UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Química

D/Dª JOSÉ ALBERTO HERRERA MELIÁN, SECRETARIO DEL DEPARTAMENTO DE QUÍMICA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA,

Que el Consejo de Doctores del Departamento en su sesión de fecha 12 de noviembre de 2015 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "ANÁLISIS DE POLIFENOLES EN ALGAS. IMPLICACIÓN DE ESTOS COMPUESTOS EN MECANISMOS PARA DISMINUIR LA TOXICIDAD DEL COBRE Y REGULAR LA DISPONIBILIDAD DEL HIERRO EN MICROALGAS" presentada por la doctoranda Dª AROA LÓPEZ MONZÓN y dirigida por la Doctora MILAGROS RICO SANTOS.

Y para que así conste, y a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, defensa, tribunal y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a 12 de noviembre de dos mil quince.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Química

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

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Título de la Tesis Doctoral

"ANÁLISIS DE POLIFENOLES EN ALGAS. IMPLICACIÓN DE ESTOS COMPUESTOS EN MECANISMOS PARA DISMINUIR LA TOXICIDAD DEL COBRE Y REGULAR LA DISPONIBILIDAD DEL HIERRO EN MICROALGAS".

Tesis Doctoral presentada por Dª AROA LÓPEZ MONZÓN

Dirigida por el Dra. Dª MILAGROS RICO SANTOS
Codirigida por el Dr. D ARGIMIRO RIVERO ROSALES

La Directora, **El Codirector,** **La Doctoranda,**

Las Palmas de Gran Canaria a 6 de noviembre de 2015.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Química

D. ARGIMIRO RIVERO ROSALES, DIRECTOR DEL DEPARTAMENTO DE QUÍMICA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

HACE CONSTAR:

Que la presente memoria titulada "ANÁLISIS DE POLIFENOLES EN ALGAS. IMPLICACIÓN DE ESTOS COMPUESTOS EN MECANISMOS PARA DISMINUIR LA TOXICIDAD DEL COBRE Y REGULAR LA DISPONIBILIDAD DEL HIERRO EN MICROALGAS", que presenta la doctoranda Dña. Aroa López Monzón para optar al grado de Doctor por esta Universidad, ha sido realizada en los laboratorios del Departamento de Química de la Universidad de Las Palmas de Gran Canaria. Tras su registro y trámite autorizado por el Departamento, autorizo con ésta fecha de presentación.

Y para que así conste a los efectos oportunos, firmo la presente en Las Palmas de Gran Canaria, a 12 de noviembre de 2015.

REGLAMENTO DE ESTUDIOS DE DOCTORADO DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA Aprobado por el Consejo de Gobierno el 17 de diciembre de 2012 (BOULPGC de 9 de enero de 2013) y modificado por el Consejo de Gobierno de 23 de octubre de 2013 (BOULPGC de 4 de noviembre de 2013)

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

TESIS DOCTORAL

ANÁLISIS DE POLIFENOLES EN ALGAS. IMPLICACIÓN DE ESTOS COMPUESTOS EN MECANISMOS PARA DISMINUIR LA TOXICIDAD DEL COBRE Y REGULAR LA DISPONIBILIDAD DEL HIERRO EN MICROALGAS

Aroa López Monzón

LAS PALMAS DE GRAN CANARIA

2015

A mis padres
y a Miguel.

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A todos gracias.

ÍNDICE

Capítulo 1:

OBJETIVOS

Este trabajo se centra en la optimización de métodos de identificación y cuantificación de polifenoles, principalmente en macroalgas recolectadas en las costas de las Islas Canarias y en microalgas cultivadas en presencia y en ausencia de altas concentraciones de metales a partir de cepas proporcionadas por el Banco Español de Algas. Un objetivo primordial del trabajo consistió en evaluar la influencia de las condiciones de cultivo en el perfil polifenólico de las microalgas y la interacción de dichos compuestos con el cobre (tóxico a altas concentraciones) y el hierro (fertilizante) disueltos en el agua de cultivo para determinar su posible papel en los mecanismos de detoxificación de metales y en los equilibrios de especiación.

1.1. Objetivos del trabajo

Objetivo I: desarrollar y optimizar una metodología para la extracción, identificación y cuantificación de compuestos polifenólicos en macroalgas mediante cromatografía líquida de alta resolución en fase reversa (RP-HPLC) acoplada a un detector de Diodo Array (DAD).

- Optimizar la extracción de los compuestos polifenólicos de algas mediante el uso de diferentes disolventes.

- Realizar el análisis cromatográfico de los polifenoles extraídos con cada disolvente de manera rápida, sensible y con alta reproducibilidad y comparar así, la capacidad de

cada disolvente para extraer un grupo seleccionado de polifenoles.

- Determinar la actividad antioxidante de los extractos preparados con diferentes disolventes y su potencial uso como fuente de polifenoles.

Todos estos objetivos fueron alcanzados y publicados en el siguiente artículo:

López A (autora principal), Rico M, Rivero A, Suarez de Tangil M. The effects of solvents on the phenolic contents and antioxidant activity of *Stypocaulon scoparium* algae extracts. *Food Chemistry* (2011) 125:1104-1111.

Objetivo II: adaptar las metodologías optimizadas en el objetivo I para la identificación y cuantificación de compuestos polifenólicos en microalgas utilizando técnicas de pre-concentración de muestras (extracción en fase sólida).

- Identificar y cuantificar los compuestos polifenólicos de diatomeas *Phaeodactylum tricornutum (P. tricornutum) y de* microalgas verdes *Dunaliella tertiolecta (D. tertiolecta)* cultivadas en agua de mar enriquecida con altas concentraciones de hierro (III) (fertilizante) o con altas concentraciones de cobre (II) (tóxico) y en agua de mar no enriquecida con metales (control).

- Analizar la posible implicación de los polifenoles en los mecanismos de defensa de las microalgas cultivadas en condiciones de estrés oxidativo debido a la presencia de altas concentraciones de cobre.

- Estudiar el efecto de los polifenoles en la especiación del hierro en disolución.

- Comparar el crecimiento celular de las microalgas y las diferencias que se producen en la producción de polifenoles en ambos microorganismos en función de las condiciones de cultivo.

- Estudiar la actividad antioxidante de extractos preparados con microalgas *P. tricornutum* y *D. tertiolecta*.

  Estos objetivos se han alcanzado en los siguientes artículos:

  Rico M, López A (autora principal), Santana-Casiano JM, González AG, González-Dávila M. Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress. *Limnology and Oceanography* (2013) 58:144-152.

  López A (autora principal), Rico M, Santana-Casiano JM, Gonzaléz AG, González-Dávila M. Phenolic profile of *Dunaliella tertiolecta* growing under high levels of copper and iron. *Environmental Science and PollutionResearch*

(2015) 22:14820-14828.

Santana-Casiano JM, González-Dávila M, González AG, Rico M, López A (no es autora principal), Martel A. Characterization of polyphenol exudates from *Phaeodactylum tricornutum* and their effects on the chemistry of Fe(II)-Fe(III). *Marine Chemistry* (2014) 158:10-16.

Objetivo III: aplicar la metodología desarrollada en los objetivos I y II a extractos de distintos materiales vegetales terrestres y marinos. Este objetivo se ha cumplido y se evidencia con la presentación de los siguientes artículos:

- Estudios con Aloe vera: se realizó la optimización de un nuevo método de identificación y cuantificación de cuatro polifenoles. Los objetivos se alcanzaron en el siguiente artículo:

  López A (autora principal), Suárez de Tangil M, Vega-Orellana O, Ramírez AS, Rico M. Phenolic Constituents, Antioxidant and Preliminary Antimycoplasmic Activities of Leaf Skin and Flowers of Aloe vera (L.) Burm. f. (syn. A. barbadensis Mill.) from the Canary Islands (Spain). *Molecules* (2013) 18:4942-4954.

- Estudios con plantas ornamentales *Protea*: colaboración con el Instituto de Productos Naturales y Agrobiología del

Centro Superior de Investigaciones Científicas de La Laguna en la que Aroa López realiza la identificación y cuantificación de polifenoles.

Alfayate C, Vera-Batista C, López A, Rico M, Brouard I, León F. Phenolic compounds, antioxidant activity and ultrastructural study from *Protea* hybrid 'Susara'. *Industrial Crops and Products* (2014) 55:230–237.

- Estudios con plantas terrestres de dos especies *Tolpis*: colaboración en la que Aroa López no es autora principal del trabajo:

  Triana J, López M, Pérez FJ, Rico M, López A, Estévez F, Marrero MT, Ignacio Brouard I, León F. Secondary Metabolites from Two Species of Tolpis and Their Biological Activities. *Molecules* (2012) 17:12895-12909

Los tres objetivos previamente descritos están alineados con los objetivos definidos en los siguientes proyectos de investigación en los que se enmarca el presente trabajo:

Título del proyecto: *Estudio del comportamiento biogeoquímico del Fe en un ambiente oceánico con alto contenido en* CO_2 (Referencia: CTM2009-12526 SUBPROGRAMA MAR). Duración: 2010. En este proyecto ha participado el Banco Español de Algas.

Título del proyecto: *I Taller TECNOVA de Gran Canaria de innovación y cooperación tecnológica TL-015 estudio de la actividad antioxidante de aloveria y otras especies vegetales* (Fondos FEDER). Duración: 2010 – 2011. Cuantía de la subvención: 22.000 euros

Título del proyecto*: Estudio del comportamiento biogeoquímico del Fe en ambientes marinos acidificados*. (Referencia: CTM2010-19517 SUBPROGRAMA MAR). Duración: Enero 2011-Diciembre 2013. En este proyecto ha participado el Banco Español de Algas.

Título del proyecto: *Actividad antioxidante, variación del perfil polifenólico y efecto de los metales hierro (III) y cobre (II) en cianobacterias Synechococcus sp*. Programa Innova Canarias 2020. Convocatoria 2014. Patrocinador: Servatur, S.A. Cuantía: 6000€.

1.2. Líneas de trabajo futuras

Los trabajos que se describen en el presente documento han abierto líneas de investigación relacionadas con la caracterización y cuantificación de compuestos orgánicos que se desarrollarán dentro del proyecto de investigación *Efecto de la Acidificación y del Calentamiento Oceánico en el comportamiento biogeoquímico del Fe en el Atlántico Norte (EACFe) (Referencia: CTM 2014-52342-P)*. En este proyecto se harán converger

- dos líneas de investigación lideradas por el grupo de investigación de Química Marina (QUIMA): (1) determinación del sistema del CO_2 en el océano y (2) estudio del Fe(II) en el medio marino;

- y se agrupa una tercera línea de investigación desarrollada por el grupo de Química Orgánica I, en el que ha estado integrada la licenciada Aroa López Monzón, y centrada en la determinación de compuestos orgánicos como continuación de los trabajos iniciados en el presente documento.

- Ambos grupos de investigación pertenecen a la Universidad de las Palmas de Gran Canaria.

El mencionado proyecto EACFe estudiará los efectos que tienen el pH, la temperatura, la concentración de O_2 y la materia orgánica en el comportamiento del sistema Fe(II)-Fe(III) en la región Subártica, en la sección hidrográfica CLIVAR A1E (59.5ºN), como base fundamental para contrastar los estudios realizados en laboratorio y desarrollar un modelo cinético global para el hierro en el contexto de la acidificación y calentamiento oceánico. El hierro tiene un gran impacto en el ciclo del carbono a través de las comunidades planctónicas y su productividad. La materia orgánica juega un papel esencial en el ciclo del Fe en el océano. Los compuestos orgánicos presentes en el agua de mar pueden reducir Fe(III) a Fe(II) y también estabilizar el Fe(II) a través de la

complejación. Se ha observado que, en presencia de determinados compuestos orgánicos como los polifenoles, aminoácidos o azúcares, el crecimiento de organismos eucariotas se ve favorecido. Investigar qué compuestos y mecanismos son los que determinan la presencia de Fe(II) en el medio marino y cómo se ven estos afectados por la acidificación y calentamiento oceánico es el objetivo principal de este proyecto. Para ello, se combinarán los estudios que se realizarán en la región Subártica Atlántica, una de las más afectadas por la acidificación oceánica, con los estudios en laboratorio usando compuestos orgánicos individuales y exudados procedentes de fitoplancton,en los que se trataran cada una de las variables de forma independiente, para poder definir así, la contribución de cada una de ellas al proceso. La cuantificación y caracterización de ligandos, polifenoles, sacáridos y aminoácidos, se realizará mediante cromatografía líquida de alta resolución (HPLC).

Capítulo 2:

INTRODUCCIÓN

2.1. Compuestos polifenólicos

Durante el transcurso de la evolución, los organismos vegetales han desarrollado mecanismos para enfrentarse a los cambios producidos en las condiciones ambientales haciendo uso de la gran variedad estructural de compuestos químicos producidos por sus metabolismos secundarios. Entre estos grupos de compuestos, los polifenoles o fenoles, así como los alcaloides y los terpenoides, han demostrado su actividad protectora en las plantas, contribuyendo a mantener el equilibrio ecológico entre los productores primarios y los consumidores. Los polifenoles merecen especial atención si se tienen en cuenta los múltiples beneficios que ofrecen a las plantas y, por lo tanto, a otros organismos vivos; resultado, principalmente, de sus propiedades fisicoquímicas vinculadas al grupo funcional fenol [Quideau y colaboradores, 2011].

Constituyen un grupo muy amplio de compuestos cuya forma estructural más elemental es el fenol, que consiste en un anillo aromático plano y un grupo hidroxilo. Se clasifican en polifenoles y fenoles simples dependiendo del número de subunidades de fenol. Los fenoles simples incluyen los ácidos fenólicos y los fenilpropanoides o derivados del ácido cinámico [Ignat y colaboradores, 2011] (FIGURA 1). Los polifenoles que poseen dos subunidades de fenol son los flavonoides y estilbenos, y los compuestos con tres o más subunidades de fenol se conocen como taninos. Los flavonoides constituyen el grupo de

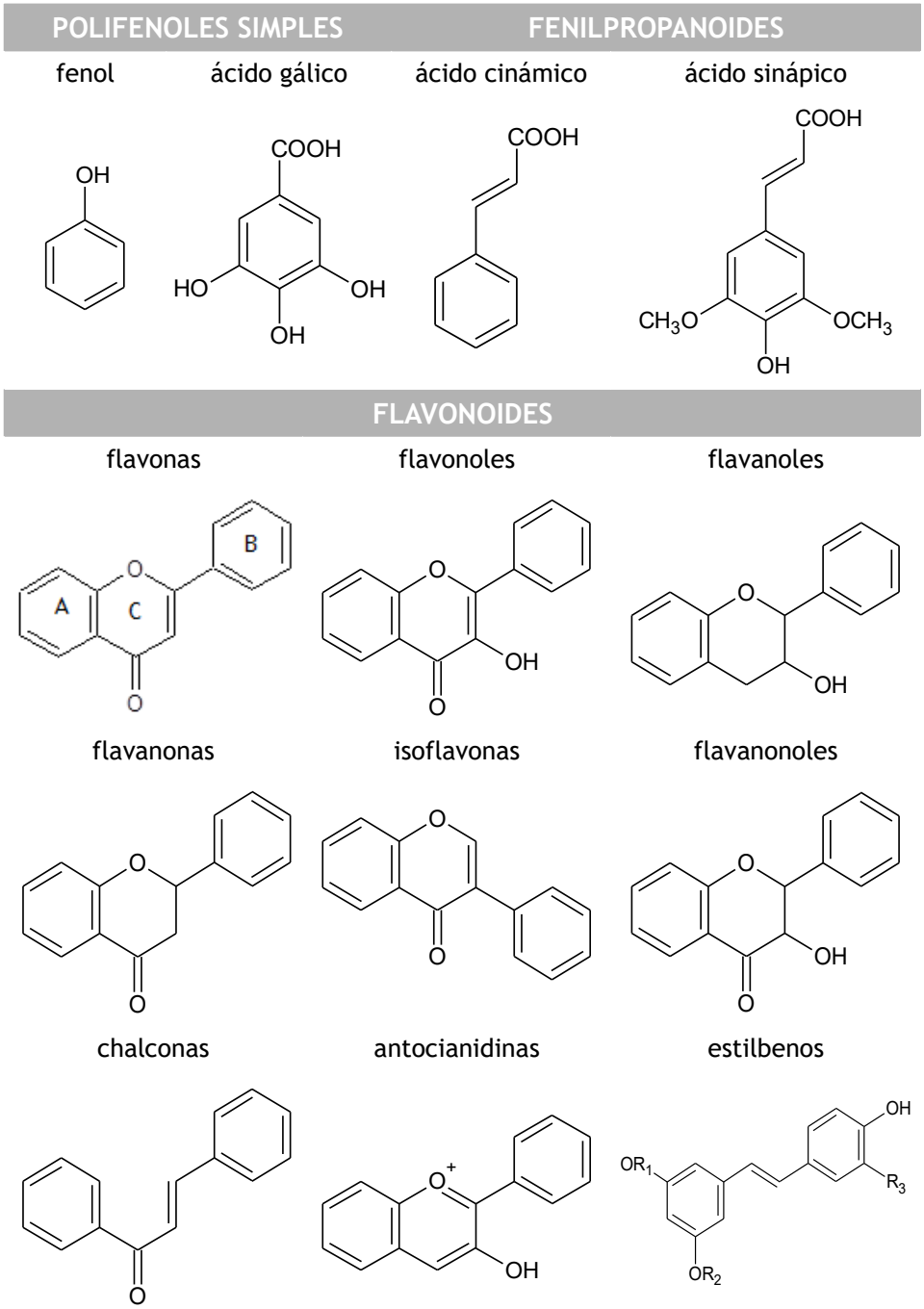


FIGURA 1.- Compuestos fenólicos.

compuestos polifenólicos más común y ampliamente distribuido en especies vegetales. Su estructura básica consiste en un sistema de dos anillos de benceno (A y C) unidos por un anillo de pi*rano (C) (ver flav*onas en FIGURA 1). Las actividades de los polifenoles están relacionad*as con su estructu*ra *y naturaleza química*, que depende de su grado de hidroxilación, sustituciones, conjugaciones y grado de polimerización [Kelly y colaboradores, 2002; Wright y co*laboradores, 2001*].

2.2. Actividad fisiológica en los organismos vegetales.

Inicialmente, los polifenoles fueron considerados productos de desecho procedentes del metabolismo secundario de los vegetales. Actualmente, se sabe que estos metabolitos tienen una amplia variedad de funciones que han sido objeto de diversas revisiones bibliográficas [Harborne y Williams, 2000; Treutter, 2006; Quideau, 2011]:

(1) Atracción de animales polinizadores: muchos flavonoides son componentes de pigmentos presentes en las flores y hojas, que les confieren coloraciones atrayentes de insectos polinizadores. Con frecuencia, el color azul de las flores se debe a la presencia de la antocianina delfinidina, presente en los pétalos, que normalmente requiere la presencia de un copigmento. El color azul es el más atractivo para las abejas polinizadoras, por lo que se observa una evolución hacia este color en la flora de clima templado, donde la abeja es el

polinizador predominante [Gottlieb, 1982].

(2) Protección de la radiación ultravioleta (UV): algunos flavonoides suelen acumularse en las capas superficiales de las plantas y captan gran parte de las radiaciones UV, evitando sus efectos nocivos en el interior. Distintas investigaciones muestran que las plantas sometidas artificialmente a la radiación UV-B (rayos UV de longitud de onda entre 280 y 320 nanómetros) responden con cambios en las rutas metabólicas de la síntesis de flavonoides. Los cambios en los niveles de estos compuestos no solo se observan en las células epidérmicas de la superficie del hazde las hojas, también se producen en la cera y en las vellosidades [Olsson y colaboradores, 1998; Gitz y colaboradores, 1998; Cuadra y colaboradores, 1997].

(3) Actividad antimicrobiana y fungicida: una de las principales funciones de los polifenoles es la protección de las plantas contra la invasión microbiana. Esto no solo implica su presencia en plantas como constituyentes sino también su acumulación como fitoalexinas en respuesta a los microbios atacantes [Harborne, 1999; Dixon y colaboradores, 1983]. En general tienen capacidad de inhibir la germinación de las esporas de patógenos.

(4) Aleloquímicos en interacciones entre plantas: a pesar de que aún no se conoce exactamente la manera en la que actúan, se ha observado actividad antimicrobiana de flavonoides

en interacciones alelopáticas entre plantas [Chou, 1999; Inderjit y Gross, 2000]. La (+)-catequina se ha identificado como un aleloquímico fitotóxico que es exudado por la planta herbácea Centaurea maculosa a través de sus raíces inhibiendo la germinación y el crecimiento de *Centaurea diffusa* y *Arabidopsis thaliana* [Bais y colaboradores, 2003]. Por otra parte, un estudio de Gao y colaboradores (2011) demostró la interacción alelopática de 3 macroalgas, *Elodea nuttallii* (Planch), *Hydrilla verticillata* (L.f.) y *Veitchia spiralis* (L.) de la familia Hydrocharitaceae, para inhibir el crecimiento de especies tóxicas y no tóxicas de la cianobacteria *Microcystis aeruginosa* por excreción de compuestos fenólicos en el agua.

