutralization (-9,0 mV). This effect originates lack of drug

and decreased PDE (Tsotas et al., 2007). Similar effects have been obtained for curcumin-loaded vesicles, in which negatively charged liposomes exhibited the highest encapsulation efficiencies compared to positively charged vesicles (Pamunuwa et al., 2016).

However, the RA ionized fraction (RA-gel) has also an important consequence in the *in vivo* antipsoriasic efficacy. In this sense, Osakabe et al. (2004), demonstrated the anti-inflammatory and anticarcinogenic activity by topical pre-treatment of RA in the mice two-stage skin model. Furthermore, studies on atopic dermatitis murine model exhibited the anti-inflammatory activity of RA by reduction of IgE, IFN-γ and IL-4, among others (Jang et al., 2011). For this reason, we supposed that the origin of the inefficacy of RA-NL in the psoriasic skin model might be due to the drug behaviour into the vesicles. Certainly, RA entrapped into the NL remains for a longer time before being released. In this case, liposomes are controlled release nanosystems, which act as reservoirs for modulating the drug delivery rate, slowing the rate compared to control solution. This finding is consistent with the results recently obtained by Budhiraja and Dhingra (2015), who developed negatively charged niosomes after loading of RA and evaluated the relative *in vivo* efficacy of niosomes as adequate nanocarriers for acne therapy. To date, the *in vivo* effectiveness or not of RA-loaded into vesicles depending of the ionization characteristics has not been demonstrated yet. The hypothesis previously planned could be related with the *in vitro* release profiles showed in Figure 2. Dex is ubiquited into the bilayer and has a significant antipsoriasic effect *in vivo*, giving rise to enhanced drug release through the dialysis membrane compared to free drug (Mourtas et al., 2007). However, RA from RA-NL-Gel remained into the vesicles, decreasing its release rate out of the liposome, compared with the control solution. Doppalapudi et al. (2017b), have recently reported the same behavior for psoralen-loaded liposomes. They pointed out a PDE higher and delayed release rate in anionic liposomes compared to cationic vesicles.

In conclusion, the use of anionic nanoliposomes for topical application allows enhancing skin permeation. Nevertheless, the molecule nature and in particular ionization properties, are crucial to retain or release the drug in the vesicles and consequently determinate its effect in time.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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CAPÍTULO V

PHOTOPROTECTIVE EFFECT OF FUCOXANTHIN AND ROSMARINIC ACID IN UVB-EXPOSED HACAT CELLS AND THEIR IMPACT ON INFLAMMASOME EXPRESSION

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ABSTRACT

Nowadays, non-melanoma skin cancer (NMSC) remains the most frequently diagnosed cancer in caucasian people worldwide. In this line, the excessive exposure to UV radiation is the main risk factor to develop skin cancer and the strategies for its prevention are highly evaluated. Natural products have been used in the prevention of oxidative stress due to their demonstrated antioxidant activities. Nevertheless, the anti-inflammatory activity and its implication in photoprevention have not yet been much studied. In this regard, the effect of one combination with rosmarinic acid (RA) and fucoxanthin (FX) has been studied on cell viability, cell cycle arrest, apoptosis induction, inflammasome mediation and antioxidative response activation in UVB-irradiated HaCaT cells. The mixture shown better anti-inflammatory and antioxidant effects than the treatment with individual compounds, by reducing cell death and the consequent decrease of apoptosis and ROS production induced by UVB exposition in pre-treated cells. Furthermore, this combination modulated the inflammatory response by diminishing the expression of inflammasome components as NLRP3 and ASC and increased the HO-1 antioxidant protein expression through Nrf2 signalling pathway.

KEYWORDS*:* fucoxanthin; rosmarinic acid; inflammasome; antioxidative; anti-inflammatory; photoprotection; UVB.

ABBREVIATIONS*:* ASC, apoptosis-attached-speck-like protein containing a CARD; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; FX, Fucoxanthin; HO-1, heme oxygenase 1; IL, Interleukin; NMSC, non-melanoma skin cancer; NLRP3, nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin domain- containing 3; Nrf2, nuclear

factor E2-related factor 2; ROS, reactive oxygen species; SRB, Sulforhodamine B; TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

INTRODUCTION

Skin is considered as the outmost protective barrier in the body protecting us from detrimental substances, mechanical damage, pathologic invasion and radiations, which could cause the perturbation of skin structure. In this sense, the skin is well-known as an essential piece of the immune system. The result of skin alteration could trigger the inflammatory process, a lower immune response or an oxidative stress, among others (Fernández-García, 2014). Several factors can contribute to the initiation and development of cutaneous alterations, in this sense, the excessive exposure to UV radiation remains the main risk factor for the skin cancer (Alam et al., 2017). Solar UV radiation consists of three broad range of wavelength UVC (100–280 nm), UVB (280–315 nm) and UVA (315–400 nm). Compared with UVA, UVB is less abundant but is more energetic causing dermal change besides affecting epidermal function and is the mainly responsible for the develop of melanoma and non-melanoma skin cancer (Katiyar et al., 2017). In this regard, a high dose of UVB-exposure promotes cutaneous inflammation, traduced in sunburn, photoaging, DNA damage, immunosuppression and the induction of skin cancer (Hatakeyama et al., 2017).

It has been extensively studied that UVB-induced ROS production leads to activation of mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB), among others, which further promote the inflammation and apoptosis in cells and increase skin aging (Subedi et al., 2017). Furthermore, this type of inflammation, resulting in the production of some cytokines as TNF-α, IL-6 and IL-1β, which are released by keratinocytes after UVB irradiation (Kondo et al., 1994). In this sense, it has been reported the relation between the secretion of IL-1β and activation of protein complexes, called inflammasome (Feldmeyer et al., 2007). Inflammasome is a wide cytosolic multiprotein complex that act as mediator of the innate immune system, which form upon activation by multiple types of tissue damages. The nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin domain- containing 3 (NLRP3), are the main components of inflammasome complex. NLRP3 activation results in activation of caspase-1, which by cleavage catalyzes the process of pro-IL-1β in cytosol into mature form, leading to production of IL-1 β into the

extracellular medium (Hasegawa et al., 2016). Furthermore, in the last years, some authors have demonstrated the implication of NLRP3 inflammasome in the tumorigenesis and development of cancer, specifically in basal cell carcinomas (Ahmad et al., 2017) (Huang et al., 2017).

Nowadays, non-melanoma skin cancer (NMSC) remains the most frequently diagnosed cancer in caucasian people worldwide and the strategies for its prevention are highly evaluated (Zink et al., 2016). In this sense, photo-chemoprevention by natural products is one of the most studied alternatives in time for skin protection, due to their antioxidative actions. Nevertheless, their anti-inflammatory properties have not yet been implemented, but it is increasingly common to find authors that studies natural products with anti-inflammatory in photo-protection models (Alam et al., 2017).

In last years, microalgae have been employed as a vast source of bioactive molecules, which highlight by their potential activity in inflammation and cancer (Talero et al., 2015). Specifically, carotenoids have shown antioxidant, anti-inflammatory or anti-carcinogenic properties in several skin inflammatory models (Oh et al., 2013)(Yoshihisa et al., 2016). Fucoxanthin (FX) is an orange carotenoid present in some brown seaweeds but also it can be isolated from microalgae. This compound is known for its antioxidant properties (D'Orazio et al., 2012), concretely, by increasing of antioxidant pathways as Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Zheng et al., 2014) or by reduction of wrinkles formation and epidermal hypertrophy in mice (Urikura et al., 2011). Also, this carotenoid highlights as photoprotective compound by stimulating restoration of the skin barrier in UVA-induced sunburn (Matsui et al., 2016). In this sense, we have previously demonstrated the benefits of topical pre-treatment with F from the microalga *Isocrhysis galbana* on skin epidermal hyperplasia, as well as on UVB-induced acute erythema model in mice (Rodriguez-Luna et al., submitted).

Polyphenols are the most popular antioxidant molecules in our diet, and exhibit several properties as antioxidant, anti-inflammatory and antineoplastic. For this reason have been considered as an important alternative for the management of skin protection (Saric and Sivamani, 2016). In this regard, rosmarinic acid (RA), is a phenolic ester that can be isolated from some plants as *Rosmarinus officinalis L. or Melissa officinalis L.*, and has been widely studied due to remarkable biological and pharmacological activities (Petersen, 2013).

This compound highlights by its antimicrobial, antioxidant and anti-inflammatory activities (Amoah et al., 2016). In addition, we have demonstrated the anti-inflammatory effect of pre-treatment with RA on Imiquimod-induced psoriasis like model in Balb/c mice (Rodriguez-Luna et al., submitted). Thus, these antecedents led to us to evaluate the possible action mechanisms of FX and RA with the aim to determinate their implication in ROS production, apoptosis prevention, cell cycle alterations, inflammasome regulation and Nrf2 pathway activation, in human keratinocytes stimulated by UVB-exposure. In addition, the combination of F and RA was evaluated to determinate a possible synergism.