(5) Interacción entre plantas y animales: algunos flavonoides protegen a las especies vegetales de los organismos herbívoros generando sabores o texturas desagradables. Muchos depredadores muestran sensibilidad frente a estos compuestos [Hoffmann-Campo y colaboradores, 2001; Haribal y Feeny, 2003; Thoison y colaboradores, 2004; Chen y colaboradores, 2004]. Sin embargo, no actúan solamente como mecanismos de defensa de amplio espectro [Forkner y colaboradores, 2004]. Algunos polifenoles confieren aromas y colores a los frutos que los hacen más apetecibles para los herbívoros y que estos actúen como dispersores de semillas. Plantas carnívoras poseen antocianinas en sus flores y hojas que atraen a los insectos que les sirven de alimento [Schaefer y Ruxton, 2008].

(6) Protección frente a condiciones climáticas extremas: en ocasiones se atribuye a los compuestos fenólicos la resistencia de las plantas a las heladas [Chalker-Scott y Krahmer, 1989] y a la sequía [Moore y colaboradores, 2005; Tattini y colaboradores, 2004] debido a las funciones que desempeñan en las membranas y paredes celulares.

(7) Protección frente a contaminantes en el medio ambiente: protegen a las plantas que viven en suelos ricos en metales tóxicos como el aluminio [Barceló y Poschenrieder, 2002]. Roitto y colaboradores (2005) comprobaron que los árboles de pino silvestre (*Pinus sylvestris* L.) sometidos a los efectos de altas concentraciones de cobre (Cu) y níquel (Ni) presentaban concentraciones más altas de taninos que los árboles de zonas no sometidas a estos metales.

2.3. Efectos sobre la salud humana.

La oxidación es un proceso natural que tiene lugar en las moléculas orgánicas con importantes implicaciones negativas en la salud humana. El metabolismo oxidativo es esencial para la supervivencia de las células con la producción de radicales libres y otras especies oxidantes. Cuando se produce un exceso de estas especies, las enzimas protectoras frente a la oxidación (catalasas, peroxidasas, superóxido dismutasa) pueden saturarse, lo que puede causar efectos destructivos y letales para las células, por oxidación de los lípidos de la membrana celular, de

proteínas celulares, de ácido desoxirribonucleico (ADN) y enzimas, etc., parando la respiración celular [Winrow y colaboradores, 1993; Bauer y colaboradores, 1999].

Los radicales libres son moléculas altamente reactivas que poseen electrones desapareados. Se trata de especies químicas deficientes en electrones que, por ello, se combinan rápidamente con un electrón de otro átomo. "Atacan" a las moléculas más cercanas para "robarles" sus electrones. Cuando la molécula que ha sido atacada pierde su electrón, se transforma ella misma en otro radical y comienza entonces una "cadena de robos de electrones". La oxidación causada por los radicales libres reduce la capacidad de combatir el envejecimiento y las enfermedades que lleva asociadas. Crece el número de investigaciones que confirman la relación entre una excesiva producción de radicales libres y algunos estados patológicos asociados al envejecimiento tales como cáncer, enfermedades cardiovasculares (arterosclerosis, obstrucción coronaria, etc.) o enfermedades neurodegenerativas como la enfermedad de Alzheimer y Parkinson. [Uttara y colaboradores, 2009; Nakabeppu y colaboradores, 2007; Halliwell, 1992].

La propiedad mejor descrita de los polifenoles es la capacidad antioxidante hacia los radicales libres y las especies reactivas de oxígeno (EROs) normalmente producidos por el metabolismo de las células o en respuesta a factores externos. Los compuestos polifenólicos tienen, por lo tanto, la capacidad

de contrarrestar los efectos dañinos de los radicales libres en los tejidos y podrían proteger el organismo contra el cáncer, arterosclerosis, enfermedades cardíacas y otras enfermedades asociadas al envejecimiento [Dillard y German, 2000; Dai y Mumper, 2010; Quideau y colaboradores, 2011]. Los polifenoles han demostrado actuar sobre varias etapas claves en mecanismos de transducción de señales relacionadas con proliferación celular, diferenciación, apoptosis, inflamación, angiogénesis y metástasis. Sin embargo, estos mecanismos moleculares de acción no están completamente caracterizados y quedan muchas cuestiones por aclarar [Ramos, 2008].

Existe actualmente una gran controversia en cuanto a la ingesta de antioxidantes naturales puros para reducir el riesgo de cáncer, enfermedades cardiovasculares, diabetes y otras asociadas al envejecimiento. Algunos autores señalan que los antioxidantes podrían reducir los radicales libres creados por la radioterapia y algunas formas de quimioterapia disminuyendo la eficacia de la terapia y estando contraindicados durante la terapéutica del cáncer [Prasad y colaboradores, 2001; Kumar y colaboradores, 2002]. Sin embargo, otros autores defienden que la administración oral de antioxidantes produce efectos beneficiosos en varios cánceres y, salvo algunos casos específicos, estudios en animales y humanos demuestran que no se produce reducción de la eficacia de la quimioterapia o la radioterapia cuando se administra con antioxidantes [Ramos, 2008; Quideau, 2004].

Los efectos potencialmente nocivos de los procesos oxidativos se pueden prevenir mediante la utilización de antioxidantes, pero su uso en la prevención de enfermedades se limita a la recomendación de consumir alimentos vegetales debido a su alto contenido en ese tipo de sustancias. La industria farmacéutica es reticente a desarrollar fármacos a partir de polifenoles dado que, como se ha comentado anteriormente, sus mecanismos moleculares de acción no están completamente caracterizados.

2.4. Efectos en la oxidación y conservación de alimentos

La oxidación puede afectar, también, a los alimentos, en especial por oxidación de los ácidos grasos [Kanner, 1994], siendo esta la mayor causa del deterioro químico que deriva en la pérdida del sabor, del valor nutricional, del color, la textura y de la seguridad de dichos alimentos [Colbert y Decker, 1991]. Se estima que la mitad de la fruta y verdura cosechada en el mundo se pierde después de la recogida debido a su deterioro [Shahidi y colaboradores, 1992]. Para evitar los efectos de la excesiva oxidación se recurre al uso de conservantes con propiedades antioxidantes. Desde comienzos del siglo pasado han sido ampliamente utilizados los antioxidantes sintéticos 3-tercbutil-4-hidroxianisol (BHA, E320) y 2,6-ditercbutil-4-metilfenol (BHT, E321). Sin embargo, en los últimos años se han impuesto restricciones en el uso de estos compuestos debido a su posible efecto carcinógeno [EFSA, 2011; EFSA, 2012].

Los efectos tóxicos del BHA y BHT con frecuencia se producen tras altas dosis y tratamientos a largo plazo. El BHA induce tumores en el estómago de animales, que son dependientes de las dosis ingeridas; el BHT induce tumores hepáticos en experimentos de larga duración. Todos los estudios publicados coinciden en que estos compuestos son promotores de tumores. Esto unido a la preocupación de los consumidores por la seguridad de los alimentos ha provocado un aumento de la demanda de antioxidantes naturales en los últimos años que pueden consistir en extractos crudos de vegetales, que reciben cada vez mayor interés para la industria alimentaria y farmacéutica [Southon, 2000].

Debido a la diversidad química de los antioxidantes, es difícil separar y cuantificar antioxidantes individuales de la matriz vegetal. A menudo es más significativo el poder antioxidante total de la mezcla de compuestos en el extracto debido a su acción conjunta [Gao y colaboradores, 2011]. Por ejemplo, con la adición de extractos de semilla de mango (con alto contenido de compuestos fenólicos) a mantequilla clarificada a un nivel igual o superior al 5% se consigue mayor efectividad en aumentar la estabilidad de dicha mantequilla que con la adición de BHA a los niveles permitidos por la normativa vigente para este producto (0.02%) [Puravankara y colaboradores, 2000]. Por otro lado, la incorporación de extractos vegetales ricos en polifenoles a macarrones ha conseguido mejorar sus propiedades

antioxidantes: el contenido de polifenoles aumenta de 0,46 a 1,80 miligramos (mg) por g de macarrones y el contenido de carotenoides aumenta de 5 a 84 µg por g de macarrones, sin afectar su textura o sus propiedades de cocción y sensoriales [Ajila y colaboradores, 2010].

2.5. Mecanismos de reacción.

Las propiedades antioxidantes de los polifenoles se explican a través de tres mecanismos principales derivados de la reacción directa con los radicales libres [Leopoldini y colabores, 2004; Wright y colaboradores, 2001] y de la quelación de metales libres, estos últimos involucrados en reacciones que generan radicales libres [Pérez-Trueba, 2003; Leopoldini y colaboradores, 2011].

• La inhibición directa de radicales libres se produce a través de dos mecanismos que en muchas ocasiones tienen lugar simultáneamente y dependen del pH [Bentes y colaboradores, 2011; Neudörffer y colaboradores, 2006]. Se trata de reacciones de transferencia de hidrógeno (1) y de transferencia de electrones (2) (FIGURA 2).

Los productos resultantes en la reacciones (1) y (2) son especies menos perjudiciales que el radical libre inicial, ya que, aunque en (1) se genera un nuevo radical, es menos reactivo debido a la estabilización por resonancia (FIGURA 3), lo que

afecta también al radical catión generado en la reacción (2). Por otra parte, la entalpía de disociación del enlace O–H fenólico es un parámetro importante en la evaluación de la acción antioxidante en el mecanismo de transferencia de átomos de hidrógeno; cuanto menor sea ese valor, más fácil será la disociación de dicho enlace y la reacción con el radical libre. En el mecanismo de transferencia de electrones, el potencial de ionización es el parámetro más importante para la evaluación de la actividad de inhibición; cuanto menor sea su valor, más fácil será la abstracción de electrones y la reacción con los radicales libres.

$$ArOH + R^{\bullet} \rightarrow ArO^{\bullet} + RH \quad (1)$$

$$ArOH + R^{\bullet} \rightarrow ArOH^{+\bullet} + R^{-} \quad (2)$$

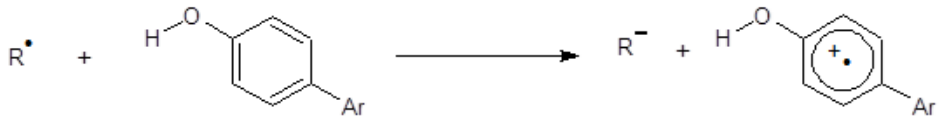


FIGURA 2.- Inhibición de especies reactivas del oxígeno.

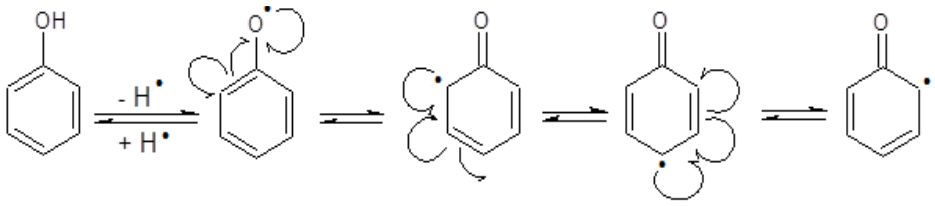


FIGURA 3.- Estabilización del radical fenoxilo por resonancia.

• Indirectamente, los polifenoles actúan como agentes quelantes de iones de metales de transición, formando compuestos complejos estables [Andjelković y colaboradores, 2006; Mira y colaboradores, 2002], lo que reduce su concentración y evita que intervengan en reacciones que producen radicales libres (FIGURA 4).

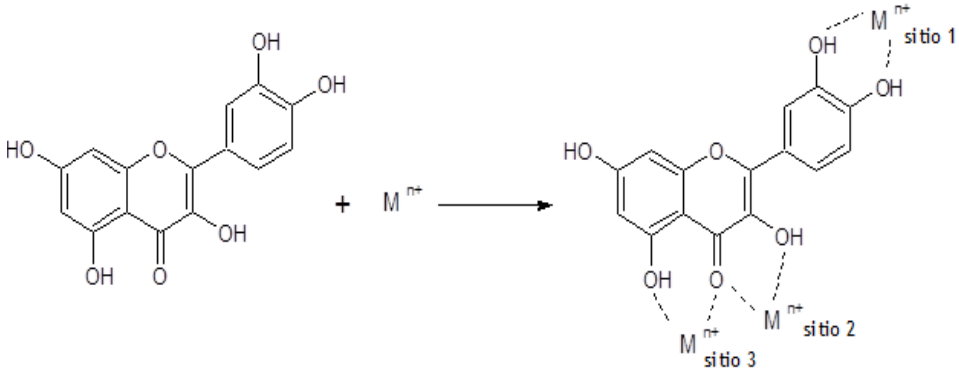


FIGURA 4.- Quelación de metales pesados

Algunos metales como el cobre, el manganeso, el cobalto y, principalmente, el Fe(II), pueden participar en la reacción de Fenton con peróxido de hidrógeno, en la que se produce el radical hidroxilo (HO•) (FIGURA 5), generalmente aceptado como

de los radicales más reactivos, con un tiempo de vida media muy corto (10^{-9} segundos). La reacción de Fenton es responsable de la acumulación de radicales libres que inician procesos de degradación de biomoléculas.

$$H_2O_2 + M^{n+} \rightarrow OH^{\bullet} + OH^{-} + M^{(n+1)+}$$

FIGURA 5.- Reacción de Fenton.

Los polifenoles no solo presentan actividad de manera individual, también se ha de tener en cuenta su comportamiento en mezclas, ya que pueden actuar de manera aditiva, sinérgica o antagónica [Jacobo-Velazquez y Cisneros-Zevallos, 2009]. Como se ha señalado anteriormente, la dificultad de separar estos compuestos de matrices vegetales y la actividad total de la mezcla, muchas veces superior a la de compuestos aislados, hace que se recurra al uso de los extractos. Tafesh y colaboradores (2011) evaluaron la acción de inhibición del crecimiento de varias cepas de bacterias (gram positiva *Streptococcus pyogenes* y *Staphylococcus aureus* y gram-negativa *Escherichia coli* y *Klebsiella pneumoniae*) por hidroxitirosol y ácido gálico. Demostraron la actividad sinérgica positiva de mezclas de hidroxitirosol y ácido gálico que, en concentraciones de 100 y 200 μg mL^{-1} respectivamente, provocaron una inhibición completa de las cuatro cepas bacterianas. Cuando cada compuesto se utilizó por separado fueron necesarias concentraciones muy superiores, de 400 μg mL^{-1} de hidroxitirosol para la inhibición total de las

cuatro cepas. El ácido gálico produjo la inhibición del crecimiento de las dos cepas gram positivas *S. aureus* and *S. pyogenes* a concentraciones de 200 y 400 μg mL^{-1} respectivamente, no inhibiendo el crecimiento de las cepas gram negativas.

2.6. Polifenoles en algas

Las macroalgas son los principales productores primarios en los ecosistemas costeros y se emplean, principalmente, como alimento, pero también como fertilizante, en la medicina tradicional o en la industria cosmética, entre otras.

Las algas se consideran una fuente de compuestos bioactivos, ricas en distintos tipos de antioxidantes [Lim y colaboradores, 2002; Yan y colaboradores, 1999]. Los extractos de varias especies de algas han mostrado actividades biológicas relacionadas con su contenido en florotaninos [Holdt y Kraan, 2011; Wijesinghe y Jeon, 2011]. Los roles y funciones de estos compuestos han sido objeto de muchos estudios, especialmente los relacionados con las interacciones con herbívoros y anti-fouling [Cronin, 2001; Amsler y Fairhead, 2006]. Arnold y Targett (2002) sugieren que la función de los polifenoles en las plantas marinas es osmorreguladora a nivel de la pared celular. Los taninos pueden afectar a la palatabilidad debido a su sabor, actúan como potenciales agentes antioxidantes y, además, interactúan en las reacciones redox de las plantas [Luck y

colaboradores, 1994; Larson, 1997].

A pesar de las propiedades de los polifenoles y del potencial de las algas como fuente de esos compuestos [Chacón-Lee y González-Mariño, 2010], pocos estudios se han centrado en su identificación y cuantificación en algas y en su implicación en los mecanismos de defensa celular frente a especies reactivas de oxígeno [Cirulis y colaboradores, 2013]. En diferentes estudios se ha evaluado la capacidad antioxidante y el contenido total de polifenoles mediante el ensayo de Folin-Ciocaleau. Hajimahmoodi y colaboradores (2010) determinan la actividad antioxidante y el contenido total de polifenoles para 12 microalgas demostrando un importante papel de los compuestos polifenólicos como los mayores contribuyentes a la capacidad antioxidante. Goiris y colaboradores (2012) obtienen resultados similares al estudiar la capacidad antioxidante y el contenido total de polifenoles de 32 muestras de microalgas. En todos los estudios mencionados, el contenido total de polifenoles se evaluó a través del ensayo de Folin-Ciocaleau mediante espectrofotómetro de UV-visible. El reactivo de Folin-Ciocaleau es un reactivo no específico capaz de oxidar simultáneamente carbohidratos, aminoácidos y polifenoles [Apak y colaboradores, 2007; Huang y colaboradores 2005] y, por lo tanto, no refleja únicamente el contenido en polifenoles.

Existen muchas evidencias de la estimulación del metabolismo polifenólico en plantas superiores que crecen en condiciones de estrés causado por la presencia en el medio de

cultivo de altas concentraciones de metales pesados o por la ausencia de metales esenciales para la supervivencia [Michalak, 2006]. Wei y colaboradores (2007) realizaron un estudio con plantas de trébol rojo sometidas a deficiencia de hierro, observando que estas condiciones inducen la secreción de compuestos fenólicos exudados por la raíz. La toxicidad producida por la presencia de diferentes metales pesados en el medio de cultivo también aumenta la producción de compuestos polifenólicos en cultivos de manzanilla (*Matricaria chamomilla* L.), de trigo y de maíz [Díaz y colaboradores, 2001; Winkel-Shirley, 2002; Kovacik y Backor, 2007]. El importante papel de los compuestos polifenólicos también se ha observado en manglares (*Aegiceras corniculatum* L.) expuestos a altas concentraciones de metales pesados, aumentando notablemente los niveles de estos compuestos en las hojas [Guangqiu y colaboradores, 2007]. Lavid y colaboradores (2001) comprobaron en un estudio realizado con nenúfares (Nymphaeaceae) que son capaces de acumular altas concentraciones de metales pesados sin que se observe ningún síntoma de toxicidad en la planta, siendo los compuestos polifenólicos y las peroxidasas los responsables de la tolerancia e implicando, al menos, dos mecanismos: quelación directa de los metales por los polifenoles; y captura y precipitación de los metales durante el proceso de polimerización de polifenoles por peroxidasas.

Jung y colaboradores (2003) han sugerido, en estudios

realizados en plantas de altramuces (*Lupinus albus* L.), que los compuestos polifenólicos exudados por las raíces de las plantas o aquellos que se encontraron asociados a las paredes de las células de la raíz, producidos como respuesta a las altas concentraciones de cobre en el medio de cultivo, son responsables de la quelación del cobre y, por lo tanto, de restringir su toxicidad para la planta. Los autores del trabajo concluyen que la formación de polifenoles constituye un mecanismo físico-químico simple para la comprensión de las estrategias desarrolladas por los microorganismos en la lucha para controlar la toxicidad de los metales en la superficie de la célula, evitando su entrada masiva. Del mismo modo, la complejación de metales tóxicos por dichos polifenoles podría añadir nuevas dimensiones, no solo a la comprensión de los mecanismos moleculares desarrollados por microorganismos para contener la toxicidad del metal, sino también a la capacidad bioabsortiva de las paredes celulares.

Suresh y colaboradores (1998) han publicado estudios bioquímicos y biofísicos realizados con hongos *Neurospora crassa* cultivados en presencia de altas concentraciones de cobre. Dichos autores sugieren que la defensa celular contra la toxicidad del cobre implica la unión del metal a polifenoles asociados a la pared celular.

A pesar de que las algas planctónicas pueden regular la especiación y disponibilidad de metales traza a través de la producción de ligandos orgánicos [Koukal y colaboradores, 2007]

y de que estos ligandos pueden complejar más del 99% del hierro y cobre total presente en aguas naturales [Gledhill y van den Berg, 1994; Wu y Luther, 1995], no se han realizado estudios centrados en la implicación de los polifenoles como componentes de esos posibles ligandos. En particular, los grupos hidroxilo y carboxilo de los compuestos polifenólicos pueden enlazar fuertemente Cu y Fe [Martell y Smith, 1989]. Este comportamiento quelante los convierte en candidatos idóneos para la prevención de la formación de radicales libres catalizada por metales y para combatir la toxicidad de los metales [Lopes y colaboradores, 1999]. Los conocimientos relativos a las algas en este aspecto se basan, principalmente, en observaciones y estudios realizados en plantas superiores.