MATERIALS AND METHODS

Compounds

Rosmarinic acid (RA) and Fucoxanthin (FX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RA and F stocks were prepared in DMSO at 10 mM respectively, and diluted to the desired concentration with culture medium. Controls were incubated with the corresponding quantity of DMSO, which was always below 1 % and did not affect cell viability.

Cell line

Human immortalized keratinocytes HaCaT were obtained from the American Type Culture Collection (ATCC) and maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO, Grand Island, NY) containing 2 mM L-glutamine. The culture media was supplemented with 10 % heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (PAA Laboratories, Pasching, Austria). Cultures were incubated in an atmosphere of 5 % CO_2 at 37 °C.

UVB-irradiation

The CL-1000M UV Crosslinker system (UVP, Upland, CA, USA) formed by 5 UVB tubes (8W), was used to submit an energy spectrum of UVB radiation (peak intensity 302 nm, inside of the spectrum of UVB light 280-315 nm). To prevent UVB light absorption by the cell culture medium, the medium was replaced by a thin layer of PBS to cover the cells during irradiation. In relation with bibliography, tree UVB intensities were evaluated (100, 150 and 225 mJ/cm^2) to determinate the most suitable to evaluate cell cycle alterations and apoptosis

(Ascenso et al., 2016). In this sense, 100 mJ/cm^2 was selected to the study.

Cell proliferation assay

Cell viability was evaluated by the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay, which determines the formation of purple formazan in viable cells and allows estimate cellular viability (Twentyman and Luscombe, 1987). The MTT assay was performed according to protocol described with slight modifications. Briefly, HaCaT cells were seeded at a density of 10^4 cells/well in a 96-well plate for 24 h. Cells were pre-treated with different doses either RA (2.5, 5, 7.5, 10 µM) or F (5, 10, 15, 20 µM) and the sixteen possible combinations between RA and F, for 1 h. Then, the culture medium was replaced with a thin layer of PBS and exposed to a single dose of UVB radiation at 100 mJ/cm^2. Cells were supplied with fresh culture medium and incubated for 24 h. Absorbance was measured at 570 nm using a Synergy HT Multi-mode Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Intracellular ROS scavenging activity

The 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) assay was used to detect intracellular ROS levels in HaCaT keratinocytes (Wang and Joseph, 1999). Briefly, keratinocytes were seeded at 10^4 cells/well in 96-wells plates and were treated with RA (2.5 and 5 µM), FX (5 µM) and the combinations M1 (2.5 RA plus 5 µM FX) and M2 (5 µM RA plus 5 µM FX) for 1 h. After UVB exposure (100 mJ/cm^2), cells were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) solution (5 mg/mL) in PBS for 30 min at 37°C. Then, the medium was eliminated and the cells were washed with PBS. The fluorescence of the 2',7'-dichlorofluorescein (DCF) product was determined by using a fluorescence plate reader (Sinergy HT, Biotek®, Bad Friedrichshall, Germany) at 485 nm for excitation and 535 nm for emission.

Cell cycle determination

HaCaT cells were seeded at 5 x 10^5 cells/well in 6-wells plates and were treated with either RA (2.5 and 5 µM), FX (5 µM) or mixtures M1 (2.5 RA plus 5 µM FX) and M2 (5 µM RA plus 5 µM FX) for 1 h. Then, treatments were removed and cells were irradiated at 100 mJ/cm^2, and then incubated with fresh medium for 48 h. Cells were harvested by trypsinization as previously detailed (Ascenso et al., 2016), fixed in 85 % cold ethanol (5 x 10^5 cells/mL) and kept at -20 °C until further

analysis. Ethanol was eliminated and cells were resuspended in PBS, and the cell suspension was filtered through a 35 µm nylon mesh to separate aggregated cells. Then, 50 µL RNase (1 mg/mL) and 50 µL propidium iodide (PI) (1 mg/mL) were added to each sample which was then incubated for 20 min in darkness at room temperature until analysis. DNA content was determined on a Coulter Epics XL Flow Cytometer (Beckman Coulter, Hialeah, FL). Data was adquired using the SYSTEM II (v. 2.5.) software examining 10^4 events. Percentages of cells in apoptotic-sub G_1, G_0/G_1, S and G_2/M were calculated using CXP software.

Apoptosis determination

Similar procedure than cell cycle determination was carried out. Briefly, apoptosis was determinated by flow cytometry using Annexin V-FITC Apoptosis detection kit from BD Pharmingen (Franklin Lakes, NJ, USA), as reported in (Ascenso et al., 2016). After pre-treatment with RA (2.5 and 5 µM), FX (5 µM) or combinations M1 (2.5 RA plus 5 µM FX) and M2 (5 µM RA plus 5 µM FX), HaCaT cells were exposed to UVB (100 mJ/cm^2) and then incubated with fresh medium for 24 h. Cells were harvested and suspended in binding buffer at 10^5 cells/mL. Then, annexin V-FITC and PI were added as indicated in the manufacture's protocol. Subsequently, samples were keep in darkness for 15 min and 400 µL of binding buffed were added. Fluorescense intensity of PI and FITC-Annexin-V-stained cells was determinated on a Coulter Epics XL Flow Cytometer (Beckman Coulter, Hialeah, FL). Data were obtained using the SYSTEM II (v.2.5.) software examing 10^4 events. The cytogram of FITC fluorescence in log scale versus PI fluorescence in log scales allows the identification of viable cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic cells (Annexin V-FITC positive, PI positive) and necrotic cells (Annexin V-FITC negative, PI positive). Flow cytometry data were analysed by FlowJo software (Tree Star Inc., Ashland, OR).

Determination of IL-1β production

HaCaT cells were seeded at 5 x 10^5 cells/well in 6-wells plates, incubated for 24 h and treated with either RA (5 µM), FX (5 µM) or M2 (5 µM RA plus 5 µM FX), for 1 h. Then, the cells were irradiated at 100 mJ/cm^2, and incubated with fresh medium for 24 h. Supernatant fluids were collected and stored at -80 °C until use. Commercial enzyme-linked immunosorbent assay (ELISA) kits (Diaclone GEN-PROBE,

France) was used to quantify IL-1β production according to the manufacturer's protocol. The absorbance at 450 nm was read by a microplate reader (Sinergy HT, Biotek®, Bad Friedrichshall, Germany). To calculate the concentration of IL-1β (pg/mL), a standard curve was constructed using serial dilutions of cytokine standards provided with the kit.

Western blot analysis

The procedure was similar than cytokine production. Then, HaCaT cells were harvested by tripsinization, centrifuged and resuspended in lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 % glycerol, 1 % Triton-100-X, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor cocktail tablet, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate). The homogenates were centrifuged (12,000 *g*, 15 min, 4 °C), and the supernatants were collected and stored at -80 °C. Bradford colorimetric method was utilized to determinate the protein concentration of the homogenates (Bradford, 1976). Samples of the supernatants with equal amounts of protein (20 µg) were separated on 10 % acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Next, the proteins were electrophoretically transferred onto a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-ASC (Bio-Rad, USA) (1:1000), rabbit anti-NLRP3 (1:1000), rabbit anti-Nrf2 (1:1000), rabbit anti-HO-1 (1:1000), rabbit anti-caspase-1 (1:1000) (Cell Signaling, Danvers, MA, USA) overnight at 4 °C. After rising, the membranes were incubated with the horseradish peroxidase-linked (HRP) secondary antibody anti-rabbit (Cayman Chemical®, Ann Arbor, MI, USA) (1:1000) or anti-mouse (Dako®, Atlanta, GA, USA) (1:1000) containing blocking solution for 1 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (Sigma Aldrich®, MO, USA). Immunodetection was performed employing an enhanced chemiluminescence light-detecting kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, IL, USA). Then, the immunosignals were monitorized by using an Amersham Imaging 600 (GE Healthcare Life Sciences, Barcelona, Spain and densitometric data were analysed after normalization to the control (housekeeping gene). The signals were analyzed and quantified with a Scientific Imaging Systems (Biophotonics ImageJ Analysis Software, National Institute of Mental Health, Bethesda, MD, USA) and expressed as total percentage respect to UVB-exposed control group.

Statistical analysis

All values in the figures and text are expressed as arithmetic means ± standard error of the mean (S.E.M.). Data were evaluated with GraphPad Prism® Version 5.00 software (SanDiego, CA, USA).). In all cases, the Shapiro-Wilk test was used to verify the normality of the data. The Mann-Whitney U-test was chosen for non-parametric values. The parametric values groups were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. P values < 0.05 were considered statistically significant.

RESULTS

RA and FX protect against UVB radiation

First, the UVB intensity was selected in a preliminary study in which we tested three different intensities (100, 150 and 225 mJ/cm^2). As was expect, the increasing of UVB dose caused higher cell morphological changes and cell death. The intensity 100 mJ/cm^2 was chosen because let over 50 % of cell survival and showed a significant difference in comparison with non-irradiated control group ($P < 0.001$) (Fig. 1A). In addition, MTT results showed that the treatment with RA (2.5 and 5 µM), FX (5 µM), M1 and M2 significantly increased cell viability ($P< 0.01$, $P< 0.05$, $P< 0.001$ and $P<0.001$, respectively) in UVB-exposed cells, being more effective M1 and M2 treatments (Fig. 1B). The remaining tested concentrations not protected against UVB damage and were discarded for the rest of experiments (Data no shown).

Effect of the pre-treatments on UVB-induced ROS production

It is well known that the intracellular ROS levels can be increased by UVB exposition (Kovacs et al., 2009). In this sense, HaCaT cells were treated with the compounds for 1 h and then were irradiated with UVB (100 mJ/cm^2). Cells exposed to UVB showed a significant increase in ROS production in comparison with non-exposed control cells ($P<0.001$). The pre-treatment with either 2.5 and 5 µM RA and 5 µM FX significantly reduced intracellular ROS levels by 12, 12 and 8 %, respectively ($P<0.01$). Nevertheless, M1 and M2 showed a higher effect, significantly decreasing ROS production by 18 % and 19 %, respectively ($P<0.001$) (Fig. 1C).

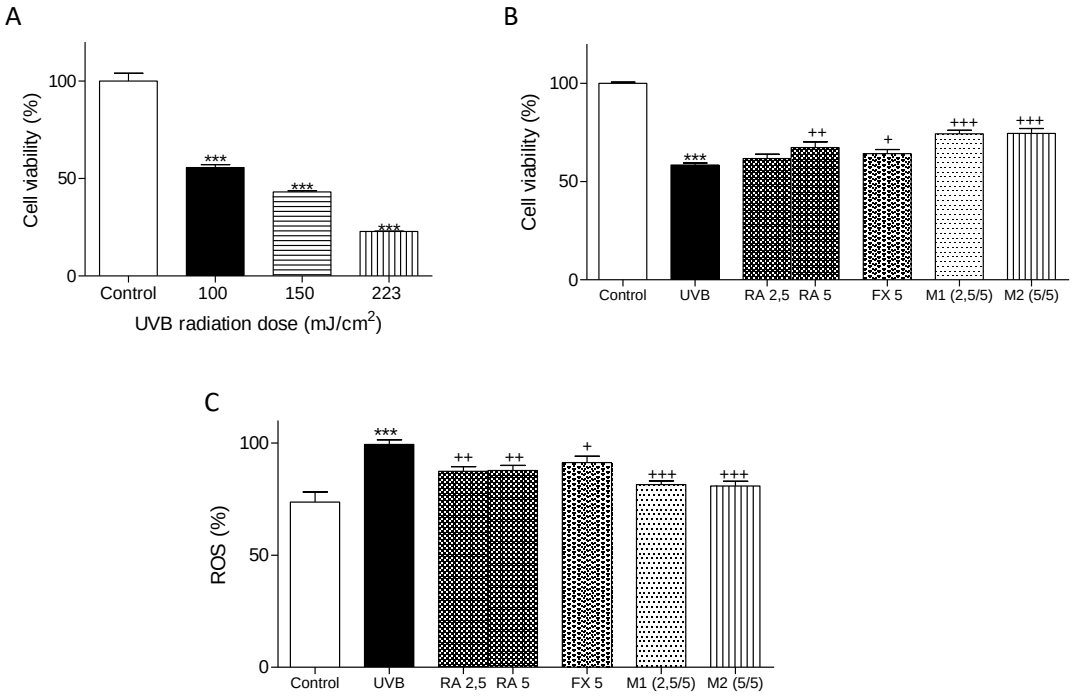

Figure 1: Selection of UVB-dose and evaluation of cell viability and ROS production in UVB-irradiated HaCaT cell. (A) Effect of different UVB doses on cell viability determined by MTT assay. Results are expressed as percentage respect to untreated control cells and bars represents mean ± SEM of four independent experiments (n = 4). *** P < 0.001 vs. untreated cells, and +++ P < 0.001 vs. UVB-irradiated cells. (B) Effect of RA, FX and their combinations on cell viability in human HaCaT keratinocytes was measured by MTT assay after 24h of UVB-exposition. (C) Intracellular ROS generation was evaluated 30 min after UVB irradiation by relative fluorescence intensity using DCF-DA assay. For experiments B and C, cells were pretreated with RA (2.5 and 5 µM), FX (5 µM) and two combinations M1 (2.5 RA plus 5 µM FX) and M2 (5 µM RA plus 5 µM FX), for 1h. Then, cells were irradiated with selected UVB dose (100 mJ/cm^2) and incubated the required times. Results are expressed as percentage respect to untreated control cells (B) or UVB-exposed control cells (C), and bars represents mean ± SEM of six independent experiments (n = 6). *** P < 0.001 vs. untreated cells; + P < 0.05, ++ P < 0.01 and +++ P < 0.001 vs. UVB-irradiated cells.

Cell cycle analysis

It is well known that UV light exposure promotes skin photodamage such as cell death, DNA damage and consequently cell cycle arrest (Lee et al., 2017). As expected, our results indicates that UVB-exposition highly affects to the cell number in G0/G1 phase, showing a reduction in the percentage of cells from 68% to 50%, accompanied by an increase in apoptotic sub-G1 subpopulations from 3% to 14%, compared with non-exposed cells (Fig. 2A). Although the S and

G2/M phases were not markedly affected by any of the pre-treatments, in UVB-exposed control group, we could observed that S phase significantly increased cell number from 16% to 24%, whereas G2/M phase kept in order to 12%, in comparison with non-irradiated control group. Pre-treatment with RA and FX at 5 µM showed an increase in cell number of G0/G1 phases, in this sense pre-treatment with M2 significantly augmented the cell number in G0/G1 phase in comparison with UVB-exposed cells (Fig.2A).

Apoptosis analysis

It is well report that, apoptosis is the most important type of programmed cell death in response to cell damaged induced by UVB exposure (Feehan and Shantz, 2016). In this sense, the use of Annexin V assay allows differentiate cells subpopulations in different stages as necrotic (R1), viable (R2), early apoptosis (R3) or late apoptosis (R4). To develop this assay, only the adherent cells were considered, which means that the necrotic cells in suspension after UVB were not analysed in the assay results, thus changes did not observed in this phase. Comparative analysis of these subpopulations between irradiated (UVB) and non-irradiated (control) HaCaT cells was performed for determinate the action of UVB (Fig.2B). As was expected, our results exhibited that UVB significantly decreased the percentage of viable cells from 88% to 20% due to the cell alterations, also increased the cells number in early apoptosis from 1% to 5% and strongly augmented the percentage of cells in late apoptosis from 8% to 72%, all of them in comparison with non-irradiated cells (Fig. 2B). Respect to pre-treatment, neither RA nor FX exhibited percentages changes in the different subpopulations, respect to UVB exposed cells. Nevertheless, the treatment with M1 and M2 allowed to reduce the percentage of late apoptotic cells ($P<0.01$) and to increase the number of viable cells ($P<0.01$), in comparison with irradiated control cells (Fig. 2B).