El principal mecanismo de toxicidad del cobre implica la reacción de Haber-Weiss [Hammond-Kosack, 1996], produciendo radical hidroxilo a través de la reacción de Fenton anteriormente mencionada (FIGURA 6). Se ha observado un incremento de la peroxidación de lípidos inducido por estrés oxidativo por Cu en plantas acuáticas [Nikookar y colaboradores, 2005].

$$Cu^{2+} + O_2^{\bullet -} \rightarrow Cu^{1+} + O_2$$

$$Cu^{1+} + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^\bullet$$

La reacción neta es:

$$O_2^{\bullet -} + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2$$

FIGURA 6.- Mecanismo de Haber-Weiss.

Por lo tanto, los microorganismos han desarrollado diversos mecanismos de protección frente al envenenamiento o toxicidad de metales [Worms y colaboradores, 2006]:

- El secuestro intracelular de metales traza es un mecanismo eficaz utilizado por microorganismos (principalmente eucariotas) para atrapar los iones metálicos tóxicos, como el cobre, impidiendo que produzca radicales libres o para atrapar aquellos iones esenciales para la vida y menos biodisponibles. Wells y colaboradores (2005) demostraron que las diatomeas del género *Pseudo-nitzschia* tenían una inusual capacidad de adaptación a la limitación de hierro en medio de cultivo sintético a través de la producción de un fuerte ligando orgánico secuestrante de hierro, el ácido domoico.

- Los efectos del exceso de iones metálicos en el citosol también pueden reducirse por emanación o compartimentación. Los metales tóxicos que son complejados por fitoquelatinas, ácidos orgánicos, etc. pueden almacenarse en compartimentos internos como vacuolas o cloroplastos.

- Los microorganismos (principalmente procariotas) tienen mecanismos de eflujo eficaces para metales. Por ejemplo, la bacteria *Cupriviadus metallidurans* es capaz de activar la síntesis de una bomba de eflujo de níquel, una vez que el níquel ha entrado en el periplasma [Gras y colaboradores, 2005]. El alga unicelular verde *Chlorella vulgaris* excluye cobre mientras que

algunas diatomeas y cianobacterias son capaces de excluir complejos de los metales [Foster, 1977].

- Los microorganismos también pueden excretar compuestos capaces de complejar los iones metálicos en el medio extracelular con el fin de reducir su biodisponibilidad. Por ejemplo, la bioacumulación de Cd por bacterias gram negativas *Rhodospirillum rubrum* disminuyó gravemente por la producción de un ligando extracelular no identificado. Las algas son capaces de producir y exudar agentes secuestrantes como polisacáridos, proteínas, péptidos y ácidos orgánicos pequeños que disminuyen la concentración de metales biodisponibles en las inmediaciones de la célula [Gortzi y colaboradores, 2008].

Los metales traza esenciales son, a menudo, altamente regulados para evitar ambas situaciones de deficiencia del metal traza y de saturación. Las estrategias son variadas, según el organismo, el metal y la fisicoquímica del metal en disolución. Ninguno de los procesos es totalmente independiente. Los nuevos avances para entender los procesos biológicos, químicos y físicos que ocurren en la inter-fase biológica permitirán una mejor comprensión de la biodisponibilidad de los metales traza en el medio natural.

Es bien sabido que el hierro actúa como fertilizante en el océano y, en especial, en áreas de alta concentración de clorofila y baja concentración de nutrientes, mientras que el cobre, un

nutriente esencial en baja concentración, llega a ser tóxico en altas concentraciones [Stumm y Morgan, 1996; Franklin y colaboradores, 2002]. El hierro es necesario para la mayoría del fitoplancton [Ho y colabradores, 2003] y debido a sus propiedades redox, es un componente de muchas enzimas y de proteínas participantes en la fotosíntesis y en la respiración [Raven y colaboradores, 1999]. Los compuestos polifenólicos pueden formar complejos débiles con el hierro y modificar la especiación química y biodisponibilidad de este metal traza [Mira y colaboradores, 2002; Andjelković y colaboradores, 2006]. Algunos polifenoles también pueden formar complejos fuertes con hierro con constantes cuyo logaritmo, log K, va desde 18 a 22 [Hynes y O'Coinceanainn, 2001; 2004], aunque la afinidad de estos compuestos por Fe (III) es mucho menor que la de sideróforos naturales como enterobactina o deferoxamina que forman complejos hexadentados [Elhabiri y colaboradores, 2007].Los compuestos fenólicos pueden influir en la química redox del hierro y favorecer la persistencia del hierro (II) proporcionando los mecanismos posibles para que esté disponible para abastecer los requerimientos de la célula.

Determinar cómo cambian las concentraciones de polifenoles en microalgas en respuesta a altos niveles de cobre y hierro (dos metales con efectos diferentes) podría demostrar el papel de los polifenoles como herramientas defensivas para evitar el envenenamiento celular y podría ser útil para ayudar a

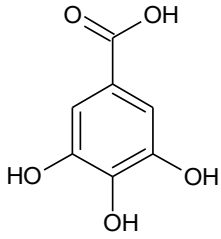
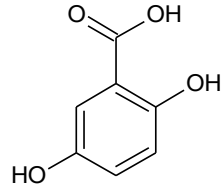
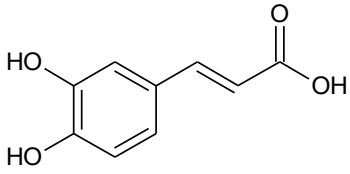
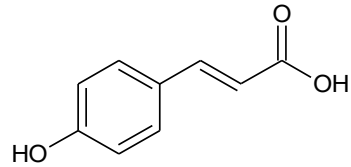
explicar la dinámica de esta importante clase de compuestos en el agua de mar [Santana-Casiano y colaboradores, 2014].

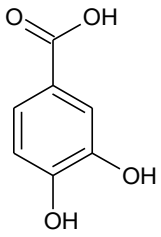
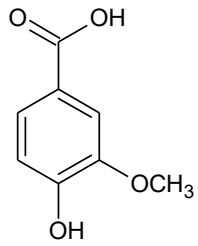
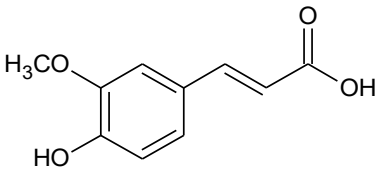
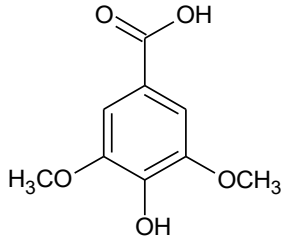
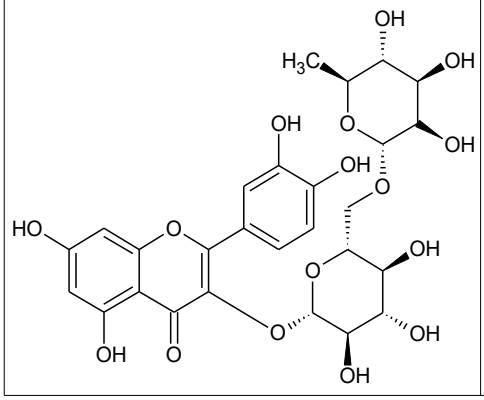
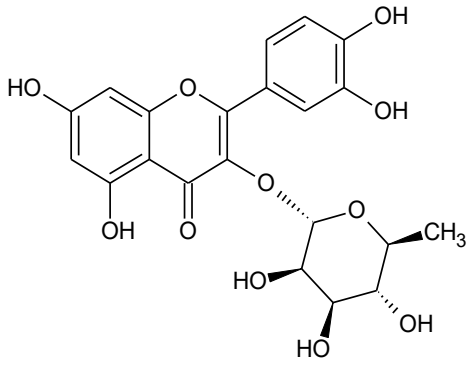
Capítulo 3:

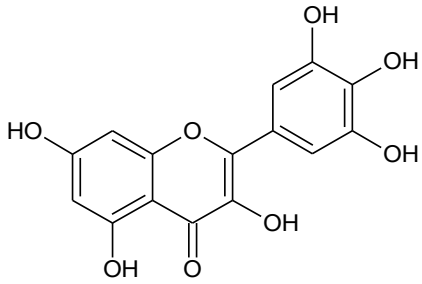
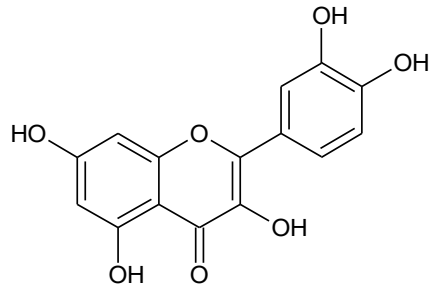
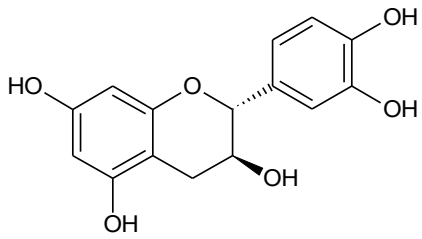
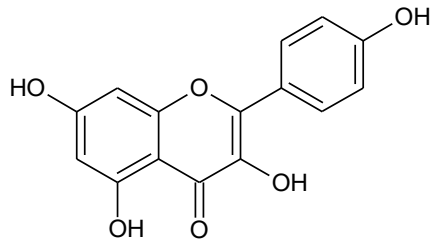
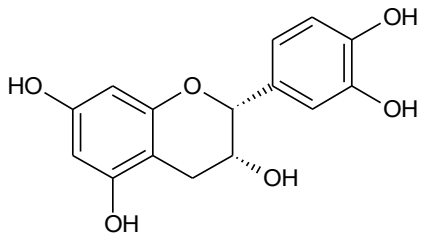
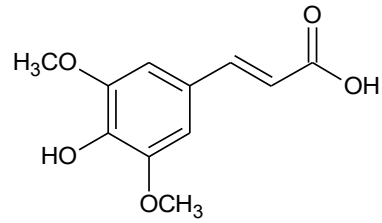
MATERIALES Y MÉTODOS APLICADOS EN EL ANÁLISIS DE POLIFENOLES

3.1. Polifenoles estudiados.

En este trabajo se optimizaron y aplicaron las metodologías necesarias para la identificación y cuantificación de dieciocho polifenoles en extractos de macroalgas y microalgas, tal y como se describe en los siguientes apartados. Los polifenoles objeto de estudio fueron: ácido gálico, ácido cafeico, ácido gentísico, ácido p-cumárico, ácido protocatecuico, ácido ferúlico, ácido vainíllico, quercetina, ácido siríngico, miricetina, rutina, kaempferol, (+)-catequina, quercitrina, (-)-epicatequina, ácido sinápico, ácido clorogénico, y apigenina (FIGURA 7).

ÁCIDO GÁLICO Ácido 3,4,5-trihidroxibenzoico $C_7H_6O_5$ 	ÁCIDO GENTÍSICO Ácido 2,5-dihidroxibenzoico $C_7H_6O_4$ 
ÁCIDO CAFEICO Ácido 3-(3,4-dihidroxifenil)-2-propenoico $C_9H_8O_4$ 	ÁCIDO p-CUMÁRICO Ácido 3-(4-hidroxifenil)-2-propenoico $C_9H_8O_3$ 

ÁCIDO PROTOCATECUICO Ácido3,4-dihidroxibenzoico $C_7H_6O_4$ 	ÁCIDO VAINILLICO Ácido 4-hidroxi-3-metoxibenzoico $C_8H_8O_4$ 
ÁCIDO FERÚLICO Ácido 3-(4-hidroxi-3-metoxifenil)-2-propenoico $C_{10}H_{10}O_4$ 	ÁCIDO SIRINGICO Ácido 4-hidroxi-3,5-dimetoxibenzoico $C_9H_{10}O_5$ 
RUTINA 3-[[6-O-(6-deoxi-α-L-manopiranosil)-ß-D-glucopiranosil]oxi]-5,7-dihidroxi-2-(3,4-dihidroxifenil)-4*H*-1-benzopiran-4-ona $C_{27}H_{30}O_{16}$ 	QUERCITRINA 3-[(6-deoxi-α-L-manopiranosil)oxi]-5,7-dihidroxi-2-(3,4-dihidroxifenil)-4*H*-1-benzopiran-4-ona $C_{21}H_{20}O_{11}$ 

MIRICETINA 3,5,7-trihidroxi-2-(3,4,5-trihidroxifenyl)-4*H*-1-benzopiran-4-ona $C_{15}H_{10}O_8$ 	QUERCETINA 3,5,7-trihidroxi-2-(3,4-dihidroxifenil)-4*H*-1-benzopiran-4-ona $C_{15}H_{10}O_7$ 
(+)-CATEQUINA (2R,3S)-2-(3,4-dihidroxifenil)-3,4-dihidro-2*H*-1-benzopiran-3,5,7-triol $C_{15}H_{14}O_6$ 	KAEMPFEROL 3,5,7-trihidroxi-2-(4-hidroxifenil)-4*H*-1-benzopiran-4-ona $C_{15}H_{10}O_6$ 
(-)-EPICATEQUINA (2R,3R)-2-(3,4-dihidroxifenil)-3,4-dihidro-2*H*-1-benzopiran-3,5,7-triol $C_{15}H_{14}O_6$ 	ÁCIDO SINÁPICO Ácido 3(4-hidroxi-3,5-dimetoxifenil)-2-propenoico $C_{11}H_{12}O_5$ 

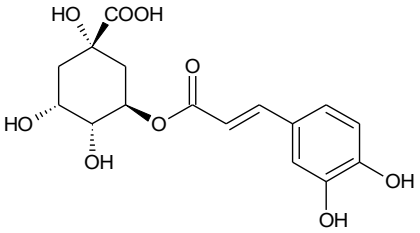
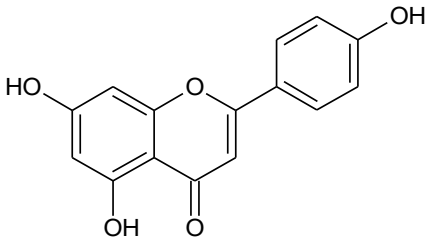
ÁCIDO CLOROGÉNICO	APIGENINA
Ácido (1*S*,3*R*,4*R*,5*R*)-3-[[3-(3,4-dihidroxifenil)-1-oxo-2-propenil]oxi]-1,4,5-trihidroxiciclohexanocarboxílico $C_{16}H_{18}O_9$ 	5,7-dihidroxi-2-(4-hidroxifenil)-*4H*-1-benzopiran-4-ona $C_{15}H_{10}O_5$ 

FIGURA 7.- Compuestos fenólicos sometidos a estudio

3.2. Material vegetal

Macroalga *Stypocaulon scoparium* (Linnaeus) Kützing (1843) [división: Heterokontophyta, clase: Phaeophyceae, orden: Sphacelariales, familia: Stypocaulaceae, género: Stypocaulon].

Es un alga parda filamentosa de talo arbuscular, con aspecto de escoba, de color marrón y de hasta 20 centímetros de alto. Se fija al sustrato por un disco basal fibroso-esponjoso. Con ramificación densa, irregularmente alterna y dística. Está distribuida por gran parte del océano Atlántico y el mar Mediterráneo y su presencia es muy frecuente en las Islas Canarias [Price y colaboradores, 1978], en las zonas rocosas y grietas batidas por las mareas y bien iluminadas, hasta 15 metros de profundidad. Se presenta en plataformas rocosas y charcos del eulitoral y en los primeros metros del sublitoral.

Las algas analizadas en el presente trabajo fueron recolectadas en Gran Canaria, entre 0 y 0,1 metros de profundidad. Las algas se enjuagaron con agua de mar y posteriormente se congelaron. Estas muestras congeladas se liofilizaron (liofilizador Cryodos 30, Telstar) y pulverizaron en una batidora (Moulinex 600W). Finalmente, se reservaron en el congelador a -20ºC en total oscuridad hasta el momento de hacer los análisis.Microalga *Phaeodactylum tricornutum* [división: Heterokontophyta, clase: Bacillariophyceae, orden: Naviculales, familia: Phaeodactylaceae, género: Phaeodactylum]

Son algas unicelulares presentes en abundancia en el fitoplancton y se caracterizan por poseer una pared celular impregnada en sílice. La diatomea *Phaeodactylum tricornutum* es la única especie que compone el género *Phaeodactylum*. Se encuentra en distintas zonas costeras de todo el mundo con grandes variaciones de salinidad. Debido a que fue de las primeras diatomeas en la que se describió la secuencia genómica completa ha sido utilizada en multitud de ensayos. Puede existir en diferentes morfologías (trirradiada, fusiforme y ovalada) (FIGURA 8). Las condiciones ambientales influyen en los cambios en la morfología y el tamaño de estas algas [Martino y colaboradores, 2007], pudiendo producirse esos cambios en medios con altas concentraciones de cobre [Markina y Aizdaicher, 2006].

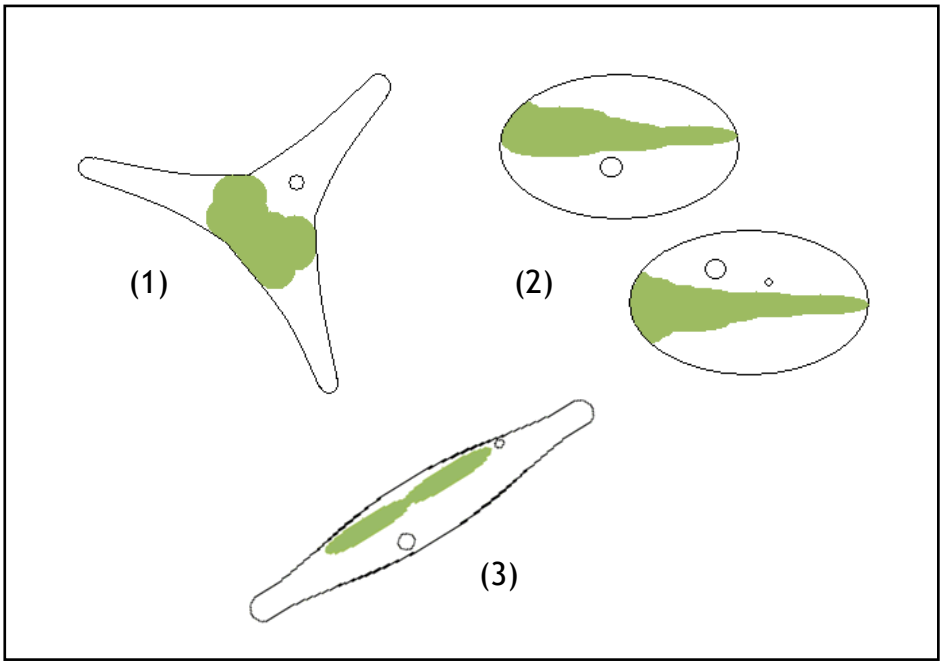


FIGURA 8.- Distintas morfologías de *P. Tricornutum*: (1)trirradiada, (2)ovalada y (3)fusiforme

Microalga *Dunaliella tertiolecta* [división: Chlorophyta, clase: Chlorophyceae, orden: Volvocales, familia: Dunaliellaceae, género: Dunaliella]

Es un alga verde unicelular de forma normalmente ovalada y simetría radial, de tamaño entre 5 y 18 micrómetros (μm) y con flagelos que duplican su longitud [Butcher, 1959]. Presenta una alta tolerancia a la salinidad y su principal característica morfológica es que no posee pared celular de polisacáridos. Es una cepa de alto crecimiento, por lo que tiene una alta tasa de secuestro de dióxido de carbono [Nozaki y colaboradores, 2002].

3.3. Cultivo de microalgas

Las microalgas utilizadas en los trabajos que se exponen en el presente documento fueron cultivadas a partir de cepas suministradas por el Banco Español de Algas. Para realizar los cultivos de microalgas (FIGURA 9) se toma agua de mar offshore enriquecida con los siguientes nutrientes de cultivo f/2 ($[NO^{3-}]$ = 883 µmol L^{-1}, $[HPO_4^{2-}]$ = 29,3 µmol L^{-1}, $[SiO_3^{2-}]$ = 142 µmol L^{-1}) [Guillard, 1975]; tratada con luz UV y filtrada con un filtro de 0,45µm. Para garantizar la ausencia de polifenoles en el agua de cultivo, se realizó la identificación de los compuestos polifenólicos antes de cultivar en ella las distintas especies de microalgas. Los cultivos se mantuvieron en una cámara de cultivo (Friocell FC111) a temperatura constante de 24ºC con iluminación permanente, 24 horas de 8000 lux. La densidad inicial de células utilizada en todos los experimentos fue de $2 \cdot 10^7$ células por litro.