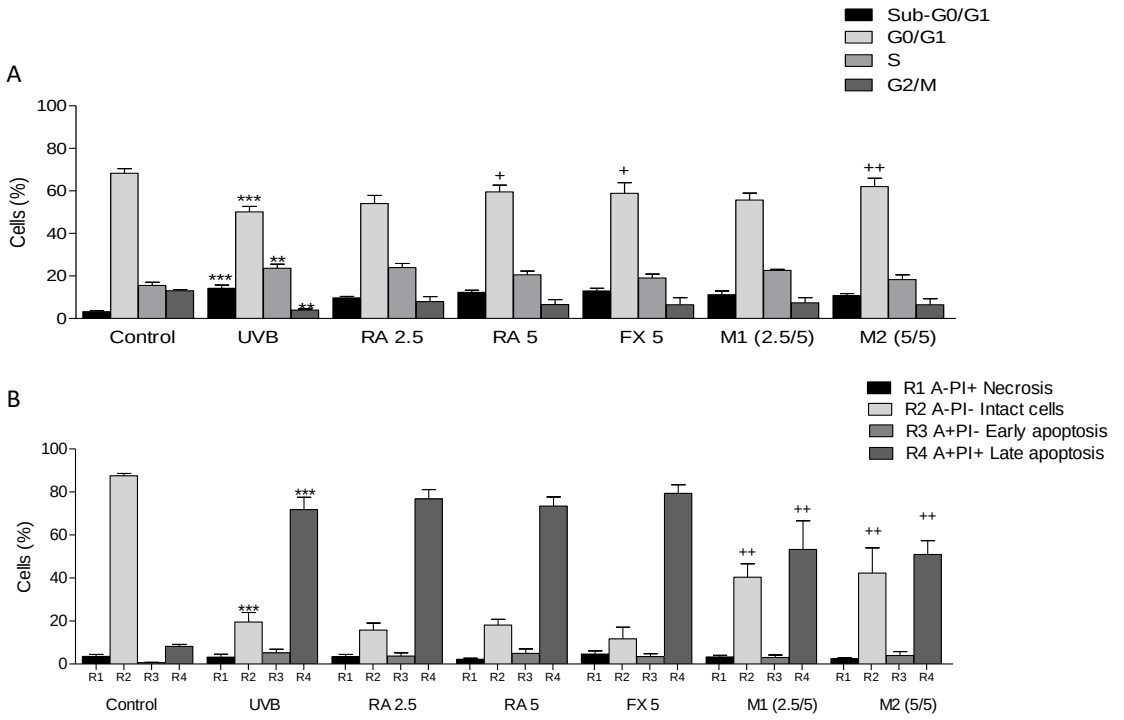

Figure 2: Effect of RA, F and combinations in cell cycle and apoptosis studied by flow cytometry. (A) Cell cycle phase distribution of UVB-exposed HaCaT cells. Cells were pretreated with RA (2.5, 5 µM), FX (5 µM) and two M1 (2.5 RA plus 5 µM FX) and M2 (5 µM RA plus 5 µM FX), then were irradiated with selected UVB dose (100 mJ/cm^2) and incubated for 48h. Results are expressed as percentage and bars represents mean ± SEM of four independent experiments (n = 4). *** P < 0.001 vs. untreated cells; + P < 0.05, ++ P < 0.01 and +++ P < 0.001 vs. UVB-irradiated cells. (B) Results of Annexin V-FITC assay as percentage of cells in different apoptotic phases; R1: necrotic cells (Annexin V-FITC negative, PI positive), R2: viable cells (Annexin V-FITC negative, PI negative), R3: early apoptotic cells (Annexin V-FITC positive, PI negative), R4: late apoptotic cells (Annexin V-FITC positive, PI positive). Data are expressed as percentage and bars represents mean ± SEM of four independent experiments (n = 4). *** P < 0.001 vs. untreated cells, and ++ P < 0.01 vs. UVB-irradiated cells.

Effect of RA and FX on UVB-induced inflammasome activation

Inflammasome is a multiprotein complex, that can be activated by several factors, among them UVB-exposure (Ahmad et al., 2017). Upon activation, NLRP is joined with apoptosis-attached-speck-like protein containing a CARD (ASC) so as to recruit pro-caspase 1 to the inflammasome (Haitao Guo, Justin B. Callaway, 2015). In consequence, caspase-1 is activated conducting to the conversion of pro-IL-1β in its active form IL-1 β (Drexler et al., 2012). In this sense, we evaluated the effect of studied compounds on inflammasome activation.

HaCaT human keratinocytes were treated with RA (5 µM), F (5 µM) and M2 for 1 hour and then exposed to UVB irradiation at 100 mJ/cm^2. Twenty-four hours after irradiation, HaCaT cells showed a higher NLRP3, ASC and caspase-1 expression when were compared with non-exposed control cells (Fig. 3A-C). Pre-treatment with RA and FX not modified the expression of these proteins. Nevertheless, M2 reduced NLRP3 and ASC expression levels but not caspase-1 levels in comparison with UVB-exposed cells. On the other hand, the activation of inflammasome results in an increment of IL-1β production. In this sense, HaCaT cells exposed to UVB lead to a raise of IL-1β production (P<XX) in comparison with control cells. Nevertheless, the pre-treatment with XX reduced IL-1β production in a significant manner (P<XX) (Fig. 3D).

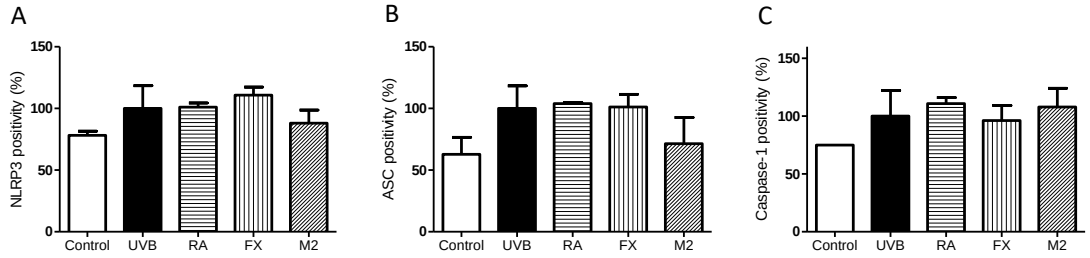

Figure 3: Pre-treatment with RA, F and M2 modulates inflammasome activation on UVB-irradiated HaCaT cells. Cells were pretreated with RA (5 µM), FX (5 µM) and M2 (5 µM RA plus 5 µM FX) for 1h, then were UVB-irradiated (100 mJ/cm^2) and incubated for 24h. Densitometry analysis of (A) nucleotide-binding domain, leucine-rich-repeat-containing family, pyrin domain- containing 3 (NLRP3), (B) inflammasome adaptor protein (ASC) and (C) caspase-1 positivity were performed following normalization to the control (β-actin housekeeping gene). (D) IL-1β production was evaluated by ELISA assay. Results are expressed as percentage respect to UVB-irradiated control and bars represents mean ± SEM of four independent experiments (n = 4). *** P < 0.001 vs. untreated cells; + P < 0.05, ++ P < 0.01 and +++ P < 0.001 vs. UVB-irradiated cells.

Effect of RA and FX on Nrf-2 antioxidant signaling pathway

Nrf2 is a transcriptional factor that detects variation in cellular oxidative stress and promotes the transcription of antioxidant genes and detoxification enzymes such as Heme-oxygenase 1 (HO-1) to protect against an oxidative damage as UVB-exposure (Furue et al., 2017). Our results showed that UVB exposure significant decreased the Nrf-2 and HO-1 expression (P < XX), (P < 0.05), respectively (Fig. 4A-B). HaCaT cells treated with RA, FX and M2 and exposed to UVB increased Nrf2 and HO-1 levels in a similar way than control (P < XXX).

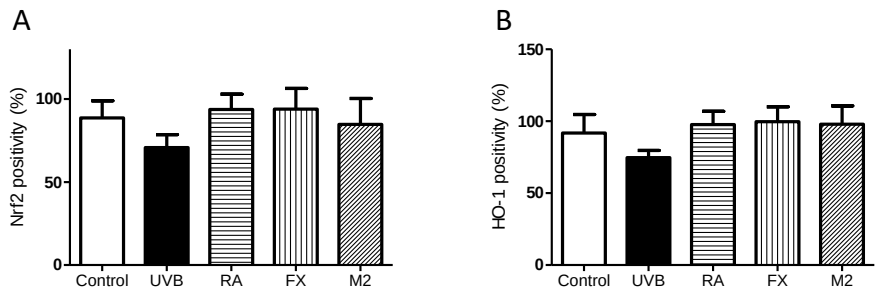

Figure 4: Effect of RA, F and M2 on up-regulation of Nrf2 and HO-1 protein expression in UVB-exposed keratinocytes. Cells were pretreated with RA (5 µM), FX (5 µM) and M2 (5 µM RA plus 5 µM FX) for 1h, then were UVB-irradiated (100 mJ/cm^2) and incubated for 24h. Densitometry analysis of (A) nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and (B) heme oxygenase (HO-1) were performed following normalization to the control (β-actin housekeeping gene). Data shown are expressed as percentage respect to UVB-irradiated control and bars represents mean ± SEM of four independent experiments (n = 4). *** P < 0.001 vs. untreated cells; + P < 0.05, ++ P < 0.01 and +++ P < 0.001 vs. UVB-irradiated cells.

DISCUSSION

Skin is considered the largest organ in the body and represents the first barrier that protects us from the deleterious effects of solar ultraviolet (UV) radiation, which is the main cause for skin cancer. Nevertheless, human skin is habitually exposed to UV irradiation, while as a short-term UV exposure, it might suppress immune function or triggers an inflammatory response, a chronic exposition, it could conduce to photoaging, DNA damage, or carcinoma (Sajo et al., 2017). Skin disposes of an antioxidant system, composed by enzymatic and non-enzymatic antioxidants, to previse against alteration by levels of toxic exogenous/endogenous ROS. Nowadays, is well known the use of natural substances that support the endogenous antioxidant systems of the skin via diet or dermatological preparations (Chen et al., 2012). To develop new skin photoprotective agents is necessary to understand the molecular mechanism of UV-induced cellular responses and determine how these products may act in the skin cells. In this sense, many treatments with an unique compound have demonstrated successful results against photodamage, but more hopeful impact were obtained when a combination of several compounds were used (Psotova et al., 2006). The efficacy of combinations could be due to the synergism of their component properties, in this sense natural products are ideal candidates for photoprotection of human skin (Dreher et al., 1998). Thus, the present study aimed to evaluate

the preventive effect of RA and FX and their combination in HaCaT human keratinocytes exposed to UVB.