Se cultivaron bajo condiciones experimentales diferentes: para el cultivo de referencia (control) se utilizó agua de mar con nutrientes f2; tres cultivos fueron preparados con agua de mar con nutrientes f2 y enriquecida con 315 nmol L^{-1} de Cu(II); 790 nmol·L^{-1} de Cu(II); y 900 nmol·L^{-1} de Fe(III). En los cultivos enriquecidos con cobre se seleccionaron estas cantidades de cobre porque están por debajo y por encima, respectivamente, de la concentración de 551 nmol L^{-1} que produce un 50% de inhibición del crecimiento celular en los trabajos realizados por Moreno-Garrido y colaboradores (2000) para la misma diatomea y

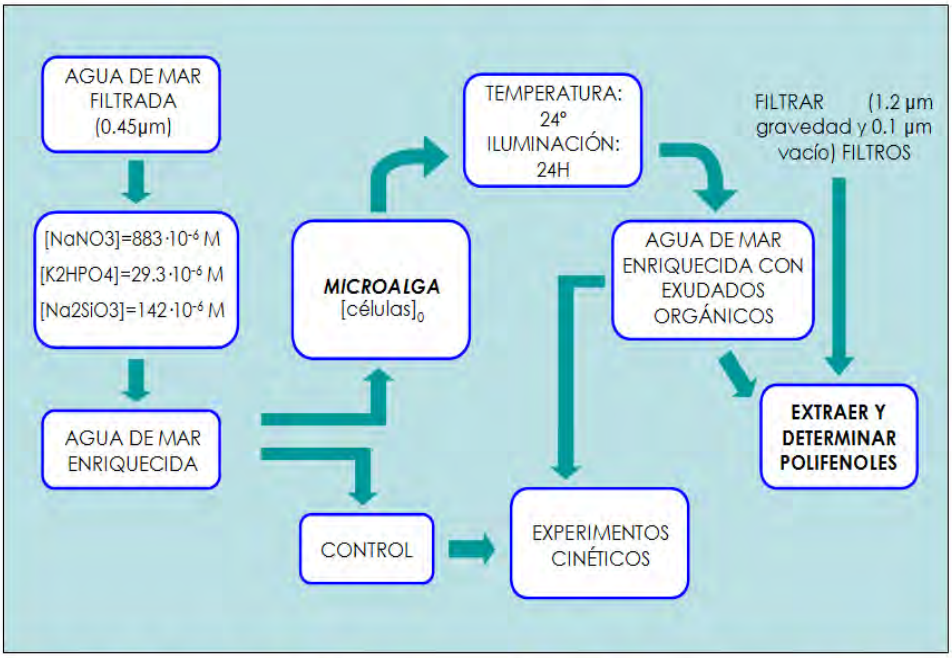


FIGURA 9.- Esquema de cultivo de microalgas

utilizando la misma densidad celular inicial. En todos los casos, las células se contaron diariamente haciendo uso de un microscopio con hemocitómetro (Microbiotest, Inc.) y midiendo la absorbancia a 670 namómetros (nm) con un espectrofotómetro(USB4000). Los cultivosse filtraron por etapas: separación por gravedad con filtros de 1,2 µm para recoger las células y mediante filtración con vacío en filtros de 0,1 µm para evitar la rotura de las células. El agua de mar enriquecida con exudados orgánicos y las células que quedaron en los filtros, a las que se les extrajo el agua por liofilización (Cryodos 30, Telstar), se reservaron a -20ºC hasta su posterior análisis.

3.4. Extracción

Los polifenoles pueden degradarse por acción enzimática cuando el material vegetal permanece fresco; por lo que es recomendable utilizar muestras en seco, liofilizadas o congeladas. Cuando se utiliza material vegetal seco generalmente se tritura hasta conseguir polvo. La mayor parte de las extracciones de polifenoles del material vegetal se realizan por extracción directa con el disolvente y, generalmente, con agitación magnética o vibración para aumentar la velocidad del procedimiento [Marston y Hostettmann, 2006].

El rendimiento de la extracción química depende del tipo de disolvente y de su polaridad, del pH, del tiempo de extracción y de la temperatura, así como de la composición de la muestra. Si se mantienen las condiciones de tiempo y temperatura, los factores más determinantes son el tipo de disolvente y las propiedades químicas de las muestras [Hayouni y colaboradores, 2007].

El disolvente se elige en función del tipo de polifenol que se necesita extraer. Se ha estudiado el efecto de distintos disolventes en la extracción de polifenoles de materiales vegetales [Pinelo y colaboradores, 2004]. Algunos trabajos [Khokhar y Magnusdottír, 2002] indican que el etanol acuoso da mejores resultados en la extracción de flavonoides del té que el metanol y la acetona. Para la extracción de compuestos

polifenólicos de algas pardas y rojas se utilizan comúnmente metanol, etanol, acetona, cloroformo o agua [Duan y colaboradores, 2007; Ganesan y colaboradores, 2008; Chandini y colaboradores, 2008; Yuan y Walsh, 2006]. Los polifenoles menos polares (isoflavonas o flavonoles, por ejemplo) se extraen con cloroformo, diclorometano, éter dietílico o acetato de etilo; mientras los compuestos más polares, como los glicósidos, se extraen con alcoholes o mezclas de alcohol-agua donde es mayor su solubilidad. Con frecuencia catequinas, proantocianidinas y taninos condensados pueden extraerse directamente con agua [Hussein y colaboradores,1990]. Los disolventes utilizados en nuestros trabajos fueron metanol, agua, etanol y una mezcla de agua con metanol al 50%. Para la extracción de microalgas se utilizó metanol.

3.5. Preparación de muestras. Extracción en fase sólida

La filtración y la extracción líquido-líquido se emplean con frecuencia en la preparación de las muestras de polifenoles [Tura y colaboradores, 2002]. Actualmente, también se usan otras técnicas como la extracción en fase sólida (SPE) que permite eliminar las sustancias interferentes de la matriz vegetal, reteniendo y concentrando los analitos de interés. Es una técnica rápida y sensible en la que se emplean distintos tipos de cartuchos con gran variedad de adsorbentes para sustancias polares, hidrofóbicas e iónicas. Con la SPE se consigue preparar y

concentrar las muestras en un único paso, con un bajo consumo de disolvente, ahorro de tiempo y un alto grado de automatización del proceso. Entre las numerosas aplicaciones de la SPE está la separación de ácidos fenólicos y flavonoides de vinos y zumos de frutas [Chen y colaboradores, 2001], la identificación de flavonoides antimutagénicos en extractos acuosos de espinacas, tras la extracción de sustancias lipófilas por SPE [Edenharder y colaboradores, 2001]. En el presente estudio, se utilizó como adsorbente un copolímero de divinilbenceno-poliestireno modificado especialmente recomendado para muestras acuosas: cartuchos Chromabon Easy (Macherey-Nagel, 500 mg, tamaño de partícula 93 μm).

3.6. Contenido total de polifenoles

Otro paso importante es la medida de la concentración total de polifenoles mediante el uso del reactivo de Folin-Ciocalteu [Julkunen-Tiitto, 1985], que consiste en una mezcla de los ácidos fosfomolíbdico y fosfotúngstico, en los que el molibdeno y el tungsteno se encuentran en el estado de oxidación 6+. Este método describe la capacidad antioxidante en base a la transferencia de un solo electrón desde el compuesto fenólico al Mo (VI) en el reactivo. En este proceso el molibdeno (VI) se reduce a molibdeno (V), de color azul y con un máximo de absorción a 765 nm (FIGURA 10). Se debe preparar una curva de calibrado, generalmente con ácido gálico, de manera que los

resultados se presentan como equivalentes de ácido gálico (EAG).

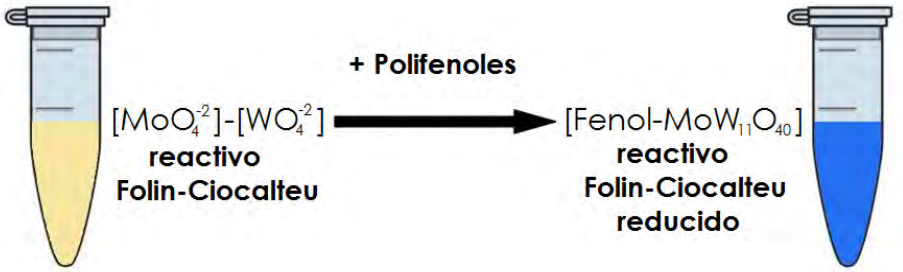


FIGURA 10.- Ensayo Folin Ciocalteau

El ensayo fue desarrollado en 1927 para la medición de la tirosina y de otros aminoácidos y péptidos y se ha utilizado durante muchos años por la industria alimentaria y agrícola para determinar el contenido fenólico de los productos vegetales. Sin embargo, este reactivo no solo mide los fenoles totales, sino que reaccionará con cualquier sustancia reductora y, en consecuencia, el reactivo mide la capacidad reductora total de una muestra, no solo el nivel de compuestos fenólicos.

La medición de los polifenoles totales a través del método Folin-Ciocalteu no muestra una imagen completa de la cantidad o la calidad de los compuestos polifenólicos de los extractos, ya que se desconoce la química exacta y el potencial redox del reactivo Folin [Huang y colaboradores, 2005] que puede actuar como un reactivo no especifico oxidando otro tipo de compuestos como azúcares o aminas [Apak y colaboradores, 2007].

3.7. Determinación de la actividad antioxidante

La actividad antioxidante de los extractos no se puede medir directamente, por lo que es necesario recurrir a métodos indirectos en los que se miden los efectos del antioxidante en el control de la extensión de la oxidación. En este estudio se han seleccionado los métodos que se describen a continuación.

3.7.1. Capacidad de inhibir radicales libres

La actividad antioxidante se puede evaluar midiendo el descenso de la concentración del radical DPPH [Chu y colaboradores, 2000]. El radical DPPH es un radical estable de coloración violeta que puede ser neutralizado por transferencia de átomos de hidrógeno y por transferencia de un electrón. La reducción de este radical puede ser medida por resonancia spin electrón o por decrecimiento de la absorbancia medida a una longitud de onda de 515 nm. El método de la decoloración propuesto por Bondet y colaboradores (1997) mide la actividad antioxidante de los compuestos a través de la pérdida de coloración del radical DPPH, tras la reacción con dichos compuestos (FIGURA 11). Los resultados se expresan en porcentaje de inhibición que se calcula según la siguiente ecuación:

$$\text{Inhibición del radical}(\%) = (1 - \text{Abs muestra} / \text{Abs } 0) \times 100$$

(**Ecuación 1**)

La absorbancia inicial (Abs_0) se corresponde con la máxima absorbancia de la disolución inicial del radical DPPH, y la absorbancia de la muestra ($Abs_{muestra}$) es la que se obtiene al añadir a la disolución de DPPH el extracto o los compuestos que son objeto de estudio al cabo de un tiempo t.

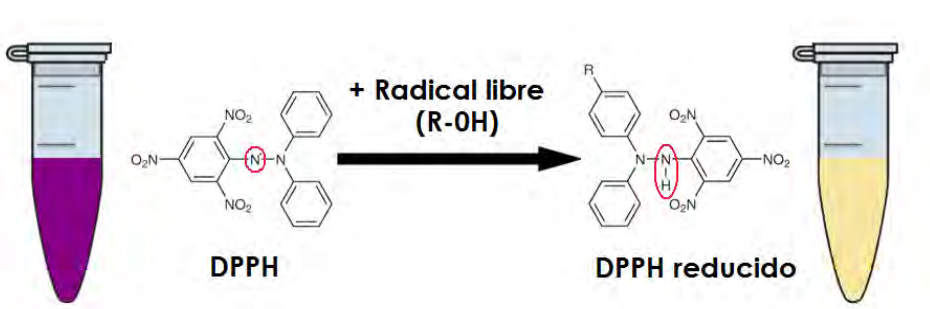


FIGURA 11.- Ensayo DPPH

3.7.2. Capacidad de reducir hierro

Este método para determinar el poder reductor del plasma sanguíneo fue desarrollado por Benzie y Strain (1996), pero el ensayo se adaptó posteriormente para antioxidantes en materiales de origen vegetal. La reacción mide la reducción en medio ácido del complejo tripiridiltriazina-Fe^{3+} (TPTZ- Fe^{3+}) al complejo de color azul TPTZ-Fe^{2+} por la presencia de antioxidantes. Cuando se produce la reacción de reducción del hierro, el color azul que toma la disolución es medido con un espectrofotómetro a una longitud de onda de 593 nm. Esta variación de la coloración se toma como una relación lineal con el total de la capacidad de reducción de los antioxidantes donantes

de electrones y se puede cuantificar en función de una curva de calibrado realizada con anterioridad en el rango adecuado de concentraciones conocidas con disoluciones de Fe^{2+} y tripiridiltriazina. Con esta técnica no se pueden determinar aquellos compuestos que actúan por transferencia de átomos de hidrógeno.

3.8. Análisis cualitativo y cuantitativo de polifenoles

Las técnicas cromatográficas y, en particular, la cromatografía líquida de alta resolución (HPLC), con un cromatógrafo acoplado a un detector de Diodo Array, a un espectrómetro de masas o a un instrumento de resonancia magnética nuclear, han tenido un enorme impacto en los estudios estructurales de polifenoles. Este método se encuentra entre las técnicas más populares en la separación de este tipo de compuestos. Continuamente se introducen mejoras en la instrumentación y en la tecnología de la columna, factores que la hacen aún más atractiva.

Se han desarrollado diversos métodos para identificar los polifenoles de materiales vegetales (plantas, frutas o verduras) utilizando esta técnica [Andre y colaboradores, 2007; Keyhanian y Stahl-Biskup, 2007; Khokhar y Magnusdottír, 2002]. En el trabajo de Merken y Beecher (2000) se muestran las aplicaciones de la HPLC, incluyendo todos los detalles relacionados con las condiciones de separación para la determinación de flavonas,

flavonoles, flavanonas, isoflavonas, antocianidinas, catequinas y sus respectivos glucosidos en los alimentos. En general, no hay un único método de HPLC que pueda abarcar todos los problemas de separación de polifenoles. Sin embargo, Sakakibara y colaboradores (2003) afirman haber encontrado un método capaz de cuantificar cada compuesto polifenólico presente en verduras, frutas y tés.El sistema de detección empleado con más frecuencia para HPLC es la espectrofotometría UV (LC-UV). La detección se basa en la medida de la absorción UV. Una única longitud de onda no es lo ideal para la detección de todos los polifenoles, ya que estos compuestos presentan máximos de absorción a longitudes de onda muy distintas. La introducción de la tecnología de Diodo-Array permite que en la prueba cromatográfica se escaneen y almacenen todos los datos en el espectro UV-visible, para posteriormente ser comparados con una biblioteca e identificar el pico [George y Maute, 1982]; además se pueden guardar simultáneamente cromatogramas a diferentes longitudes de onda. Mabry y colaboradores (1970) publican datos del espectro UV de polifenoles en diferentes disolventes. Comúnmente, las flavonas y flavonoles se encuentran de 76 a 270 nm y de 330 a 365 nm, las flavononas en 290 nm, las isoflavonas a 236 o 260 nm, en 340 y 360 nm las chalconas,a 502 o 520 nm las antocianinas y a 210 o 280 nm las catequinas.

En nuestros trabajos, cada compuesto estándar fue analizado individualmente para determinar su tiempo de retención (RT):

- Método 1: ácido gálico (RT: 5,3 min), ácido protocatecuico (RT: 10,0 min), (+)-catequina (RT: 12,7 min), ácido clorogénico (RT: 14,9 min), ácido gentísico (RT: 17,1 min), ácido vainíllico (RT: 17,7 min), (-)-epicatechin (RT: 17,9 min), ácido cafeico (17,9 min), ácido siríngico (RT: 18,9 min), ácido p-cumárico (RT: 23,4 min), rutina (RT: 28,1 min), ácido ferúlico (RT: 24,3 min), miricetina (RT: 30,6 min), y quercetina (RT: 34,6 min). Se realizó la monitorización simultánea de los ácidos gálico, protocatecuico, vainíllico y siríngico, (+)-catequina y (-)-epicatequina a 270 nm; de los ácidos clorogénico, gentíssico, cafeico, p-cumárico y ferúlico a 324nm y de los compuestos rutina, miricetina y quercetina a 373 nm.

- Método 2: los compuestos ácido sinápico (RT: 6,9 min), quercitrina (RT: 7,6 min), kaempferol (RT: 10,8 min) and apigenina (RT: 11,3 min). La longitud de onda se fijó a 254 nm.

Capítulo 4:

DISCUSIÓN Y RESULTADOS

4.1. *Stypocaulon scoparium*. Efecto de los disolventes de extracción sobre el contenido fenólico y la actividad antioxidante de extractos del alga parda *Stypocaulon scoparium*.

Se prepararon extractos de alga parda *Stypocaulon scoparium* utilizando diferentes disolventes (agua, metanol, etanol y una mezcla de agua y metanol al 50%) para determinar el contenido total de polifenoles mediante el ensayo de Folin-Ciocalteu [Julkunen-Tiitto, 1985]. También se realizó el análisis de la actividad antioxidante de los extractos a través del ensayo de inhibición del radical DPPH [Chu y colaboradores, 2000]. Los resultados mostraron correlación significativa entre la actividad antioxidante y el contenido total de polifenoles, donde la mayor actividad antioxidante y el contenido fenólico más alto correspondieron al extracto acuoso. Se desarrolló, además, una metodología para la identificación y cuantificación de 14 polifenoles de manera rápida y precisa en todos los extractos anteriormente mencionados para comparar la capacidad de cada disolvente para extraer un grupo seleccionado de polifenoles. La presencia de polifenoles en los extractos se confirmó por comparación de los tiempos de retención y por solapamiento de los espectros de ultravioleta de los compuestos estándar. Cada extracto mostró la presencia de los catorce polifenoles, entre los que la quercetina, el ácido cafeico y el ácido cumárico aparecen en menor cantidad que el resto. El ácido gálico fue el compuesto

predominante en cada extracto analizado. También se encontraron cantidades significativas de (+)-catequina y (-)-epicatequina en todos los extractos.

Los cambios de polaridad del disolvente alteran su capacidad para extraer un grupo seleccionado de compuestos antioxidantes. Un aumento en la polaridad del disolvente dio un aumento en la cantidad de ácido gálico en los extractos. Teniendo en cuenta la suma total del resto de polifenoles identificados: 32,2 mg en el extracto acuoso; 24,2 mg en la mezcla de agua y metanol al 50%; 25,2 mg en metanol y 23,3 mg en etanol (todas las cantidades referidas a 100 gramos de alga seca), parece que la actividad antioxidante se correlaciona con el contenido de ácido gálico, el antioxidante que presentó mayor actividad en inhibir el radical DPPH de los ensayados (FIGURA 12).

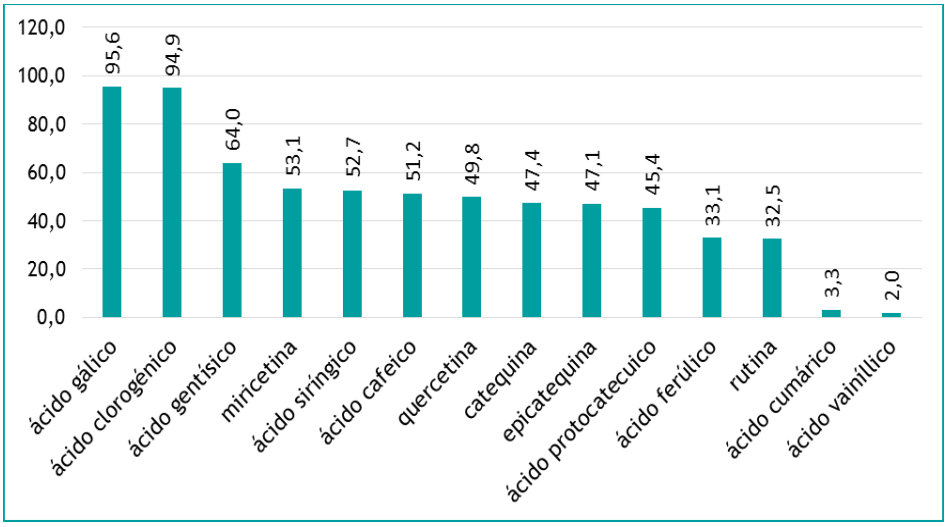


FIGURA 12.- Inhibición del radical DPPH (%) por los compuestos estándar

Es difícil describir la correlación entre rendimiento, contenido fenólico total y actividad antioxidante de los extractos, ya que cada extracto puede tener diferentes clases de compuestos con diferentes actividades antioxidantes (FIGURA 12) y que dan diferente repuesta al ensayo Folin Ciocalteau (FIGURA 13). Algunos autores han señalado que existe una estrecha relación entre la actividad antioxidante y el contenido total de polifenoles [Duan y colaboradores, 2007; Zhao y colaboradores, 2006]. Otros autores han indicado en sus trabajos que no han encontrado tal correlación y que, por lo tanto, en esos casos los polifenoles no son los responsables de la actividad antioxidante de las muestras [Kähkönen y colaboradores, 1999; Patthamakanokporn y colaboradores, 2008; Rapisarda y colaboradores, 2008]. Aunque estas interpretaciones son válidas en algunos casos, no consideran factores tales como las diferencias en el perfil fenólico entre las distintas muestras y las interacciones entre sus componentes (actividad sinérgica, aditiva o antagónica) [Jacobo-Velázquez y Cisneros-Zevallos, 2009].

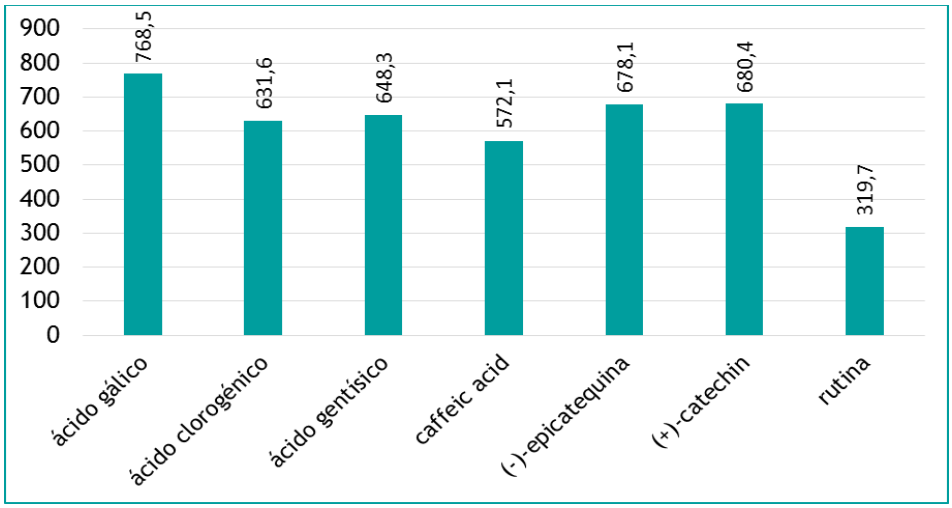


FIGURA 13.- Respuesta de los compuestos estándar al ensayo de Folin-Ciocalteau

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Analytical Methods

The effects of solvents on the phenolic contents and antioxidant activity of *Stypocaulon scoparium* algae extracts

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ABSTRACT

Water, water/methanol (1/1), methanol and ethanol crude extracts from a brown alga *Stypocaulon scoparium* were examined for total phenolic contents (TPC) using Folin–Ciocalteu method. DPPH scavenging assay was performed to measure the radical scavenging activities (RSA) of the extracts. Results showed a significant association between the antioxidant potency and the TPC. The aqueous extract showed both, the highest antioxidant activity and highest phenolic contents. The identification and quantification of phenolic antioxidants were carried out with a rapid and simple method of reverse phase high performance liquid chromatography (RP-HPLC). This method was developed for the simultaneous analysis of 14 polyphenols, namely gallic acid, catechin, epicatechin, rutin, *p*-coumaric acid, myricetin, quercetin and protocatechuic, vanillic, caffeic, ferulic, chlorogenic, syringic and gentisic acids. The chromatographic separation of 14 polyphenols was achieved in less than 40 min by RP-HPLC (Varian, Pursuit XRs C18 column, 5 μm, 250 mm × 4.6 mm) using linear gradient elution of methanol and water (0.1% formic acid) with a flow rate of 1 ml/min. Gallic acid was by far the predominant polyphenol.

1. Introduction

Recently phytochemicals in herbal plants have attracted a great deal of attention mainly concentrated on their role in preventing diseases caused as a result of oxidative stress (Southon, 2000). Dietary antioxidants from plants are believed to help prevent ageing and many degenerative diseases such as cardiovascular diseases and cancers through radical scavenging activity (Dillard & German, 2000; Prior & Cao, 2000; Steinmetz & Potter, 1996; Virgili & Scaccini, 2003; Wargovich, 2000; Yang, Landau, Huang, & Newmark, 2001). There is considerable interest in preventive medicine in the development of natural antioxidants obtained from botanical sources (Kaur & Kapoor, 2002). Seaweeds are considered to be a rich source of antioxidants (Cahyana, Shuto, & Kinoshita, 1992; Lim, Cheung, Ooi, & Ang, 2002), and different types of antioxidants from various species of seaweeds have been reported (Yan, Chuda, Suzuki, & Nagata, 1999).

The high level of solar radiation and the high temperature prevailing in the regions of Canary Islands (African Northwestern Coast) forces plants to develop defence mechanisms against ultraviolet radiation and excessive production of free radicals through the accumulation of antioxidant substances. This prompted us to evaluate total phenolic content of the crude extracts derived from *Stypocaulon scoparium* collected from Canary Islands.

The effects of different extracting solvents have been tested for the extraction of polyphenols from plant material (Pinelo, Rubilar, Sineiro, & Nunez, 2004). Extraction yield is dependent on the solvent and the method of extraction (Hayouni, Abedrabba, Bouix, & Hamdi, 2007). Khokhar and Magnusdottír (2002) reported that aqueous ethanol was superior to methanol and acetone for extracting flavonoids from tea. Thus, the objective of this study was to investigate the effect of several extracting solvents on the total phenolic compounds of the crude extracts derived from *S. scoparium*. It is obvious that total phenolics measured by Folin–Ciocalteu procedure do not give a full picture of the quantity or quality of the phenolic constituents in the extracts. Several methods have been developed to identify polyphenols from plants, fruits and vegetables using RP-HPLC analysis (Andre et al., 2007; Keyhanian & Stahl-Biskup, 2007; Khokhar & Magnusdottír, 2002). We report here the rapid determination of 14 polyphenols in crude extracts derived from a brown alga *S. scoparium*, to compare the ability of each solvent to dissolve a selected group of polyphenols. In addition, we will investigate the correlation between TPC and RSA of the extracts. Several reports have convincingly shown a close relationship between antioxidant activity and

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total phenolic content (Duan, Wu, & Jiang, 2007; Zhao, Yang, Wang, Li, & Jiang, 2006). However, other reports indicated that this correlation doesn't exist and it was concluded that phenolic compounds are not responsible of the antioxidant activity (Kähkönen et al., 1999; Patthamakanokporn, Puwastien, Nitithamyong, & Sirichakwal, 2008; Rapisarda, Lo Bianco, Pannuzzo, & Timpanaro, 2008). Although valid in some cases, this interpretation does not consider factors such as differences in the phenolic profiles between samples (Jacobo-Velazquez & Cisneros-Zevallos, 2009).

2. Materials and methods

2.1. Chemicals

Methanol (Panreac, Barcelona) and ethanol (Panreac, Barcelona) were of HPLC grade. Water was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Formic acid provided by Merck (Darmstadt, Germany) was of analytical quality. Folin–Ciocalteu's phenol reagent and sodium carbonate were from Sigma-Aldrich Chemie (Steinheim, Germany).

Polyphenol standards were supplied as follows: gallic acid, protocatechuic acid, chlorogenic acid, (−) epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+) catechin, by Sigma–Aldrich Chemie (Steinheim, Germany); rutin and gentisic and caffeic acids by Merck (Darmstadt, Germany).

2.2. Plant material

The brown alga *S. scoparium* used for this study was freshly collected from Canary Island, Spain, at 0–0.1 m depth between March and April 2008. The algae were rinsed carefully in fresh seawater and then frozen. The frozen samples were lyophilised, pulverised into powder by a blender (Moulinex, 600 W, France) and were kept in the dark at −20 °C under nitrogen.

2.3. Preparation of seaweed extracts for both, DPPH assays and TPC determinations

Dried powders (2.0 g) were separately extracted for 2 h at room temperature in 30 ml of one of these solvents: absolute methanol, absolute ethanol, water and a mixture water/methanol (50%) by mixing using a magnetic stirrer.

Each extract was filtered for removal of alga particles. After centrifugation at 2000*g* for 20 min, the supernatant was collected and filtered through 0.45 μm filter paper and stored (10 ml) at 4 °C.

Extraction solutions (20 ml) were dried by vacuum-evaporator. The dried residues were weighed and the yield for extractable substances was calculated based on the weight of dry alga powder.

2.4. Preparation of seaweed extracts prior to HPLC injection

Approximately 50 mg of powdered freeze-dried material was mixed separately with 1.0 ml of each solvent (methanol, ethanol, water and methanol/water 50%). The mixture was homogenised using a vortex for 30 s and shaken for 60 min at room temperature in the darkness. After centrifugation at 2000*g* for 20 min at 4 °C, the supernatant was collected. Supernatants were evaporated to dryness and residues were suspended in 500 μl of water and filtered through a 45 μm nylon syringe filter prior to injection.

2.5. Determination of total phenolics

The amount of total phenolics in extracts was determined according to the Folin–Ciocalteu assay (Julkunen-Tiitto, 1985). Samples (100 μl) were introduced into test tubes containing 8.4 ml of water; 0.5 ml of Folin–Ciocalteu's reagent and 1 ml of sodium carbonate (20%) were added. The tube were mixed and allowed to stand for 1 h in the darkness at room temperature. The absorbance was measured at 765 nm using a SHIMADZU 1700 UV–vis spectrophotometer. The estimation of phenolic compounds was carried out in triplicate, and the results were averaged. A calibration curve of gallic acid (ranging from 0.050 to 0.9 mg/ml) was prepared (in methanol), and the results, determined by the regression equation of the calibration curve ($y = 0.00029x - 0.00025$); correlation coefficient $r = 0.9992$), were expressed as gallic acid (GA) mg equivalents/100 g dry alga powder.

2.6. DPPH scavenging activity

The free radical scavenging activity was measured using the method of Chu, Chang, and Hsu (2000) with some modification. One ml of 0.1 mM DPPH (1,1-diphenyl-2-pikryl-hydrazin) solution in methanol was added to 100 μl of the sample solution. The decline in absorbance was recorded at 515 nm against a methanol blank over a period of 20 min. The scavenging activity (%) (RSA) on DPPH radicals was calculated as follows: RSA (%) = (1 − absorbance of sample/absorbance of control) × 100.

2.7. Analytical data

Chromatographic analysis was performed on a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostatted column compartment and a diode array detector (DAD), connected to a ChemStation software. The separation was performed with a reverse phase Pursuit XRs C18 (250 mm × 4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian, Barcelona). A gradient system was used involving two mobile phases. Eluent A was water with 0.1% formic acid and eluent B methanol. The flow rate was 1.0 ml/min, and the injection volume was 60 μl of crude extracts. The system operated at 27 °C. The elution conditions applied were: 0–5 min, 20% B isocratic; 5–30 min, linear gradient from 20% to 60% B; 30–35 min, 60% B isocratic; 35–40, linear gradient from 60% to 20% B and finally, washing and reconditioning of the column. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin and syringic acid), 324 nm (chlorogenic acid, gentisic acid, caffeic acid, coumaric acid and ferulic acid) and 373 nm (rutin, myricetin, and quercetin) for quantification.

2.7.1. Calibration curves

Stock solution containing standards was prepared and diluted with methanol to appropriate concentration in the range of 1.0–200 μg/ml for establishing calibration curves. For quantitative analysis, five different concentrations of fourteen analytes were injected in triplicate. The calibration curves were constructed by plotting the peak areas versus the concentration of each analyte.

2.7.2. Selectivity

The selectivity of the method was determined by analysis of standard compounds and samples. The peaks of polyphenols were identified by comparing their retention times and overlaying of UV spectra with those of standard compounds.

2.7.3. Linearity

Previous experiments showed us the correct ranges for concentrations to construct calibration curves. Stock solution was prepared containing 200, 100, 50, 10, 2.0 μg/ml for gallic acid; 75, 40, 20, 10 and 5.0 μg/ml for catechin; 50, 25, 10, 5.0 and 1.0 μg/ml for rutin; 25.0, 10.0, 5.0, 3.0 and 1.0 μg/ml for caffeic acid; 75,

50, 25, 10 and 2.0 μg/ml for epicatechin, myricetin, quercetin, protocatechuic acid, ferulic acid, coumaric acid, chlorogenic acid, gentisic acid, vanillic acid and syringic acid. The linearity was assessed by linear regression analysis, which was calculated by the least square method. Each point on the calibration plot was the mean from two area measurements. All correlation coefficients were not less than 0.9982 (Table 1).

2.7.4. Limit of detection and quantification

Limits of detection (LOD) and limits of quantification (LOQ) were estimated from signal-to-noise ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10, respectively (Taverniers, De Loose, & Van Bockstaele, 2004). The detection and the quantification limits are shown in Table 2.

2.7.5. Accuracy and precision

Reproducibility, expressed as relative standard deviation (RSD), was obtained by analysing six replicate samples containing the following concentrations: 50 μg/ml for epicatechin, 35 μg/ml for catechin, 30 μg/ml for quercetin, 25 μg/ml for gallic and syringic acids; 20 μg/ml for rutin, protocatechuic acid, and gentisic, ferulic and coumaric acids; 15 μg/ml for myricetin and vanillic acid and 10 μg/ml for caffeic and chlorogenic acids. RSDs values ranged from 1.91% to 5.81%. The accuracy was expressed as the recovery of standard compounds added to the pre-analysed sample (Shabir, 2003). The recovery was found to be in the range of 87.97–115.79%. RSD values and recoveries are shown in Table 2.

Table 1
Linearity data for calibration curves of fourteen polyphenols determined by RP-HPLC.

Polyphenol	Linearity range (μg/ml)	Regression equation	Correlation coefficient (r)
Gallic acid	2–200	$y = 175{,}500x + 41{,}647$	0.9987
Protocatechuic	2–75	$y = 179{,}055x - 6369$	0.9995
Catechin	5–75	$y = 40{,}283x + 4319$	0.9993
Vanillic acid	2–75	$y = 165{,}648x + 12{,}613$	0.9994
Epicatechin	3–75	$y = 82{,}583x + 5895$	0.9984
Syringic acid	2–75	$y = 202{,}997x - 6454$	0.9999
Chlorogenic acid	2–75	$y = 127{,}234x + 4450$	0.9999
Gentisic acid	2–75	$y = 98{,}060x - 4968$	0.9992
Caffeic acid	1–25	$y = 364{,}361x - 2983$	0.9992
Coumaric acid	2–75	$y = 424{,}019x - 2775$	0.9997
Ferulic acid	2–75	$y = 407{,}619x - 4259$	0.9994
Rutin	1–50	$y = 90{,}597x - 3719$	0.9994
Myricetin	2–75	$y = 274{,}627x - 4760$	0.9982
Quercetin	2–75	$y = 408{,}705x - 1420$	0.9984

Table 2
Method validation data for the quantitative determination of fourteen polyphenols in extracts of *S. scoparium* alga by RP-HPLC.

Compounds	LOD[a] (μg/ml)	LOQ[a] (μg/ml)	Recovery[b] (%)	RSD[c] (%)
Gallic acid	0.0003	0.0008	99.92 ± 3.13	5.37
Protocatechuic	0.0015	0.0050	97.85 ± 3.04	4.88
Catechin	0.0069	0.0230	95.96 ± 0.64	2.93
Vanillic acid	0.0117	0.0390	97.28 ± 4.74	5.43
Epicatechin	0.0165	0.0550	102.7 ± 3.42	3.65
Syringic acid	0.1020	0.3400	99.85 ± 3.40	1.94
Chlorogenic acid	0.0032	0.0105	89.34 ± 1.52	3.60
Gentisic acid	0.0156	0.0520	95.45 ± 2.22	3.25
Caffeic acid	0.0267	0.0890	99.42 ± 3.56	5.81
Coumaric acid	0.0207	0.0690	102.9 ± 4.27	5.51
Ferulic acid	0.0315	0.1050	98.98 ± 3.87	3.95
Rutin	0.1230	0.4100	115.8 ± 4.18	3.17
Myricetin	0.0528	0.1760	91.28 ± 4.11	4.65
Quercetin	0.0410	0.0410	87.97 ± 3.48	1.91

[a] Detection limits are calculated as signal-to-noise ratio of six times.
[b] Means ± standard deviation of three measurements.
[c] $n = 6$.

3. Results and discussion

3.1. Extraction yields

It is well known that the yield of chemical extraction depends on the type of solvents with varying polarities, pH, extraction time and temperature as well as on the chemical compositions of the sample. Under the same conditions of time and temperature, the solvent and the chemical properties of the sample are two most important factors. Earlier, solvents such as methanol, ethanol, butanol, acetone, chloroform and water have been commonly used for the extraction of phenolics from brown and red seaweeds (Chandini, Ganesan, & Bhaskar, 2008; Duan, Zhang, Li, & Wang, 2006; Ganesan, Kumar, & Bhaskar, 2008; Lim et al., 2002; Yuan & Walsh, 2006). Yields of different extracts of *S. scoparium* were examined and presented in Table 3. The highest yield for extractable substances was achieved by the polar solvents. The order of the yields from high to low was: water/methanol > water > methanol > ethanol. As compared to results of the present study, Chandini et al. (2008) observed lower yields of total methanol extracts of three brown seaweeds (12.31%, 5.76% and 5.45%).

3.2. Amounts of total phenolics

The amount of total phenolics varied from 123.2 to 328.7 mg equivalent GA/100 g of dry alga powder (Table 3). TPC was strongly affected by the extracting solvent with the following order from high to low: water > water/methanol > methanol > ethanol. Similar findings were also reported by Kuda, Tsunekawa, Goto, and Araki (2005). No correlation was found between yield and TPC, which agrees with earlier reported results (Chandini et al., 2008). In spite of that water extract showed higher TPC than water/methanol extract, both extracts gave the same yield. By other way, water extraction gained only 2.7 times more total phenolics than ethanol extraction, although water gave 7.0 times higher yield than ethanol. That means that ethanol extract gave relatively high TPC, although yield was too low.

3.3. DPPH radical scavenging activity

Antioxidant activities of the extracts are shown in Table 3. The highest activity (47.9%) was observed in the aqueous extract. Solvent polarity was significantly effective on the DPPH inhibition of crude extracts. Extracts with higher amounts of polyphenols gave also higher values of RSA. The lowest activity and phenolic content were observed when ethanol was used for maceration. This present finding corroborates well with earlier reports in other plants materials including brown/red seaweeds (Alothman, Bhat, & Karim,

Table 3
Comparative analysis of extraction yields, total phenolic contents and DPPH radical scavenging activities of *S. scoparium* extracts prepared using different solvents.

Solvent	Extraction yield[a] (%)	TPC[b]	RSA[c]
Water	16.6	328.7 ± 2.87	47.9 ± 0.67
Water–methanol	16.8	292.3 ± 5.14	38.6 ± 0.95
Methanol	13.3	255.2 ± 1.59	34.8 ± 0.55
Ethanol	2.36	123.2 ± 3.36	17.0 ± 0.11

[a] EY: values expressed as % of dry algae powder.
[B] TPC: values are expressed as mg GA equivalents/100 g dry alga powder (means ± standard deviation of three measurements).
[c] RSA: values represented means of triplicate determinations ± standard deviation.

2009; Kuda et al., 2005; Sun & Ho, 2005). However, some authors reported that no significant correlation could be found between the antioxidant activity and total phenolic content (Bozan & Karakaplan, 2007; Kähkönen et al., 1999). Ethanol extract gave also relatively high RSA as happened with TPC, although yield was too low.

3.4. HPLC analysis

To achieve better resolution in a short period a mobile phase consisting of methanol/water with 0.1% formic acid as a gradient eluent was selected. Each standard was individually tested to determine its retention times (RT). Fourteen phenolics, namely gallic acid (RT: 5.3 min), protocatechuic (RT: 10.0 min), catechin (RT: 12.7 min), chlorogenic acid (RT: 14.9 min), gentisic acid (RT: 17.1 min), vanillic acid (RT: 17.7 min), epicatechin (RT: 17.9 min), caffeic acid (17.9 min), syringic acid (RT: 18.9 min), coumaric acid (RT: 23.4 min), rutin (RT: 28.1 min), ferulic acid (RT: 24.3 min), myricetin (RT: 30.6 min) and quercetin (RT: 34.6 min) were well resolved (Fig. 1). At 270 nm, epicatechin and caffeic acid were detected with a retention time of 17.9 min (peak 5). At 324 nm, only caffeic acid (peak 9) was detected whit this retention time. Epicatechin amount was determined subtracting the area of peak 9 from peak 5 area and using the corresponding calibration curve. The LODs were found to be in the range of 0.0003–0.1230 μg/ml and the LOQs were observed in the range of 0.0008–0.4100 μg/ml. This indicated that the proposed method gave a good sensitivity for the quantification of polyphenols.

The presence of polyphenols in the extracts was confirmed by comparison of their retention times and overlying of UV spectra with those of standard compounds. Each extract showed the presence of all the fourteen polyphenols, among which quercetin, caffeic acid and coumaric acid were observed in lower amount than the other. In this analysis, gallic acid was by far the predominant polyphenol for each tested extract. However, an exception has been found, the ethanolic extract contain gallic acid as a minor constituent. In comparison to other polyphenols, significant amounts of catechin and epicatechin were also found in all the extracts.

Few reports are available on the characterisation of polyphenols by HPLC techniques from algal material to compare results. Some of the first polyphenols found in algae were florotannins (Parys et al., 2007).

As compared to results of the present study, Onofrejová et al. (2010) reported lower amounts of protocatechuic, *p*-coumaric, vanillic, caffeic and chlorogenic acids extracted from *in vitro* culture of two freshwater algae and from food products of marine macroalgae.