It is well reported that UVB radiation induces increase of ROS production and DNA damage, as well as a strong inflammatory response, characterized by the production of pro-inflammatory mediators, which leads to premature skin aging and carcinomas (Leerach et al., 2017). In this regard, apoptosis is the most important type of programmed cell death due to its magnitude in development homeostasis and pathogenesis of different diseases, such as cancer. Thus, the activation of apoptosis after UVB exposure led to the elimination of irreversibly damaged cells that could harbour oncogenic mutation (Feehan and Shantz, 2016). In this sense, FX has demonstrated an antioxidant role in HaCaT keratinocytes due to its DNA protection against oxidative stress and the prevention of apoptosis (Heo and Jeon, 2009) (Zheng et al., 2013). In a similar way, several studies have reported that RA reduces ROS production and protects against DNA damage and apoptotic markers (Psotova et al., 2006) (Vostálová et al., 2010). In this line, we observed that the pre-treatment of HaCaT keratinocytes with FX and RA significantly attenuated UVB-induced damage by increase of cell viability, inhibition of ROS production, but did not modify the number of cell in apoptosis after UVB exposure. Nevertheless, the combination of both compounds not only improved the cell viability and ROS results, but also ameliorated the protection of cell reducing the number of cells in late apoptosis induced by UVB. In addition to cellular responses previously detailed, UVB induces cell cycle arrest. Previous studies have reported that UV-irradiation activates cell cycle arrest both at G1 and G2 phases (Pavey et al., 2001). In this line, our results indicates that UVB-irradiation affects more the percentage of cells in G0/G1, showing a significant reduction of number of cells in this phase in comparison with non-exposed cells. Furthermore, G2-phase check-point control did not shown to be highly affected. The pre-treatment with RA, FX and M2 significantly reversed UVB-induced G0/G1 phase cell cycle arrest in HaCaT cells.

In terms of inflammation, it is well-known that NLRP3 inflammasome activation by UVB leads to increase not only IL-1β, but also other pro-inflammatory cytokines as IL-1α, IL-6, TNF-α or the mediator PGE_2 (Hasegawa et al., 2016). Recently, it has been reported that pro-IL-1β is cleaved and IL-1β is secreted from cells as a pro-inflammatory cytokine after NLRP3 inflammasome activation (Jang et

al., 2015). It can be concluded that in keratinocytes, IL-1β production is inflammasome dependent finally (Feldmeyer et al., 2007) . In this line, NLRP3 inflammasomes may play an important role in skin inflammatory responses induced by UVB, and consequently have a critical function in initiation of skin pathologies as skin cancer (Ahmad et al., 2017). In addition, the inflammasome adaptor protein ASC, has showed anti-tumor activity in keratinocytes by interaction with p53 on UVB-irradiated keratinocytes (Drexler et al., 2012). Due to the high connection between inflammasome, inflammation and cancer, the therapeutic strategies from inflammasome-related products are currently in development and suppose a pivotal tool for skin cancer prevention (Lin and Zhang, 2017). In this regard, the anti-inflammatory activity of fucoxanthin have been well-reported due to its implication of NF-κB and MAPKs pathways (Choi et al., 2016), and consequently some studies have evidenced an upregulation of the production of IL-1β, TNF-α, iNOS, and COX-2 (Tan and Hou, 2014) (Gong et al., 2014). Nevertheless, the relation with inflammasome has not yet been studied. In this sense, our results indicated that the pre-treatment with FX did not markedly reduced the inflammasome proteins NLRP3 and ASC, but manifested a tendency to reduce the levels of caspase-1 in keratinocytes UVB-irradiated. Respect to RA activity, has been showed the inhibition of pro-inflammatory cytokines, including IL-1β, and the downregulation of NF-κB signaling pathway in human keratinocytes. Furthermore, the implication of RA to modulating inflammasome activation has been demonstrated by inhibition of caspase-1, NLRP3 and ASC expression in poly(I:C)-induced inflammatory reaction on epidermal keratinocytes (Zhou et al., 2016). Also, the anti-inflammatory role of RA inhibiting capsase-1, has been elucidated by others authors that show the RA reduces caspase-1 downstream signal pathway, in addition to prevents apoptosis cisplatin-induced in chemotherapy (Jeong et al., 2011). In addition, due to its anti-inflammatory activity, RA has been proposed to ameliorate allergic reactions in allergic rhinitis and rhinoconjunctivitis (Oh et al., 2011).

Besides of anti-inflammatory effects, the role of these compounds as antioxidants has been highly demonstrated. FX has showed to promote AKT/Nrf2/GSH-dependent antioxidant response in keratinocytes (Zheng et al., 2014) (Liu et al., 2016), as well as diminish wrinkles formation and epidermal hypertrophy in mice (Urikura et al., 2011) and supress melanogenesis and prostaglandin synthesis (Shimoda et al., 2010). In this sense, due to antioxidant activity has

been proposed as a photoprotective compound by stimulating restoration of the skin barrier in UVA-induced sunburn (Matsui et al., 2016). Furthermore, our previous studies with this compound has evidenced to increase Nrf2 and HO-1 expression on UVB-induced acute erythema in SKH-1 hairless mice model (Rodríguez-Luna et al., submitted), according with other authors that suggest that this compound could be an interesting strategy to counteract UVB-induced oxidative damage in skin (Sun et al., 2016a). In this line, similar properties have been demonstrated with RA applications, confirming the antioxidant activity of this polyphenol via activation of Nrf2-antioxidant system (Sun et al., 2016b) (Lu et al., 2017).

In conclusion, according to data obtained from all experimental assays, we demonstrate that the combination of these natural products have several benefits upon keratinocytes irradiated with UVB. It is due to increase of cell viability, reduction of ROS production and anti-inflammatory activity by inflammasome modulation. In addition, this work, are in accordance with previous reports that observe an up-regulation of the Nrf2-antioxidant system previous pre-treatments with these compounds in UVB-exposed HaCaT cells. For this reason, we propose the combination of these compounds as a promising tool in prevention of skin UVB-induced alterations as photoaging, skin inflammation and its derivation in pre-cancerous lesions and skin carcinomas.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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DISCUSIÓN

La piel es el principal órgano que actúa como defensa frente a agresiones externas, protegiendo al organismo de sustancias tóxicas, daño físico, e invasión por patógenos o radiaciones, suponiendo una pieza indispensable del sistema inmunológico. La exposición crónica de la piel a la luz UV induce la producción de ROS, así como una respuesta inflamatoria, la cual juega un papel importante en el fotoenvejecimiento, en el desarrollo de lesiones pre-cancerosas, como es el caso de la queratosis actínica, y en el cáncer de piel. Por otro lado, las enfermedades inflamatorias de la piel tienen un alto impacto en la calidad de vida de los pacientes; una de ellas es la psoriasis, enfermedad crónica mediada por el sistema inmune, de afectación predominantemente cutánea, y evolución imprevisible, con periodos libres de enfermedad y exacerbaciones. Si bien los últimos años han sido testigos de una auténtica revolución en la farmacoterapia de la psoriasis con el uso de agentes biológicos, que han permitido el tratamiento efectivo de las formas más severas de la enfermedad, antes tratadas con fármacos inmunosupresores potentes, a día de hoy no se dispone de una cura definitiva.

Dada la necesidad de encontrar nuevas alternativas terapéuticas en el tratamiento de la psoriasis, la queratosis actínica o el cáncer de piel, la quimioprevención con compuestos naturales está tomando un importante papel en los últimos años. Así, compuestos antiinflamatorios capaces de reducir las manifestaciones clínicas de la psoriasis o de impedir el desarrollo frecuente de recidivas son excelentes alternativas complementarias a la terapéutica actual. Del mismo modo, la búsqueda de estrategias encaminadas a retrasar el fotoenvejecimiento de la piel para prevenir el desarrollo de lesiones pre-cancerosas presenta un gran interés en la actualidad. En los últimos años, las microalgas han emergido como fuente de compuestos bioactivos, entre los que cabe destacar los glicolípidos y carotenoides como la FX. Estos compuestos han atraído el interés de la industria farmacéutica en base a sus actividades como antioxidantes, anti-inflamatorios y anti-carcinogénicos. Sin embargo, son escasas las publicaciones que evalúan estos compuestos en patologías inflamatorias de la piel.

En base a estos antecedentes, el objetivo de nuestras investigaciones fue evaluar la actividad biológica de ciertos glicolípidos y el carotenoide FX, aislados de la microalga *Isochrysis galbana*, en diferentes modelos inflamatorios de piel, tanto *in vitro*

como *in vivo*, y estudiar los posibles mecanismos implicados en sus efectos.