As it can be observed in Table 4, changes on solvent polarity alter its ability to dissolve a selected group of antioxidant compounds. An increase in the polarity of the solvent gave an increase in the quantity of gallic acid in the tested extract. No significantly differences were found when the sums of the rest of identified polyphenols in Table 4 were calculated: 32.2 mg (water); 24.2 mg (water/methanol); 25.2 mg (methanol) and 23.3 mg (ethanol). It is difficult to describe the correlation between yield, total phenolic content and antioxidant activities, since each extract might have different classes of phenolics which have varying antioxidant potential. In this study we reported the extreme changes in the concentration of gallic acid when solvent

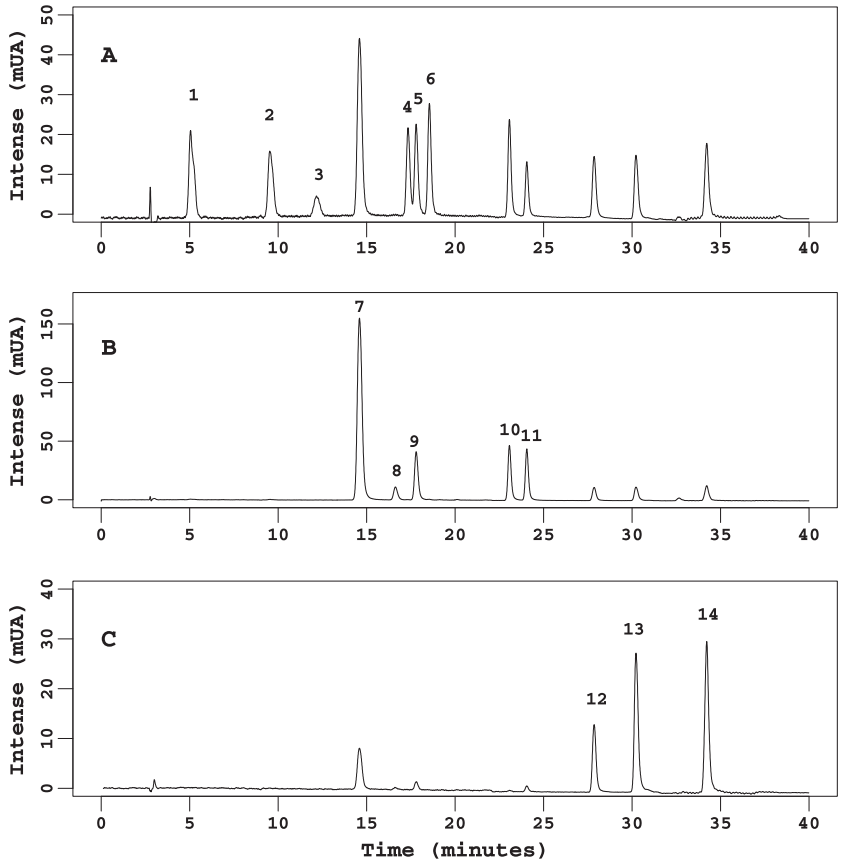

Fig. 1. HPLC chromatograms of standard polyphenols: (A) 270 nm: peak 1 = gallic acid, peak 2 = protocatechuic acid, peak 3 = catechin, peak 4 = vanillic acid, peak 5 = epicatechin + caffeic acid, and peak 6 syringic acid; (B) 324 nm: peak 7 = chlorogenic acid, peak 8 = gentisic acid, peak 9 = caffeic acid, peak 10 = coumaric acid, and peak 11 = ferulic acid; and (C) 373 nm: peak 12 = rutin, peak 13 = myricetin, and peak 14 = quercetin.

Table 4
Polyphenol amounts in extracts of *S. Scoparium* algae determined by RP-HPLC analysis.

Compound	*S. scoparium* (mg/100 g dry alga powder)[a]			
	Ethanol	Methanol	Aqueous methanol	Water
Gallic acid	2.798 ± 0.081	34.42 ± 0.155	71.45 ± 2.455	90.62 ± 1.549
Protocatechuic	1.719 ± 0.104	1.153 ± 0.068	0.967 ± 0.052	0.904 ± 0.011
Catechin	4.995 ± 0.225	6.908 ± 0.001	5.906 ± 0.298	6.548 ± 0.223
Vanillic acid	1.271 ± 0.023	1.166 ± 0.081	1.467 ± 0.053	2.074 ± 0.037
Epicatechin	4.434 ± 0.126	5.069 ± 0.024	5.452 ± 0.045	7.649 ± 0.035
Syringic acid	1.276 ± 0.039	1.797 ± 0.080	1.439 ± 0.001	4.437 ± 0.102
Chlorogenic acid	1.767 ± 0.032	1.751 ± 0.018	1.841 ± 0.066	1.862 ± 0.008
Gentisic acid	1.953 ± 0.065	1.932 ± 0.005	2.157 ± 0.026	2.792 ± 0.146
Caffeic acid	0.659 ± 0.006	0.643 ± 0.016	0.659 ± 0.037	0.691 ± 0.014
Coumaric acid	0.747 ± 0.020	0.739 ± 0.046	0.676 ± 0.003	0.742 ± 0.019
Ferulic acid	1.188 ± 0.074	1.324 ± 0.087	0.668 ± 0.021	1.242 ± 0.003
Rutin	0.956 ± 0.025	0.804 ± 0.022	0.860 ± 0.002	1.176 ± 0.062
Myricetin	2.076 ± 0.017	1.851 ± 0.025	1.861 ± 0.057	1.838 ± 0.026
Quercetin	0.291 ± 0.023	0.079 ± 0.001	0.235 ± 0.007	0.211 ± 0.010

[a]Means ± standard deviation of three measurements.

polarity increases. However, solvent polarity does not change drastically the total amounts of a phenolics group, but the phenolic profile.

4. Conclusion

The RP-HPLC method developed in this study was shown to be rapid, sensitive, and accurate in simultaneously detecting and quantifying of 14 polyphenols with good reproducibility. On the basis of this study, it can be concluded that the extracting solvent significantly affected total polyphenol content and antioxidant activity of several extracts from *S. scoparium*. The HPLC analysis showed that solubility of polyphenols changes with the polarity of the solvent but not always in the same way: with some of them it increases and with other ones it decreases. Thus, gallic acid was found to be increased in extracts prepared with polar solvents, while protocatechuic acid amount increases when polarity of solvent decreases. Ethanol extract gave relatively high RSA and TPC, although yield was too low. It seems that ethanol might dissolve more radical scavenging active polyphenols than other solvents, as happened with protocatechuic acid, and may be, a lower amount of no antioxidant active substances.

Extracting solvents, phenolic content and antioxidant activity must be discussed when evaluating the antioxidant activity of extracts. In addition, the typical approach used to analyse correlations between total antioxidant activity and total phenolics do not reflect the characteristics of phenolics, which can act synergistically, additively or antagonistically (Jacobo-Velazquez & Cisneros-Zevallos, 2009). Differences in the phenolic profiles should be too considered. Our proposed analytical method can be used for the rapid analysis of bioactive phenols algae materials and their derived food products.

Algae represent a source of interesting natural bioactive compounds for human nutrition. The result of this experiment may show *S. scoparium* as a natural source of well known antioxidant compounds such as gallic acid, catechin and epicatechin.

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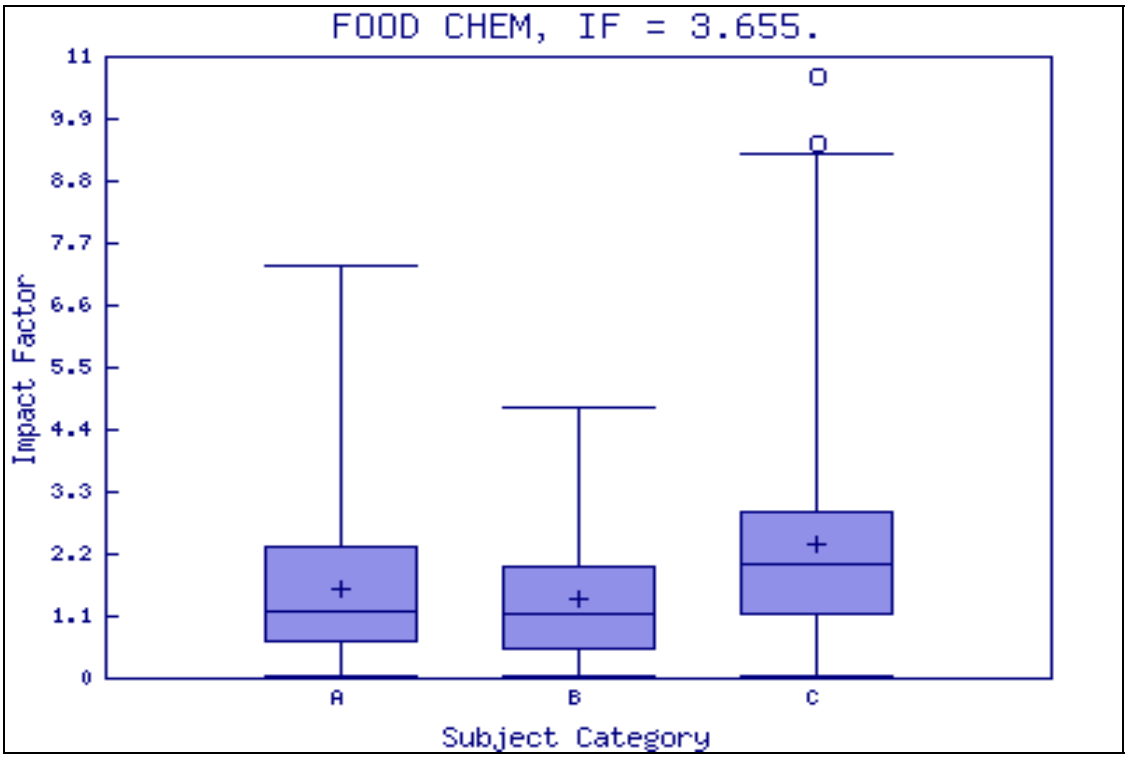

Key

A - CHEMISTRY, APPLIED

B - FOOD SCIENCE & TECHNOLOGY

C - NUTRITION & DIETETICS

4.2. *Phaeodactylum tricornutum*.

4.2.1 Variación del perfil fenólico de la diatomea *Phaeodactylum tricornutum* cultivada en condiciones de estrés por hierro y cobre.

Se identificaron y cuantificaron catorce compuestos fenólicos en los extractos preparados con diatomea *Phaeodactylum tricornutum* cultivada en agua de mar natural (control) y en agua de mar natural enriquecida con cobre (Cu) (315 nmol L^{-1} y 790 nmol L^{-1}) o con hierro (Fe) (900 nmol L^{-1}). La densidad celular para las diatomeas expuestas al hierro creció 471% (expresado como porcentaje de la concentración celular en el control) y 80% y 52.5% para las diatomeas expuestas al cobre en concentraciones de 315 nmol L^{-1} y 790 nmol L^{-1}, respectivamente.

En cada extracto se detectó la presencia de los 14 compuestos fenólicos, con las excepciones del ácido gálico, que fue detectado solo en las diatomeas enriquecidas con hierro, y quercetina, que no fue detectada en los exudados de la diatomea cultivada en ausencia de metales (control).

Las diatomeas *P.tricornutum* exudan polifenoles capaces de complejar el cobre presente en el agua de cultivo para disminuir su efecto tóxico.

La cantidad de polifenoles exudados por cada célula se

duplicó (pasando de 41,3 amol por célula en el experimento controla 82,4 amol en el ensayo realizado con la máxima concentración de cobre) (FIGURA 14). La concentración de los polifenoles analizados en el agua de mar enriquecida con cobre fue 1,6 veces mayor que en el control a pesar de que la densidad celular era prácticamente la mitad respecto al control. La diatomea, por tanto, debe hacer un esfuerzo metabólico extra para poder vivir en estos niveles de concentraciones de cobre y debe producir cantidades importantes de polifenoles para disminuir la toxicidad del metal en la solución, actuando como un mecanismo de protección.

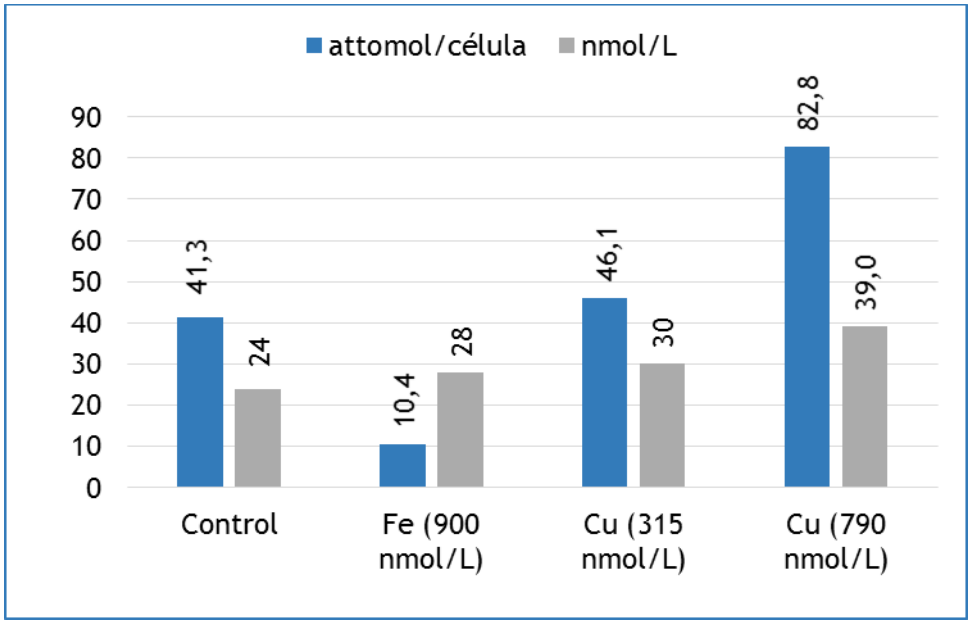

FIGURA 14.- Compuestos fenólicos totales excretados por las células

El contenido fenólico de la célula (2,4 veces mayor en el experimento con mayor concentración de cobre que en el control) se duplica al aumentar los niveles del cobre, lo que puede reflejar la participación de estos compuestos en alguno de los mecanismos de defensa para paliar la toxicidad del cobre a nivel del interior de la célula (FIGURA 15).

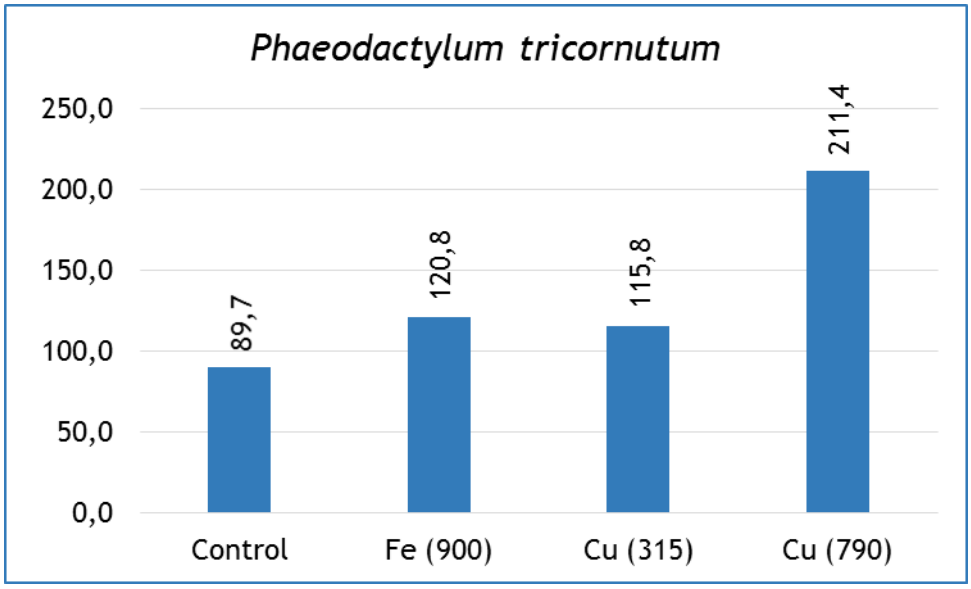

FIGURA 15.- Compuestos fenólicos totales en el interior de la célula (amol cel^{-1})

El contenido fenólico de la célula también se incrementa respecto al control en presencia de altas concentraciones de hierro, debido, probablemente, a la capacidad complejante de estos compuestos que puede evitar o reducir la reacción de Fenton. Sin embargo, la cantidad de compuestos fenólicos exudados por cada célula disminuye drásticamente (cuatro veces menor que en el

control) debido a la alta densidad celular en el cultivo (crecimiento celular de 471% respecto al control) que ayuda a reducir al mínimo el estrés sufrido por cada célula, disminuyendo el esfuerzo metabólico necesario para la asimilación de hierro (FIGURA 14). Aunque la cantidad de polifenoles exudados por cada célula disminuyó, la concentración de hierro en el agua de mar se mantiene prácticamente constante (24 nmol L^{-1} en el control y 28 nmol L^{-1} en el ensayo con hierro).

Se encontraron diferencias significativas en el perfil fenólico de la diatomea *Phaeodactylum tricornutum* cultivada en agua de mar (control) y en agua de mar enriquecida con metales pesados (hierro y cobre) en función de las distintas necesidades de las células. Los extractos de las diatomeas expuestas a cobre presentaron mayores actividades antioxidantes que el resto, tanto mayor al incrementar la concentración de cobre en el agua de cultivo, probablemente, debido al requerimiento de la célula en esas condiciones de mayor estrés. Los perfiles fenólicos para estos extractos más activos fueron muy similares. Sin embargo, las algas expuestas a hierro mostraron diferente perfil fenólico que debe variar el potencial antioxidante.

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Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress

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Abstract

Fourteen phenolic compounds were identified and quantified in extracts derived from in vitro culture of *Phaeodactylum tricornutum* diatom growing in natural seawater (control) and in natural seawater containing the following added copper (Cu) and iron (Fe) metals: Cu(II) (315 nmol L^{-1} and 790 nmol L^{-1}) and Fe(III) (900 nmol L^{-1}). The cell concentration was 471% for the last day of culture (expressed as a percentage of the control) for diatoms exposed to iron, while diatoms exposed to copper showed cell densities of 80% and 52.5% at concentrations of 315 nmol L^{-1} and 790 nmol L^{-1}, respectively. Each extract revealed the presence of 14 phenolic compounds, with the exceptions of gallic acid, which was detected only in the iron-enriched diatoms, and quercetin, which was not detected in the control diatom exudates. Significant differences in the phenolic profiles were found depending on the metal added to the culture media. These differences seem to be the response to the different needs of diatoms exposed to copper and iron concentrations. These results show that increasing levels of metal result in a general increase in levels of total phenolic contents in the cells of *P. tricornutum*. The great increase in phenolic compounds in the cells at the highest copper concentration was 2.4 times higher than in the control, which may reflect the involvement of these compounds in protection against copper toxicity. All the extracts revealed radical scavenging activity against the stable radical 1,1-diphenyl-2-picrylhydrazyl, with the most active extracts from the copper enrichments.

Phenolic compounds are an important group of natural products involved in responses to different kinds of biotic and abiotic stresses (Treutter 2006). The main chemical process where polyphenols are involved is in the scavenging of reactive oxygen species (Neudorffer et al. 2006; Bentes et al. 2011), but phenolic compounds from plants can also act as antioxidants by chelating metal ions (Mira et al. 2002). Therefore, plants under metallic stress conditions accumulate elevated amounts of phenolic compounds for protection and recovery from heavy-metal injury. There is considerable evidence with respect to the implications of phenolic compounds in plants growing in conditions of metal deficiency and stress (Michalak 2006). Phenolic compounds are frequently reported as the main components of root exudates in response to iron deficiency (Wei et al. 2007). Stimulation of phenolic metabolism in response to the toxicity produced by several heavy metals has also been found in chamomile (*Matricaria chamomilla* L.), wheat (*Triticum aestivum*), and in maize (*Zea mays*) (Díaz et al. 2001; Winkel-Shirley 2002; Kovacik and Backor 2007). Studies carried out in order to evaluate the effects of high soil concentrations of copper (Cu) and nickel on phenolics in Scots pine (*Pinus sylvestris* L.) showed that trees exposed to nickel had higher concentrations of condensed tannins than controls. However, concentrations of several other phenolic compounds decreased when seedlings were exposed to high levels of copper or to a combination of nickel and copper (Roitto et al. 2005). Loponen et al. (2001) reported both increased and decreased levels of individual phenolic compounds in mountain birch *Betula pubescens* ssp. *czerepanovii* leaves from forest sites polluted by copper and nickel. The important role of phenolic compounds has also been observed in mangroves (*A. corniculatum*) exposed to heavy metals where the total foliar phenolics were markedly enhanced (Guangqiu et al. 2007) and in waterlily (Nymphaeaceae), capable of accumulating heavy metals with no observed toxicity to the plant (Lavid et al. 2001). In addition, polyphenols and peroxidases seem to play a major role in heavy-metal accumulation and tolerance in *Nymphaea* plants, involving at least two mechanisms: direct chelation by polyphenols and binding and trapping of heavy-metal precipitates during the process of polyphenol polymerization by peroxidases.