En primer lugar, se evaluó la actividad antioxidante y fotoprotectora de diferentes glicolípidos aislados de la especie *Isochrysis galbana*, empleando un modelo *in vitro* de queratinocitos humanos HaCaT irradiados con UVB. La irradiación UVB induce citotoxicidad celular a través de la pérdida de la integridad de la membrana celular, lo que conduce a la liberación de la enzima LDH del citosol al medio de cultivo (Podda et al., 1998). Además, esta radiación causa una mayor producción de ROS y daño en el ADN, así como una fuerte respuesta inflamatoria, caracterizada por la producción de citocinas inflamatorias, como la IL-6, lo que conduce al envejecimiento prematuro de la piel y al desarrollo de carcinomas (Aoki-Yoshida et al., 2013). El pre-tratamiento de los queratinocitos con los compuestos puros con estructuras de MGMGs y DGDGs, así como las fracciones ricas en MGDGs o DGDGs redujo significativamente el daño celular inducido por UVB, mediante la disminución de la liberación de LDH y la producción de ROS e IL-6. Estos resultados coindicen con estudios previos que muestran una reducción de marcadores relacionados con el estrés oxidativo por glicolípidos bacterianos (Matsufuji et al., 2000), o bien procedentes de espinacas (Shiota et al., 2010) o de la microalga *Porphyridium cruentum* (Bergé et al., 2002). Este estudio preliminar nos permitió la selección de los compuestos más activos, que fueron la fracción de glicolípidos MGDG y el glicolípido puro MGMG-A, para su estudio posterior en modelos *in vivo*.

Como se ha mencionado anteriormente, la psoriasis no dispone en la actualidad de tratamiento curativo. Además, muchas de las terapias empleadas no se recomiendan para el tratamiento de las formas más leves de la enfermedad debido a sus efectos adversos. Por lo tanto, se necesitan otras alternativas que impliquen el uso de tratamiento tópicos para los casos leves-moderados, que suponen el 90% de los pacientes diagnosticados. Dada la interesante actividad antiinflamatoria de los glicolípidos observada en el estudio *in vitro* y que no existen en la literatura datos previos que evalúen su efecto en enfermedades inflamatorias como la psoriasis, nos planteamos realizar un primer experimento *in vivo* con los compuestos más activos. Así, se estudiaron la fracción MGDG y el glicolípido puro MGMG-A, en un modelo de hiperplasia epidérmica inducida por TPA en ratones Swiss CD-1. Son numerosas las evidencias que muestran que la exposición de la piel a un activador de la proteína kinasa C, como es el TPA, induce

una respuesta pleiotrópica en el tejido, dando lugar al desarrollo de lesiones macroscópicas como descamación y eritema, lo cual se asemeja al fenotipo aparente de la psoriasis. En primer lugar, examinamos los efectos anti-inflamatorios de la aplicación tópica de MGDG y MGMG-A, disueltos en acetona (10 µg/µL, 200 µg por zona), comparándolos con la Dex como compuesto de referencia. Nuestros resultados mostraron que el tratamiento con los glicolípidos disminuyó el edema, el espesor de la epidermis y la producción de las citocinas pro-inflamatorias TNF-α, IL-1β, IL-6 y IL-17. En este sentido, tanto el compuesto puro como la fracción, proporcionaron resultados similares, de modo que, la elección de la fracción para continuar los estudios *in vivo* se llevó a cabo teniendo en cuenta su capacidad de aislamiento, ya que el rendimiento al obtener MGDG de *I.galbana* era mayor y permitía disponer de mayor cantidad.

Bien es sabido que la aplicación tópica de compuestos requiere una adecuada incorporación de las moléculas bioactivas en una fórmula que ofrezca estabilidad, que permita la permeación y que permanezca el suficiente tiempo en la piel. De modo que, desarrollamos diferentes estudios de permeación *in vitro*, *ex vivo* e *in vivo*, para comparar el perfil de tres formulaciones diferentes (gel, pomada y crema). Los estudios *in vitro* y *ex vivo* se desarrollaron en células de Franz y se completaron con un estudio de permeabilidad *in vivo*, usando gel, pomada y crema con rodamina, lo que permitió evaluar la estabilidad de las formulaciones y las propiedades de permeación de las mismas a través de las capas de la piel. Los estudios de permeabilidad mostraron que la crema era la formulación con el mejor perfil de permeación. Por tanto, estos resultados nos llevaron a evaluar una crema que contenía la fracción MGDG (MGDG-crema) (0.2 % p/p), en el modelo de hiperplasia en ratón, comenzando esta vez el tratamiento 2 días antes de la primera aplicación de TPA. En concordancia con la actividad anti-inflamatoria demostrada *in vitro* con esta fracción, la aplicación de la crema disminuyó el edema provocado por el TPA, mejoró los daños histopatológicos y redujo el infiltrado celular. Cabe destacar que estos resultados fueron más significativos con la aplicación de la crema que con la fracción disuelta en acetona.

La respuesta inflamatoria causada por la aplicación tópica de TPA activa señales de transducción intracelular con el consiguiente aumento de la expresión de la enzima COX-2 en la piel (Kundu et al., 2009). Con el objeto de estudiar el mecanismo responsable de los efectos anti-inflamatorios de la crema, el siguiente paso fue evaluar la expresión de esta proteína por inmunohistoquímica. Estos resultados

pusieron de manifiesto una importante reducción en la expresión de COX-2, alcanzando niveles similares a la Dex. Por tanto, nuestros datos muestran por primera vez el efecto preventivo de los glicolípidos en patologías inflamatorias de la piel como la psoriasis y sugieren que la fracción formulada en crema podría ser una interesante estrategia terapéutica para el tratamiento de esta patología.

Además de en compuestos lipídicos, las microalgas son ricas en otros compuestos como carotenoides. En la presente tesis, nos hemos centrado en la FX, que ha demostrado una actividad anti-inflamatoria y antioxidante en estudios tanto *in vitro* como *in vivo*, pero los estudios en piel son escasos. Por tanto, nuestro siguiente objetivo fue valorar la actividad biológica de la FX aislada de *Isochrysis galbana* en diferentes modelos inflamatorios de piel. En primer lugar, se evaluó la actividad anti-inflamatoria en macrófagos THP-1 estimulados con LPS y en queratinocitos HaCaT estimulados con TNF-α, demostrándose una reducción de las citocinas TNF-α, IL-6 e IL-8.

Seguidamente, estos datos nos llevaron a evaluar el posible efecto preventivo de este carotenoide en dos modelos *in vivo*: un modelo de hiperplasia epidérmica inducida por TPA en ratones Swiss CD-1, y un modelo de eritema agudo inducido por UVB en ratones sin pelo SKH-1 expuestos a una irradiación de 360 mJ/cm^2. Con el objeto de evitar los efectos negativos derivados del uso de disolventes como la acetona y de aumentar la permeabilidad de la FX a través de la piel, se formuló una crema que contenía el carotenoide (FX-crema) para su aplicación a los animales (0.2 % p/p). Los resultados del modelo de TPA mostraron que el pre-tratamiento con la FX-crema efectivamente mejoraba la hiperplasia en ratón inducida por TPA, reduciendo significativamente el edema de la piel, el espesor de la epidermis y la actividad de la MPO, así como la expresión epidérmica de los niveles de COX-2. Los datos relacionados con la inhibición de COX-2 concuerdan con un estudio *in vitro* en macrófagos RAW 264.7, en el que además, el carotenoide redujo consecuentemente los niveles de PGE_2. (Kim et al., 2010) En otro artículo más reciente *in vivo*, la FX redujo los niveles de esta enzima en un modelo de obesidad inducida por una dieta alta en grasa (Tan and Hou, 2014).

El modelo experimental usado en nuestros estudios, ha revelado por primera vez la actividad anti-inflamatoria de una formulación tópica que contiene FX en la hiperplasia cutánea inducida por TPA, lo que podría sugerir su uso en patologías inflamatorias como la

psoriasis o la dermatitis. Además, vale la pena destacar que dado que estas condiciones se benefician de la exposición terapéutica al sol, es muy importante asegurar la fotoprotección de la piel expuesta. Los protectores solares se utilizan para prevenir los efectos perjudiciales de la radiación UVB; sin embargo, su eficacia limitada apoya la necesidad de encontrar nuevas estrategias terapéuticas dirigidas contra la inflamación y el estrés oxidativo producidos por la exposición a UVB. Por lo tanto, nuestro siguiente objetivo fue examinar los efectos fotoprotectores de la FX en un modelo de daño celular inducido por UVB (50 mJ/cm^2) en queratinocitos HaCaT, así como en un modelo de eritema inducido por UVB en ratones sin pelo.

Recientemente, se ha demostrado que el papel antioxidante de la FX en queratinocitos HaCaT está relacionado con la protección del ADN contra el estrés oxidativo y la prevención de la apoptosis (Heo and Jeon, 2009; Zheng et al., 2013). De acuerdo con estos resultados, hemos demostrado el efecto fotoprotector de la FX en las células HaCaT, mediante el aumento de la viabilidad celular y la inhibición de los niveles de ROS e IL-6. Por otra parte, la radiación UVB aguda promueve la aparición de eritema, edema y pérdida de elasticidad y del contenido de humedad de la piel, así como el aumento de la producción de melanina, que está implicada en la melanogénesis (Shimoda et al., 2010). Varios estudios han revelado los efectos protectores de la FX en ratones irradiados con UVB mediante la reducción de los parámetros de melanogénesis (Shimoda et al., 2010) y en las quemaduras de sol inducidas por UVA mediante la promoción de la formación de la barrera cutánea (Matsui et al., 2016). Además, este carotenoide impidió el fotoenvejecimiento de la piel en ratones sin pelo a través de sus efectos antiangiogénicos y antioxidantes (Urikura et al., 2011). Los resultados del modelo *in vivo* demostraron que la FX-crema reducía el eritema inducido por UVB en ratón, así como el edema y la actividad MPO. Además, en concordancia con los resultados obtenidos en el modelo de TPA, la crema con el carotenoide inhibió la expresión de COX-2 en la piel expuesta a UVB.