In addition, planktonic algae are an important component of natural surface waters. They can regulate the speciation and bioavailability of trace metals through the production and release of organic ligands (Koukal et al. 2007). These organic ligands bind $> 99\%$ of the total iron (Fe) and copper in natural waters (Gledhill and van den Berg 1994; Wu and Luther 1995). Wells et al. (2005) demonstrated that diatoms of the genus *Pseudo-nitzschia* have an unusual capacity for adapting to iron limitation in synthetic growth medium through the production of the strong iron-complexing organic ligand, domoic acid. Few reports have focused on the analysis of bioactive phenolic acids, flavonoids, or similar polyphenols and their implications in algae growing under metal stress (Onofrejová et al. 2010; López et al. 2011). Recently, there have been reports of increases in the total phenolic content and flavonoids in submerged macrophyte *Vallisneria natans* exposed to lead (Pb) stress (Wang et al. 2011).

The aim of this study was to determine the differences in the phenolic profile of the diatom *Phaeodactylum tricornutum*

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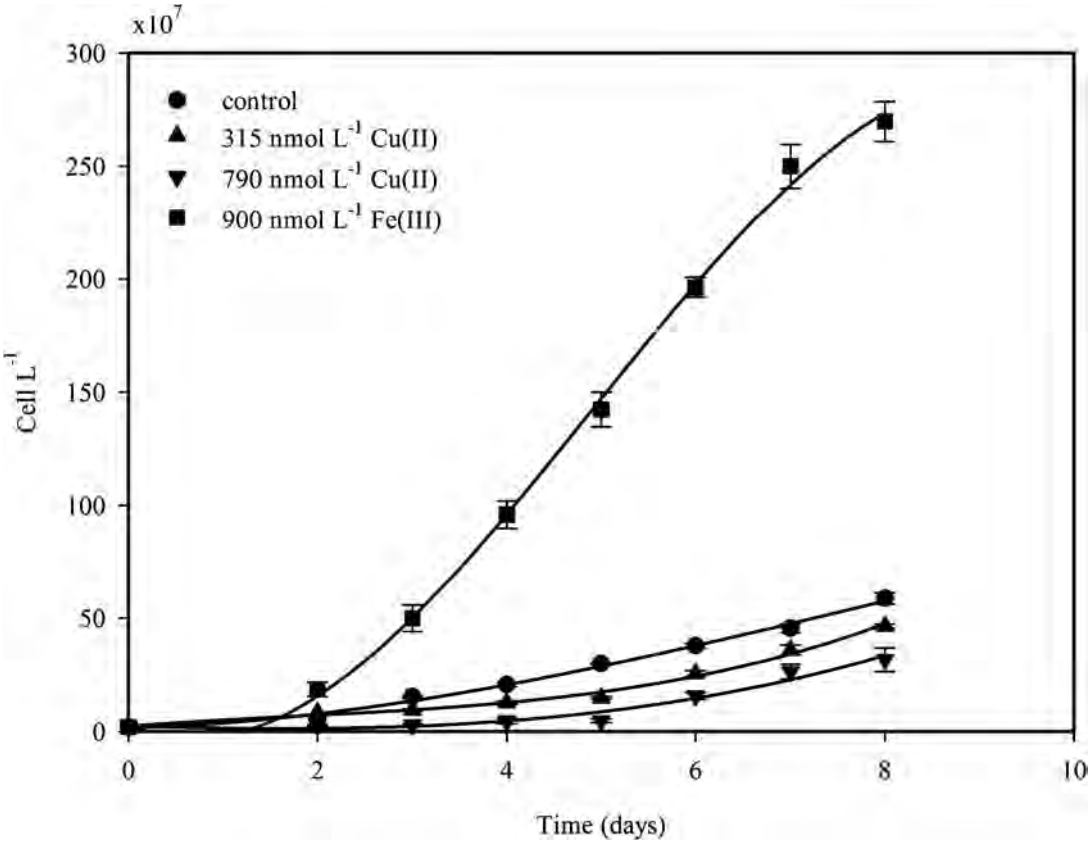

Fig. 1. Growth rates (cell division) of diatoms exposed to iron and copper.

harvested in natural seawater (control) and in natural seawater enriched with heavy metals: Cu(II) (315 nmol L^{-1} and 790 nmol L^{-1}) and Fe(III) (900 nmol L^{-1}). These two metals were chosen because of their opposite effects. It is well known that iron acts as fertilizer in the ocean and in special in high-nutrient low-chlorophyll areas, whereas copper, a nutrient at low concentration, becomes toxic at high concentrations (Stumm and Morgan 1996; Franklin et al. 2001). Iron is needed by most phytoplankton (Ho et al. 2003) and, due to its redox properties, is a component of many enzymes and electron transferring proteins in photosynthesis and respiration (Raven et al. 1999). Copper, with its two oxidation states, is also a valuable cellular constituent, performing an important role in respiration and in the photosynthetic apparatus in some marine diatoms (Peers and Price 2006). Copper replaces iron in some metabolic functions and is also involved in the high-affinity iron transport system of some iron-limited diatoms (Maldonado and Price 2001). Thus, due to iron limitation, copper is important for the growth of oceanic phytoplankton. At physiological and molecular level, this interaction is, however, not well studied. The differences in the phenolic profile may have their roots in the response of the diatoms when exposed to different metal conditions. The effect of those metal concentrations on the growth of the diatoms (cell division) was also studied. A solid-phase extraction method for isolation of the phenolic compounds was developed. Reverse phase high-performance liquid chromatography (RP-HPLC) was applied for the analysis and quantification of 14 phenolic compounds. The antioxidant activity of diatom extracts in scavenging of the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was also determined.

Methods

Chemicals—Methanol, hexane, and acetone were HPLC grade (Panreac). Milli-Q water (18MQ, Millipore) was used throughout the entire study. Formic and acetic acids (Merck) were analytical quality reagent. DPPH and polyphenol standard gallic acid, protocatechuic acid, chlorogenic acid, (−)-epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+)-catechin, sinapic acid, quercitrin, kaempferol, and apigenin were purchased from Sigma-Aldrich Chemie (Steinheim); rutin and gentisic and caffeic acids were supplied by Merck.

The SPE cartridges used were Chromabon with polar modified polystyrene divinylbenzene (500 mg) from Macherey-Nagel.

The stock solutions of nutrients were prepared using sodium nitrate (Sigma) (0.88 mol L^{-1}), potassium hydrogen phosphate (Sigma) (0.03 mol L^{-1}), and sodium silicate (Sigma) (0.14 mol L^{-1}). Both the copper and iron were used direct from the stock solution for atomic absorption spectroscopy (Fluka).

Algae cultivation and preparation—Axenic cultures of *P. tricornutum* were given support by the Spanish Bank of Algae at Taliarte (Gran Canaria, Spain). The main culture of

diatom was kept in a clean culture chamber (Friocell FC111) using f/2 media (Guillard 1975) at constant temperature (24°C) with permanent illumination at 8000 lux.

Experimental cultures were carried out in seawater with f/2 nutrients (Guillard 1975). Seawater was ultraviolet treated (off the coast of Gran Canaria) and filtered by 0.45 μm. The f/2 nutrients ($[NO_3^-]$ = 883 μmol L^{-1}, $[HPO_4^{2-}]$ = 29.3 μmol L^{-1}, $[SiO_3^{2-}]$ = 142 μmol L^{-1}) were added to the seawater. Under these conditions, *P. tricornutum*, with an initial cell density of 2×10^7 cells L^{-1}, reached the stationary phase after 8 d of growth (Fig. 1). This time of culture was used throughout to collect the samples in the same conditions under different conditions of metal concentrations.

The experimental cultures were produced under four different conditions: (1) at the reference culture, in seawater with f/2 nutrients. The effect of copper was tested (2) with 315 nmol L^{-1} of Cu(II) and (3) 790 nmol L^{-1} of Cu(II). The effect of iron (4) was studied with 900 nmol L^{-1} of Fe(III). All of the experiments were triplicated.

The cell concentration was counted daily using a light microscope (Microbiotest) with a hemocytometer and measuring the absorbance (640 nm) with a spectrophotometer (USB4000). Once the culture reached the stationary phase, the cell was collected by filtration in two steps: gravity (1.2 μm) and vacuum (0.1 μm). This treatment avoids rupturing cells. Both cells and enriched seawater were used in this study for the analysis of the phenolic compounds.

Preparation of P. tricornutum *diatom extracts for isolation and quantification of the phenolic compounds*—The algae were freeze-dried and 1 g of the dry material was extracted by stirring with methanol (25 mL) for 1 h. After centrifugation at 3500 revolutions per minute (rpm) for 30 min, the supernatant was collected and evaporated. The dry residue (100 mg) and 4 mL of acetone : hexane (1 : 4) were mixed and stirred for 10 min. The supernatant was discarded and the residue was mixed and homogenized with 5 mL of methanol using a vortex (10 min). After centrifugation at 3000 rpm, the supernatant was separated and the residue was extracted twice with 5 mL of methanol. All the methanolic fractions were collected and evaporated in a rotary vacuum evaporator (Eppendorf, Concentrator plus) at 30°C (6800 rpm). The residues were resolved in 5 mL of HCl (1 mol L^{-1}) and the samples were hydrolyzed at room temperature, 3800 rpm for 30 min. The hydrolysates were purified using solid-phase extraction (SPE).

Solid-phase extraction—The cartridge Chromabon Easy (Macherey-Nagel, 500 mg, particle size 93 μm) was preconditioned by successive elution with 3 mL of methanol and 3 mL of deionized water. The hydrolysates (5 mL) were passed through the cartridges at a flow of 2.5 mL min^{-1}. The cartridge was then rinsed using 2% acetic acid in a 5% methanol solution (Onofrejová et al. 2010). The retained analytes were subsequently eluted using aqueous methanol solutions (5%, 10%, and 20% methanol, 2 mL for each). Prior to the analyses, the fractions were evaporated to dryness in a rotary vacuum evaporator at 25°C, 3500 rpm, resolved in 500 μL of methanol, filtered through a 45 μm nylon syringe filter, and injected directly into the HPLC system.

Seawater enriched with diatom exudates (2.5 L) were passed through the conditioned cartridges (as described above) at a flow of 2.5 mL min^{-1} (~ 800 mL per cartridge, using three cartridges), following the same elution procedure described above for each cartridge. The fractions from the three cartridges were collected, evaporated as above, and the residue resolved in 500 μL of methanol, and then filtered to be injected into the HPLC system.

Quantification of the phenolic compounds by RP-HPLC—Chromatographic analysis was performed on a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostatted column compartment, and a diode array detector, connected to a ChemStation software. A reverse phase Pursuit XRs C18 (250 mm × 4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian) with a gradient system involving two mobile phases were used. The flow rate was 1.0 mL min^{-1} and the injection volume was 60 μL of crude extracts. The system operated at 27°C. The phenolic compounds gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin and syringic acid, chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid, rutin, myricetin, and quercetin were quantified in line with previously reported methods (López et al. 2011). In brief, the eluent A was Milli-Q water with 0.1% formic acid and eluent B was methanol. The elution conditions applied were 0–5 min, 20% B isocratic; 5–30 min, linear gradient from 20% to 60% B; 30–35 min, 60% B isocratic; 35–40 min, linear gradient from 60% to 20% B; and, finally, washing and reconditioning of the column. Each standard was individually tested to determine its retention times (RT) as follows: gallic acid (RT: 5.3 min), protocatechuic (RT: 10.0 min), catechin (RT: 12.7 min), chlorogenic acid (RT: 14.9 min), gentisic acid (RT: 17.1 min), vanillic acid (RT: 17.7 min), epicatechin (RT: 17.9 min), caffeic acid (17.9 min), syringic acid (RT: 18.9 min), coumaric acid (RT: 23.4 min), rutin (RT: 28.1 min), ferulic acid (RT: 24.3 min), myricetin (RT: 30.6 min), and quercetin (RT: 34.6 min) were well resolved. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, (+)-catechin, vanillic acid, (−)-epicatechin, and syringic acid), 324 nm (chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid, and ferulic acid), and 373 nm (rutin, myricetin, and quercetin) for quantification. Limits of detection (LODs) and limits of quantification (LOQs) were estimated from signal-to-noise ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10, respectively. The LODs were found to be in the range of 1.59×10^{-3} nmol mL^{-1} to 0.52 nmol mL^{-1} and the LOQs were observed in the range of 4.25×10^{-3} nmol mL^{-1} to 0.17 nmol mL^{-1}.

SPE accuracy and precision—The presence of polyphenols in the extracts was confirmed by comparison of the retention times and overlapping ultraviolet spectra with those of standard compounds. The precision of the SPE-RP-HPLC method was estimated by measuring the repeatability and the relative standard deviations of 10 replicate samples

Table 1. Solid-phase extraction recoveries and relative standard deviations (RSD) ($n = 10$).

Compound	Recovery* (%)	RSD (%)
Gallic acid	94±1	4.2
Protocatechuic acid	95.1±0.7	2.6
Catechin	94±1	2.6
Vanillic acid	108±1.0	3.9
Epicatechin	105.3±0.8	3.2
Syringic acid	96.8±0.8	3.1
Chlorogenic acid	93.6±0.5	2.9
Gentisic acid	91.1±0.5	2.1
Caffeic acid	106.5±0.8	6.4
Coumaric acid	97±1.0	5.6
Ferulic acid	93.4±0.8	3.9
Rutin	112±1	3.4
Myricetin	89.4±0.6	3.0
Quercetin	87.3±0.6	4.5

* Means ± standard deviation of 10 measurements.

containing the following concentrations: 146.9 μmol L^{-1} for (+)-catechin; 117.6 μmol L^{-1} for gallic acid, 129.8 μmol L^{-1} for protocatechuic acid, 118.9 μmol L^{-1} for vanillic acid, 100.9 μmol L^{-1} for syringic acid, 129.8 μmol L^{-1} for gentisic acid, 32.7 μmol L^{-1} for rutin, and 73.4 μmol L^{-1} for (−)-epicatechin; 41.3 μmol L^{-1} for chlorogenic acid, 91.4 μmol L^{-1} for *p*-coumaric acid, 77.2 μmol L^{-1} for ferulic acid, 47.1 μmol L^{-1} for myricetin, 55.5 μmol L^{-1} for caffeic acid, and 33.1 μmol L^{-1} for quercetin. Relative standard deviation (RSD) values ranged from 2.1% to 6.4%. The accuracy was expressed as the recovery of the standards and was found to be in the range of 87.3–107.5%. RSD values and recoveries are shown in Table 1.

Preparation of P. tricornutum *diatom extracts for DPPH assay*—The algae were freeze-dried and 1 g of the dry material was extracted by stirring with methanol (25 mL) for 1 h. After centrifugation at 3500 rpm for 30 min, the supernatant was collected and evaporated. The dry residue (100 mg) was extracted for 1 h at room temperature with 3 mL of methanol by mixing with a magnetic stirrer. Each extract was centrifuged at 7500 rpm for 20 min, and the supernatant collected and filtered through 0.45 μm filter paper.

Free radical scavenging activity on DPPH—The reducing ability of antioxidants on DPPH radical was evaluated by measuring the loss of DPPH color at 515 nm after reaction with the test extracts (Bondet et al. 1997). The sample solution (100 μL) was rapidly mixed with 1 mL of a solution of 0.1 mmol L^{-1} DPPH. After 25 min incubation time in the dark at ambient temperature (23°C), the decline in absorbance (Abs) was measured against a methanol blank. The inhibition percentage values, expressed in terms of radical scavenging activity (RSA), were calculated by the equation:

$$\mathrm{RSA} = 100 \times [1 - (\text{Abs in the preence of sample}) / (\text{Abs in the absence of sample})] \quad (1)$$

Results

Effects of copper and iron on the growth of P. tricornutum—The cultures of *P. tricornutum* were produced at three metal concentrations in order to stimulate the production of exudates. To this effect, the diatom was harvested in seawater with f/2 nutrients (control seawater) as the reference growth (Fig. 1) in close concordance with recent studies (Vasconcelos and Leal 2008; González et al. 2012). At these conditions, the cell density increased from 2×10^7 cells L^{-1} to 5.9×10^8 cells L^{-1} after 8 d of culture. The slope for the exponential phase was 8.9×10^7 cells L^{-1} d^{-1}. The growth rate during the 8 d of culture was 7.6×10^7 cells L^{-1} d^{-1}.

The growth of *P. tricornutum* was also carried out in the presence of 315 nmol L^{-1} of Cu(II) (Fig. 1). The cell density increased to 4.7×10^8 cells L^{-1}, with a growth rate of 2.6×10^7 cells L^{-1} d^{-1} over the 8 d of culture, meaning that the *P. tricornutum* cell density decreased 20% with respect to the reference culture. In addition, during the initial 5 d of culture, the growth curve showed a linear relationship with time, where the slope was 2.26×10^7 cells L^{-1} d^{-1}. Between the sixth and eighth days of culture, the cell density reached a slope of 10.4×10^7 cells L^{-1} d^{-1}, comparable to the reference culture in the absence of copper.

The third culture of *P. tricornutum* was carried out under conditions of 790 nmol L^{-1} of Cu(II) (Fig. 1). This concentration allows for the determination of the polyphenolic profile under high copper stress. The growth curve showed that the cell density increased to 3.1×10^8 cells L^{-1}. The cell concentration decreased 47.5% with respect to the reference culture. In this experiment, a similar growth pattern was found as in the study at 315 nmol L^{-1} of Cu(II). The growth slope was only 0.53×10^7 cells L^{-1} d^{-1} through to the fifth day of culture and increased to 8.01×10^7 cells L^{-1} d^{-1} over the following days.

The iron effect was studied at 900 nmol L^{-1} of Fe(III) added to the culture of *P. tricornutum.* Under these conditions, the cell density reached a maximum concentration of 2.70×10^9 cells L^{-1}, one order of magnitude higher than the reference culture. The growth slope in the exponential phase was 46.9×10^7 cells L^{-1} d^{-1}, and the growth rate was 5.4×10^8 cells L^{-1} d^{-1} for the 8 d of culture (the cell density increased 471% with respect to the reference culture).

Polyphenolic profiles—The polyphenolic profile of *P. tricornutum* was determined both in the extracts of cells collected from the cultures and in seawater enriched with exudates. Tables 2 and 3 show all the polyphenols quantified in this work.

The proposed polyphenols were identified in the extracts of cells, except for gallic acid, which was only detected in the iron enrichment experiment. In addition, in the seawater enriched with exudates, only quercetin was not detected.

The phenolic compound content in cell extracts of *P. tricornutum* exposed to copper was strongly affected by the metal concentration (Table 2). As compared to the control,

Table 2. Phenolic compounds contents in extracts of diatom *Phaeodactylum tricornutum* exposed to high iron and copper concentrations.

Phenolic compound	Control	[Fe(III)] 900 nmol L^{-1}	[Cu(II)] 315 nmol L^{-1}	[Cu(II)] 790 nmol L^{-1}
Gallic acid*	nd†	43±4	nd	nd
Protocatechuic acid*	46.9±0.4	65±4	59±3	144±10
Catechin*	236±3	277±1	208±4	670±42
Vanillic acid*	53.8±0.8	59±4	83±6	95±2
Epicatechin*	123±5	129±6	174±13	234±15
Syringic acid*	65±4	76±3	67±2	118±3
Chlorogenic acid*	50±2	47±2	62±4	106±2
Gentisic acid*	138±4	74±5	174±8	278±10
Caffeic acid*	35±1	35.1±0.5	50±2	75±1
Coumaric acid*	38.2±0.2	38.1±0.6	60±2	81±2
Ferulic acid*	37.0±0.9	60±5	79±9	122±10
Rutin*	12.9±0.3	60±3	20±2	45±4
Myricetin*	56.1±0.5	209±9	119±9	136±6
Quercetin*	5.3±0.3	36±2	3.1±0.4	9.5±0.6
Sum‡	897±22	1208±49	1158±64	2114±108

* nmol of phenolic compound per cell × 10^{10} ± standard deviation of two measurements.
† nd, not detected.
‡ nmol of total phenolic compounds per cell × 10^{10} ± standard deviation of two measurements.

the amount of most of the identified phenolic compounds increased slightly when the copper concentration was 315 nmol L^{-1} (growth rate 80%). Significantly, the greatest amount (around double with respect to the control) of all the identified phenolic compounds was found when the diatom *P. tricornutum* was exposed to copper concentration of 790 nmol L^{-1} (Table 2). Similar findings were given in the seawater samples enriched with exudates, with double the sum of all the identified phenolic compounds exuded per cell in the culture enriched with a copper concentration of 790 nmol L^{-1} as opposed to those in the control (Table 3). The concentration of polyphenols increased in the culture seawater enriched with copper at concentration 790 nmol L^{-1} to 39 nmol L^{-1}.

In the iron enrichment experiments, the amount of the quantified phenolic compounds per cell increased under these conditions of high cell growth (471%). The sum of all the identified compounds was 1208 nmol of polyphenols, 1.3 times higher than the control values of 897 nmol of polyphenols (Table 2). In these experiments, myricetin and (+)-catechin were the predominant phenolic compounds, and gallic acid was detected only in the diatoms exposed to iron. The contents of polyphenols such as rutin, myricetin, and quercetin increased by 365%, 273%, and 579%, respectively, whereas in the copper enrichment experiment ([Cu(II)] = 790 nmol L^{-1}) the increases were inferior (249%, 142%, and 79%, respectively). However, gentisic acid decreased in the presence of iron and increased when

Table 3. Phenolic compounds contents in seawater enriched with exudates of diatom *P. tricornutum* under high iron and copper concentrations.