La generación excesiva de ROS resulta en un estado de estrés oxidativo en las células de la piel y desempeña un papel esencial en la iniciación, la promoción y la progresión del envejecimiento de la piel, la carcinogénesis y muchos trastornos inflamatorios (Kanekura et al., 2000). Nrf2 es un factor que promueve la transcripción de genes antioxidantes y enzimas de detoxificación tales como HO-1, las cuales protegen contra el daño oxidativo inducido por UVB (Zhong and Li,

2016; Furue et al., 2017). Nuestros datos muestran que la aplicación de FX-crema aumentó la expresión de HO-1, a través de la regulación de la vía del Nrf2. Los resultados obtenidos en todos los estudios realizados con la FX sugieren que este carotenoide, vehiculizado en una formulación tópica para mejorar su permeación, podría suponer una estrategia novedosa para prevenir las exacerbaciones asociadas a la psoriasis y proteger a la piel de la radiación UV.

En relación a los estudios con compuestos naturales, las plantas siguen siendo hoy en día uno de los campos más prometedores para el descubrimiento de nuevos compuestos que pueden conducir al desarrollo de fármacos. En este sentido, los polifenoles son uno de los grupos más estudiados; estos compuestos son metabolitos secundarios de las plantas y generalmente, participan en los mecanismos de defensa de éstas contra agresiones externas. Con más de 8.000 estructuras diferentes conocidas, tienen como base común la presencia de uno o más grupos fenólicos. La actividad anti radicales libres de esta familia de compuestos ha sido ampliamente descrita, de hecho son los antioxidantes más potentes que consumimos en nuestra dieta. Sin embargo, la investigación en torno a ciertas propiedades todavía continúa en desarrollo. En este sentido, la actividad anti-inflamatoria es una de las más importantes. Por lo tanto, en nuestro siguiente estudio evaluamos el efecto de un compuesto polifenólico, el RA en un modelo de psoriasis inducida por IMQ. El IMQ es un agonista de los receptores *Toll-like* 7 y 8, cuya aplicación tópica en el dorso de ratones Balb/c durante 6 días, induce una inflamación acompañada de alteraciones típicas de la psoriasis. Debido a que la efectividad del RA aplicado por vía tópica puede ser limitada por su baja liposolubilidad y estabilidad, así como por su facilidad para oxidarse o degradarse con la luz, hemos usado nanotransetosomas aniónicos (NL) para encapsular dicho polifenol. Este tipo de liposomas deformables se emplean tanto para favorecer la estabilidad de los compuestos, como para dirigir la liberación de los mismos a una zona concreta de la piel, debido a su alta capacidad de permeación, lo que permite una optimización de la dosis empleada.

El pre-tratamiento tópico con el RA disuelto en gel, así como con el RA encapsulado en NL dispersos en gel (0.2 % p/p) 30 minutos antes de la aplicación del IMQ (62.5 mg por zona durante 6 días consecutivos) inhibió el daño macroscópico y el peso de la muestra obtenida de la piel afectada, sugiriendo una reducción del edema de la piel. Además, el RA disuelto en gel disminuyó la actividad de la MPO

y los niveles de las citocinas pro-inflamatorias TNF-α, IL-1β, IL-6 e IL-17, así como el tratamiento con los NL que contenían Dex, que mostró aún un mayor poder anti-inflamatorio. Sin embargo, el RA encapsulado en NL únicamente fue capaz de reducir la producción de TNF-α e IL-6. Dada la potente actividad antinflamatoria detectada con la Dex encapsulada en NL, esperábamos encontrar resultados similares con los NL que contenían RA. Por el contrario, los resultados obtenidos mostraron un mejor perfil anti-inflamatorio tras el tratamiento con el RA sin encapsular. Una posible explicación de estos resultados podría deberse a las diferentes características del RA y la Dex. Con respecto a la constante de disociación (pk_a), el RA a pH fisiológico (pH usado en la elaboración de NL) se encuentra ionizado en su forma negativa (pk_a 3.57), mientras que la Dex, al ser una base débil, se encuentra en su forma positiva (pk_a 12.14). En consecuencia, al tratarse de un NL cargado negativamente, se produce una repulsión entre la carga negativa del RA ionizado y la carga superficial del NL, por lo que apenas se ve afectado el potencial Z (-21 mW), respecto al control sin compuesto (-17 mW). También se observó un mayor tamaño de partícula (315 nm), debido a la repulsión y un mayor porcentaje de encapsulación del compuesto (72 %). Estos resultados confirmarían la estabilidad del RA dentro del espacio acuoso, debido a que no se observan cambios en la estructura del NL respecto al control vacío. En concordancia con lo establecido, moléculas con un perfil similar, como la curcumina, han demostrado una mayor eficiencia de encapsulación en NL cargados negativamente en comparación con las vesículas cargadas positivamente (Pamunuwa et al., 2016). Así, esta alta estabilización conduce a una menor liberación del compuesto desde los NL al medio celular, y consecuentemente, a una menor eficacia biológica obtenida en el modelo *in vivo*. Sin embargo, la atracción que sufre la Dex (cargada positivamente) con la bicapa del NL, además de la similitud estructural que presenta con los lípidos que forman la bicapa (colesterol), hacen que se dirija hacia la misma, produciendo un desplazamiento de la estructura. Esto provoca una variación del potencial Z (-9.0 mW), un menor tamaño causado por la atracción (150 nm) y una disminución del porcentaje de droga encapsulada (43%). Por tanto, esto se traduce en una mayor liberación del compuesto debido a la pérdida de la estructura del NL (Tsotas et al., 2007). En este sentido, al confirmar que la estructura química del RA influye en la eficacia final de la formulación, sería necesaria una encapsulación específica para este tipo de compuestos, si se busca una liberación rápida. Estudios previos han demostrado que un alto poder de

encapsulación retrasa la liberación del compuesto en liposomas aniónicos en comparación con las vesículas catiónicas (Doppalapudi et al., 2017). En este sentido, con estos resultados se pretende aportar un mayor conocimiento sobre la encapsulación de determinados compuestos en este tipo de vesículas, ya que consideramos el uso de las mismas una innovadora herramienta para la industria farmacéutica en términos de permeación y liberación tópica.

Por otra parte, se ha demostrado en extractos de origen natural que la combinación de diferentes compuestos muestra una mayor actividad que dosis equivalentes de los componentes individuales, indicando que entre ellos hay sinergismo o potenciación de efectos (Mukherjee et al., 2011). Estas interacciones juegan un rol importante en la eficacia terapéutica y su estudio es imprescindible a la hora de desarrollar un nuevo medicamento. Por tanto, nuestro último objetivo fue evaluar un posible sinergismo entre los principales compuestos empleados en esta tesis, la FX y el RA. Para ello, se ensayaron en un modelo de queratinocitos HaCaT irradiados con UVB (100 mJ/cm^2) con el objetivo de elucidar su mecanismo de acción y determinar si una mezcla de los mismos (5 µM de FX + 5 µM de RA) podría mejorar sus actividades biológicas. En este estudio se determinó la viabilidad celular, el ciclo celular, y la inducción de apoptosis, así como se evaluaron mediadores implicados en la activación del inflamasoma y de la vía antioxidante modulada por el Nrf2. La mezcla de estos compuestos demostró un mejor efecto fotoprotector y antioxidante que los compuestos por separado, disminuyendo la muerte celular, con el consecuente aumento de la viabilidad celular, y reduciendo la apoptosis y los niveles de ROS inducidos por la acción del UVB. Estos resultados eran los esperados, puesto que el papel antioxidante de la FX ha sido ampliamente estudiado y durante este tiempo se ha relacionado con la prevención del daño en el ADN y la disminución de la apoptosis, actuando como agente protector frente al estrés oxidativo (Heo and Jeon, 2009; Zheng et al., 2013). Asimismo, debido a su estructura polifenólica, el carácter antioxidante del RA es también conocido por inhibir la producción de ROS y la apoptosis inducida por el UVB (Psotova et al., 2006; Vostálová et al., 2010). En relación a la actividad anti-inflamatoria, la combinación de ambos compuestos redujo la expresión de los componentes del inflamasoma como son el NLRP3, la ASC y la caspasa-1, así como la consiguiente producción de IL-1 β. Tras el conocimiento de que la producción de esta citocina pro-inflamatoria se lleva a cabo tras la activación del inflamasoma en

queratinocitos (Jang et al., 2015), son numerosos los estudios que centran su atención en este complejo multriproteico. En este sentido, el inflamasoma desempeña un importante papel en la inflamación inducida por UVB, y consecuentemente, tiene un impacto crítico en la aparición de patologías de la piel, así como en el cáncer de piel (Ahmad et al., 2017). Debido a que el estudio del mismo está aún en desarrollo, el papel de la FX sobre este complejo no ha sido estudiado, siendo éstos los primeros resultados obtenidos al respecto. En relación al RA, recientemente Zhou et al. (2016), han observado un efecto en la regulación del inflamasoma frente a una respuesta inflamatoria en queratinocitos. Por último, sobre el efecto protector de estos compuestos, la mezcla de ambos aumentó la expresión de los niveles de la proteína antioxidante HO-1, mediante la regulación de la vía del Nrf2. Estos datos están en concordancia con estudios que recientemente han puesto de manifiesto el papel modulador de la FX (Liu et al., 2016) y del RA (Lu et al., 2017) sobre la vía del Nrf2.

En base a lo expuesto, nuestros estudios demuestran que los productos naturales tanto glicolípidos y la FX aislados de microalgas, como el polifenol RA, presentan propiedades anti-inflamatorias y antioxidantes muy relevantes para el tratamiento de diversas patologías de la piel, entre las que se pueden destacar la psoriasis o la inflamación causada por la exposición UVB. La capacidad de estos compuestos para activar vías antioxidantes como la del Nrf2, así como para modular respuestas inflamatorias relacionadas con el inflamasoma, hace que se consideren moléculas con un perfil muy interesante para su aplicación en la prevención del eritema solar, así como para controlar recidivas en pacientes con enfermedades inflamatorias crónicas de la piel, como la psoriasis.

CONCLUSIONES

1. Los glicolípidos MGMG y DGDG, así como las fracciones estudiadas muestran una acción anti-inflamatoria, antioxidante y foto-protectora frente al daño causado por la radiación UVB en queratinocitos humanos HaCaT, debida a la disminución de la liberación de LDH y ROS, así como de la producción de IL-6. Por otro lado, el estudio de estos compuestos en un modelo de macrófagos humanos THP-1 confirma su actividad anti-inflamatoria, mediante la reducción de la producción de la citocina TNF-α.

2. El pre-tratamiento tópico con el glicolípido MGMG-A y la fracción MGDG de *I. galbana,* disueltos en acetona, reduce el edema de la piel, la infiltración neutrofílica, y la producción de citocinas pro-inflamatorias en ratones con hiperplasia epidérmica inducida por TPA, lo que apoya una importante actividad anti-inflamatoria *in vivo*.

3. La elaboración de una crema que contiene la fracción MGDG permite mejorar sus propiedades de permeación a través de la piel y, como consecuencia, mejorar los parámetros inflamatorios evaluados en el modelo de hiperplasia, en comparación con su aplicación en acetona.

4. La administración tópica de la fucoxantina vehiculizada en crema reduce la inflamación producida por TPA en un modelo de hiperplasia epidérmica en ratones. Las respuestas anti-inflamatorias están relacionadas con la disminución del edema de la piel, del infiltrado de células inmunes y la expresión de COX-2.

5. La aplicación de la crema que contiene fucoxantina protege la piel de los ratones en un modelo de eritema agudo inducido por UVB. Los efectos beneficiosos se asocian a una diminución de la actividad de la MPO, reducción en el espesor de la epidermis, y de mediadores inflamatorios como la COX-2, así como a la activación de vías de señalización antioxidantes que incluyen el factor Nrf2.

6. El uso de nanoliposomas aniónicos para su aplicación por vía tópica permite una mejor permeación a través de las capas de la piel. Sin embargo, cuando en ellos se vehiculizan compuestos ionizados, como

el ácido rosmarínico (carga negativa), la estructura del nanoliposoma aumenta la estabilidad del compuesto en su interior, dificultando la liberación del mismo al medio celular, impidiendo que ejerza su efecto biológico. En este sentido, la aplicación tópica de RA vehiculizado en este tipo de nanoliposomas no ha supuesto una mejora de la actividad anti-inflamatoria esperada en el modelo de psoriasis en ratón inducida por Imiquimod, debido a que se estima que no se ha llevado a cabo la completa liberación del compuesto.

7. El pre-tratamiento con ácido rosmarínico o bien fucoxantina presenta un efecto fotoprotector en un modelo *in vitro* de células HaCaT irradiadas con UVB. Estos compuestos disminuyen la muerte celular, la producción de ROS y estimulan la vía de señalización antioxidante Nrf2 respecto a las células irradiadas. Sin embargo, no se observan cambios significativos en el número de células en apoptosis, ni en la expresión de las proteínas que conforman el inflamasoma. Sin embargo, la combinación de ambos compuestos disminuye el número de células en apoptosis, aumentando el número de células viables, a la vez que muestra una importante actividad anti-inflamatoria, mediante la reducción de la expresión de los genes del inflamasoma como son NLRP3, ASC y caspasa-1, con la consecuente inhibición de la producción de IL-1β.

En conclusión, nuestros estudios *in vitro* e *in vivo* han demostrado los efectos beneficiosos de la FX y el RA, en enfermedades de base inflamatoria, asociadas tanto a la exposición UVB como a patologías mediadas por el sistema inmune como la psoriasis. Sus mecanismos de acción podrían estar relacionados con la prevención del estrés oxidativo a través de la disminución en la producción de ROS y la activación de la vía Nrf2, así como con la reducción de la inflamación mediante la regulación del inflamasoma y otras citocinas relacionadas con la vía del NF-κB. Por esta razón, estos compuestos podrían suponer moléculas prometedoras para su posible incorporación en productos para el cuidado de la piel como por ejemplo protectores solares, cremas anti-edad o en formulaciones tópicas para el tratamiento de patologías inflamatorias como la psoriasis.

CONCLUSSIONS

1. The study of MGMG and DGDG glycolipids and fractions reported an anti-inflammatory, antioxidant and photoprotective actions against UVB-caused damage to HaCaT keratinocytes by reduction of LDH leakage, ROS generation and IL-6 production. In the other hand, these compounds confirm their anti-inflammatory activity inhibiting the production of the pro-inflammatory cytokine TNF-α in lipopolysaccharide-stimulated human THP-1 macrophages.

2. The topical pre-treatment with the MGMG-A glycolipid and the MGDG fraction from *I. galbana*, dissolved in acetone, reduced skin edema, leucocyte infiltration and pro-inflammatory cytokines production in the inflammatory model of TPA-induced skin hyperplasia in mice, which confirm the relevant *in vivo* anti-inflammatory activity.

3. The elaboration of the MGDG-cream allows improving the permeability properties through skin and ameliorating the inflammatory profile in hyperplasia skin model, in comparison with the application of compound dissolved in acetone.

4. The topical administration of Fucoxanthin-cream reduced skin inflammation on TPA-induced skin hyperplasia in mice. The anti-inflammatory responses are related with decrease of skin edema, cell infiltrate and COX-2 expression.

5. The application of Fucoxanthin-cream protects the skin against UVB-induced acute erythema model in hairless mice. The beneficial effects are associated with decrease of MPO activity, reduction of epidermal thickness and pro-inflammatory mediators as COX-2, as well as the activation of anti-oxidative signalling pathways as Nrf2.

6. The use of anionic nanoliposomes for topical application allows enhancing skin permeation. Nevertheless, when ionized compounds are encapsulated, as rosmarinic acid (negative charge), the formulation stability increases because the drug remains negatively charged into the aqueous space of liposomes. This leads to hindering the compound release to cell medium, which result in the reduction from the biological effect. In this sense, the topical application of RA-loaded anionic nanoliposomes do not improve the expected anti-inflammatory activity on Imiquimod-

induced psoriasis-like model in mice. This could be due to the high encapsulation efficiency of RA, which makes difficult the release of compound.

7. The pre-treatment with rosmarinic acid or fucoxanthin exhibits photoprotective effect on UVB-exposed HaCaT keratinocytes *in vitro* model. These compounds reduce of cell death and ROS production, as well as the stimulation of Nrf2 anti-oxidative pathway. However, the study do not reflect a significant change on apoptotic cells neither inflammasome proteins regulation. Conversely, the combination of both compounds diminishes apoptotic cells, increasing the viable cell number and shows an important anti-inflammatory activity by reduction of NLRP3, ASC and caspase-1 expression, with the consequent inhibition of IL-1β production.

In conclusion, our *in vitro* and *in vivo* studies have demonstrated the beneficial effects of FX and RA in inflammatory pathologies, related with UVB exposure or immune diseases as psoriasis. Their mechanisms of action could be related with the prevention of oxidative stress through of activation of Nrf2 pathways as well as with the reduction of inflammation via reduction of inflammasome components and other cytokines involved in NF-κB pathway. For this reason, these compounds could be promising molecules for their incorporation in many skin care products such as sun protectors and anti-aging creams, as well as for the treatment of inflammatory skin pathologies as psoriasis.

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