Phenolic compound	Control	[Fe(III)] 900 nmol L^{-1}	[Cu(II)] 315 nmol L^{-1}	[Cu(II)] 790 nmol L^{-1}
Gallic acid*	2.3±0.1	10.3±0.5	0.58±0.07	4.8±0.5
Protocatechuic acid*	23.2±0.6	5.9±0.5	19.7±0.9	43±2
Catechin*	125±11	11.6±0.1	112±4	283±15
Vanillic acid*	19±1	4.3±0.3	18.3±0.9	36±2
Epicatechin*	55.9±0.8	11.7±0.0	66±7	107±3
Syringic acid*	32.0±0.1	7.8±0.4	24±2	58±2
Chlorogenic acid*	18.2±0.7	3.5±0.0	24±2	35.2±0.8
Gentisic acid*	48.3±0.7	6.8±0.2	61±5	93±7
Caffeic acid*	11.4±0.1	2.4±0.2	16.4±0.8	21.4±0.6
Coumaric acid*	14.8±0.3	2.9±0.2	19.6±0.7	27.3±0.6
Ferulic acid*	21.0±0.4	4.1±0.2	27±3	41±4
Rutin*	9.0±0.3	6.5±0.6	11.4±0.6	16.8±0.6
Miricetin*	32.4±0.9	21±2	59±3	60±2
Quercetin*	nd†	5.5±0.2	2.2±0.2	1.9±0.1
Sum*	413±17	104±5	461±30	828±40
Sum‡	24±1	28±2	30±2	39±2

* nmol of phenolic compound per cell × 10^{10} ± standard deviation of two measurements.
† nd, not detected.
‡ nmol L^{-1} ± standard deviation of two measurements.

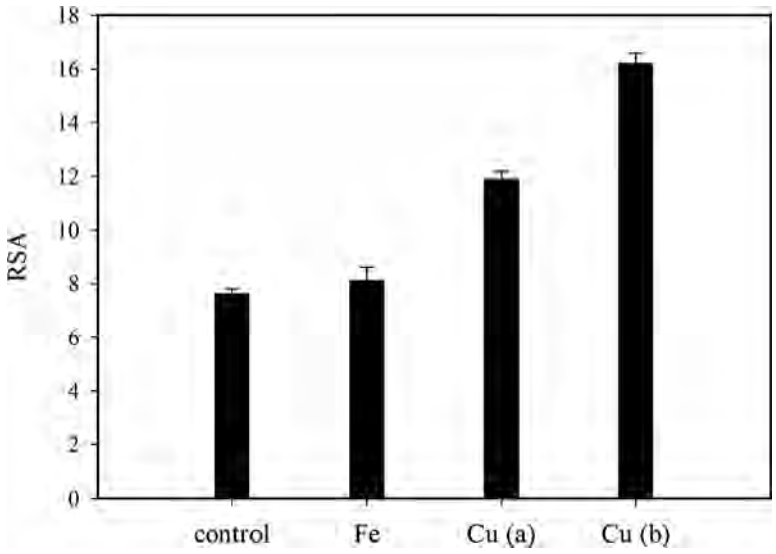

Fig. 2. Relative activity of the extracts derived from diatom *Phaeodactylum tricornutum* to scavenge the stable radical DPPH under different conditions: control seawater, [Fe(III)] = 900 nmol L^{-1}, Cu(a) was [Cu(II)] = 315 nmol L^{-1}, and Cu(b) was [Cu(II)] = 790 nmol L^{-1}. RSD values represented means of triplicate determinations ± standard deviation.

the diatom was exposed to copper, whereas the rest of the polyphenols showed major increases when the diatoms were exposed to a copper concentration of 790 nmol L^{-1}. Quantification of phenolic compounds in the seawater samples enriched with exudates from the iron enrichment experiments showed that the amount of single phenolic compounds exuded per cell declined dramatically as compared to the control, with the sum of all the identified compounds exuded per cell 4 times less than those in the control (Table 3). However, the concentration of the polyphenols in nmol L^{-1} increased in the culture seawater enriched with iron from 24 nmol L^{-1} (control) to 28 nmol L^{-1} due to the large number of cells.

Antioxidant activities of the diatom extracts—Figure 2 shows the relative antioxidant efficiency of the diatom extracts against the DPPH radical. Antioxidants suppressed the absorbance at 515 nm on a time scale dependent on the antioxidant activity of the extracts. As may be observed, the extracts derived from the diatom exposed to copper showed the highest radical scavenging activities (16.2% and 11.9%) followed by the extract derived from cells under iron enrichment conditions (8.1%) and the control (7.6%).

Discussion

Copper and iron stress affect growth and the phenolic contents of the marine diatom *P. tricornutum*, but not in the same way. The behavior of *P. tricornutum* under copper additions suggested that the diatoms metabolically produced exudates capable of complexing the copper present in the solution, thereby decreasing the toxic effect. Once the conditions were optimal, the diatom growth began. The effect of copper on diatom growth concords well with results published in previous reports with respect to the effect of copper toxicity on microalgae growth. Kagalou et al. (2002) reported that Cu(II) concentrations of 15.7 μmol L^{-1} had significant effects on the growth rate of the microalgae *Isochrysis galbana*, whereas lower concentrations (157 and 1570 nmol L^{-1}) produced less decline in algal growth. Franklin et al. (2002) concluded that the copper concentrations required to inhibit growth rate by 50% increased from 72.4 to 252 nmol L^{-1} for the tropical freshwater alga *Chlorella* sp. and from 104 to 268 nmol L^{-1} for the temperate species *Selenastrum capricornutum* as the initial cell density increased from 10^5 to 10^8 cells L^{-1}. The initial cell density is a very important parameter in toxicity tests carried out using microalgae. Moreno-Garrido et al. (2000) reported copper growth inhibition tests on four marine microalgal species including *P. tricornutum*. Diatoms were cultivated in artificial seawater enriched with a modification of f/2 medium, lacking ethylenediaminetetraacetic acid, which decreases the toxicity of heavy metals. In these conditions, Moreno-Garrido et al. (2000) observed EC_{50} values (representing the concentration of copper that caused a 50% reduction in the cell division rate compared with controls and calculated by semilogarithmic plotting of average inhibition data fits to straight line) for *P. tricornutum* of 154, 542, and 551 nmol L^{-1} for the initial cellular densities 10^6, 5×10^6, and 10^7 cells L^{-1}, respectively (after 72 h exposure to copper). Franklin et al. (2001) reported that copper had an inhibitory effect on the cell division rate of several algal species after 48 h and 72 h exposure to copper (initial cell density 2×10^7 to 4×10^7 cells L^{-1} and using filter-sterilized seawater supplemented with $[NO_3^-]$ = 242 μmol L^{-1} and $[PO_4^{3-}]$ = 15.8 μmol L^{-1} as a culture medium to maintain exponential growth over 72 h). In said study, the 48 h and 72 h EC_{50} values were 142 ± 47 and 158 ± 63 nmol L^{-1}, respectively, and complete growth inhibition was observed at 11.8 μmol L^{-1}. The cell light scatter properties of *P. tricornutum* depended on the cell size and intracellular granularity. These properties were found to be

a useful indicator of chronic copper toxicity to the marine alga *P. tricornutum*. Franklin et al. (2001) found that copper caused an increase in cell size after 24 h exposure, with 50% or more of the cells larger than the controls at 3.15 μmol L^{-1}, whereas similar increases in cell size were observed after 48 h and 72 h at 157.5 nmol L^{-1}, with an EC_{50} value of 126 $\pm$ 47 nmol L^{-1}. Similar changes in cell size and granularity were also reflected in changes in side-angle light scatter with an EC_{50} value of 189 nmol L^{-1} (after 48 h and 72 h exposure to copper). The diatoms *P. tricornutum* exposed to lower doses of copper in said study than those used by the present researchers at the same initial cell density, showed changes in cell size, granularity, and growth rate, considered to be indicators of copper toxicity.

P. tricornutum showed the highest growth rate at iron concentration 900 nmol L^{-1}. Iron is well known as a fertilizer and as an essential micronutrient participating in essential metabolism processes such as photosynthesis, respiration, and nitrogen fixation (Stumm and Morgan 1996). Morel et al. (2008) have demonstrated that unchelated Fe is highly available for uptake but that chelated Fe is necessary in order to explain the total uptake system. There is even evidence of direct internalization of siderophores by microorganisms using reductive mechanisms with varying degrees of efficiency (Hopkinson and Morel 2009). Our results align well with Kudo et al. (2000), who reported the combined effect of iron nutrition and temperature on growth (cell division) of the diatom *P. tricornutum*, concluding that the growth rate of cells cultivated with Fe(III) 2.0 μmol L^{-1} was twice as fast as the growth rate of diatoms cultivated with lower Fe(III) concentration (2.0 nmol L^{-1}) at 20°C.

In this study, increased phenolic compound content has been reported in *P. tricornutum* exposed to increasing doses of Cu(II). At the highest copper concentration (790 nmol L^{-1}), the phenolic content per cell was 2.4 times higher than in the control. In addition, the concentration of polyphenols increased in the culture seawater enriched with copper at concentration 790 nmol L^{-1} to 39 nmol L^{-1} (Table 3), despite the number of cells being much lower (52.5%), indicating that cells exposed to copper must excrete a larger amount of polyphenols. The diatom must make an extra metabolic effort in order to live in these levels of copper concentrations and must produce relevant amounts of polyphenols to slow down the toxicity of the copper in the solution, acting as a protective mechanism. This role has also been described for soil bacteria (*Pseudomonas aureofaciens*) (González et al. 2010) and plants that present mechanisms of detoxification, such as the exudation of phenols that act as chelate towards heavy metals to protect cells from induced oxidative damage (Jung et al. 2003). Increasing phenolic compounds may also contribute to enhancing the ability to cope with osmotic stress by binding metal ion to wall-associated polyphenols, countering metal toxicity at the cell surface (Suresh and Subramanyam 1998; Jung et al. 2003). Plant intracellular detoxification mechanisms may involve specific scavenging of reactive oxygen species through the action of enzymes and a wide array of metabolites, including phenolic components or direct chelate formation by polyphenols (Quideau et al. 2011). Wells et al. (2005) reported a synergistic link between the release of the iron-complexing ligand domoic acid and the limitation by iron and copper in toxigenic diatoms *Pseudo-nitzschia* spp. In said study, copper was found to be a key factor facilitating the success of these diatoms in iron-deficient waters, by inducing a high-affinity iron uptake. Iron limitation is the primary trigger for domoic acid production, which doubled under iron deficiency and increased by one order of magnitude under copper-limiting conditions. However, similar increases in domoic acid production were detected under conditions of toxic copper concentration, presumed to reduce copper toxicity.

The findings here evidenced increased phenolic compounds per cell under iron-rich media conditions ([Fe(III)] = 900 nmol L^{-1}) as compared to the control. However, the amounts of phenolic compounds exuded per cell decreased drastically, resulting four times lower than the control. This decline in excreted polyphenol concentrations per cell may have its explanation in the high cell densities in the culture that help to minimize the stress suffered per cell, making the metabolic effort needed to uptake iron to decrease.

P. tricornutum produced and exuded phenolic compounds mixtures with different phenolic profiles depending on the culture conditions. The extracts derived from the diatom exposed to copper showed the highest radical scavenging activities (16.2% and 11.9%). The phenolic profiles for these more active extracts were very similar. However, the diatoms exposed to iron showed different phenolic profiles that may vary the antioxidant potential. The antioxidant activity is not only dependent on the concentration of antioxidants, but also on the structure (Wright et al. 2001). The antioxidant activity is also dependent on the interaction among the antioxidants (phenolic compounds may act synergistically or antagonistically) (Jacobo-Velazquez and Cisneros-Zevallos 2009). Amoros et al. (1992) demonstrated the synergistic effect of binary flavone–flavonol combinations against herpes simplex virus type 1 in cell culture. Gao et al. (2011) reported that four phenolic compounds (vanillic acid, protocatechuic acid, ferulic acid, and caffeic acid) exerted additive and synergistic inhibition effects on the growth of *Microcystis aeruginosa* depending on the mix ratios. Tafesh et al. (2011) reported that hydroxytyrosol at 400 μg mL^{-1} caused growth inhibition of the four bacterial isolate gram-positive (*Streptococcus pyogenes* and *Staphylococcus aureus*) together with the gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*), whereas gallic acid at 200 and 400 μg mL^{-1} inhibited the growth of *S. aureus* and *S. pyogenes* strains, respectively (no growth inhibition was observed for the gram-negative bacteria). However, the combination of both compound gallic acid and hydroxytyrosol at lower concentrations (100 and 200 μg mL^{-1}, respectively) caused a complete inhibition of the four bacterial strains. In the present study, significant differences in the phenolic profiles were found in *P. tricornutum* diatoms growing under iron fertilization conditions and under copper stress. These differences seem to be the response to the different needs of the diatoms when grown under different stress conditions: as compared to the control, the amount of gentisic acid decreased when the culture seawater was enriched with iron

and increased when the seawater was enriched with copper; cells under iron enrichment conditions revealed a higher content of quercetin, myricetin, and rutin than the other diatom extracts and gave no change for (−)-epicatechin or chlorogenic acid, which increased in diatoms exposed to copper (790 nmol L^{-1}) (Table 2). As Gao et al. (2011) suggested, the profile of the phenolic compounds in a mixture and their mix ratios determine the joint action of the compounds, with either synergistic or additive effects. In addition, various different kinds of activities of the phenolic mixture may also depend on the mix ratios, where this mixture may be more active in complexing or reducing trace metals in solution. Therefore, further research is required to study the joint action of the phenolic compounds identified in cells exposed to multiple metal stresses and the influence of the mix ratio of said phenolic compounds on the kind and intensity of the mix activity.

Polyphenols have attracted a great deal of attention recently, mainly focused on their role in preventing diseases produced as a result of oxidative stress (Dillard and German 2000; Dai and Mumper 2010). Dietary antioxidants from plants are believed to help prevent aging and many degenerative diseases, such as cardiovascular complaints and cancers, as a result of their radical scavenging activity (Virgili et al. 2003; Quideau et al. 2011). Therefore, there is considerable interest in the field of preventive medicine in the development of natural antioxidants obtained from botanical sources. Seaweeds are considered to be a rich source of antioxidants, and recent studies have suggested using *microalgae* as "health" foods (Chacón-Lee and González-Mariño 2010). The results of this study confirm that *P. tricornutum* is a natural source of well-known antioxidant compounds (Silva et al. 2002) and afford more than sufficient arguments for researching the viability of the use of *P. tricornutum* diatom in the health and food industries in general, as well as in the pharmaceutical industry.

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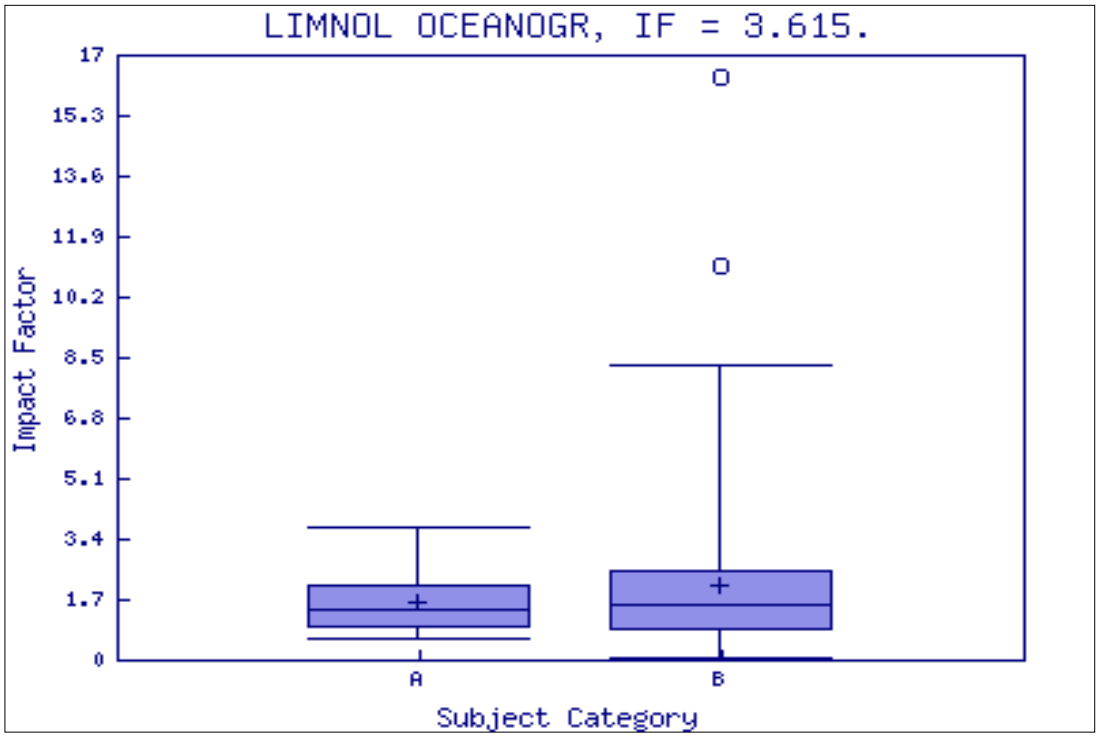

Key

A - LIMNOLOGY
B - OCEANOGRAPHY

4.2.2. Caracterización de los polifenoles exudados por la diatomea *Phaeodactylum tricornutum* y sus efectos en la química del Fe(II)-Fe(III).

Se optimizó un método para la identificación y cuantificación de cuatro polifenoles en microalgas (ácido sinápico, quercitrina, kaempferol y apigenina), además de los catorce identificados en el estudio comentado anteriormente [Rico y colaboradores, 2013]. De todos los polifenoles identificados en los exudados de la diatomea, se seleccionaron los más abundantes, (+)-catequina y ácido sinápico, y se realizó un estudio del efecto de dichos compuestos en la química del hierro en agua de mar a pH 8,0 y pH 6,0. Estos compuestos favorecieron la reducción de Fe(III) a Fe(II), que es un proceso dependiente del pH. El porcentaje de Fe(II) regenerado fue siempre mayor en presencia de (+)-catequina que en presencia de ácido sinápico.

Este estudio demostró que los compuestos polifenólicos excretados por las diatomeas pueden influir en la química del hierro en disolución y favorecer la persistencia del Fe(II) para su asimilación por la célula. Estos resultados proporcionan posibles mecanismos de las microalgas para mantener el Fe(II) en regiones de afloramiento costero y en un futuro escenario de acidificación oceánica.

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Characterization of phenolic exudates from *Phaeodactylum tricornutum* and their effects on the chemistry of Fe(II)–Fe(III)

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ABSTRACT

The effect of exudates from *Phaeodactylum tricornutum* on the iron chemistry has been studied at pH 8.0 and 7.5 in seawater and seawater enrichment with the diatom exudates. At pH 8.0 the rate constant for the oxidation of Fe(II) decreased by 29% in the presence of the exudates, while at pH 7.5, the rate constant decreased by 56%. At the stationary phase of growth, eighteen individual phenolic compounds were identified in the extracts. The role played by the two most abundant phenols, catechin and sinapic acid, in the iron chemistry from pH 8.0 to 6.0 was investigated. These phenolic compounds favored the reduction of Fe(III) to Fe(II), which is a pH-dependent process in both catechin and sinapic acid. In the presence of catechin, the Fe(III) reduction rate, log k' (k', s^{-1}) was −6.15 at pH 8.0 and −3.79 at pH 6.0, in seawater. The reduction rate was lower in seawater than in NaCl solution due to the interaction of Mg^{2+} and, to a lesser extent, Ca^{2+}, with the benzoquinone intermediate. In the presence of sinapic acid, log k' was −6.57 at pH 8.0 and −3.90 at pH 6.0, and was also lower in seawater than in NaCl solutions. This difference was explained by the interaction of the major ions, Ca^{2+} and Mg^{2+} with benzoquinone. The percentage of regenerated Fe(II) was always higher in the presence of catechin than in sinapic acid. This study showed that phenolic compounds exudated from diatoms can influence the iron redox chemistry and favor the persistence of Fe(II) in coastal upwelling region and in a future scenario of ocean acidification, providing possible mechanisms to make Fe available for their requirements.

1. Introduction

Marine microorganisms are known to release ligands into their surrounding environments that form metal complexes in order to acquire micronutrients, sequester toxic metals, and to establish electrochemical gradients (Jones, 1998) resulting in changes in the speciation, the bioavailability, and the toxicity of the trace metals in the media (Moffet et al., 1990). Relatively little is known about the chemical characteristics of these organic compounds present in seawater and their effects on the iron chemistry (van der Berg, 1995). According to their binding affinities for iron, organic ligands have been operationally defined into two classes, strong and weak ligands (Gledhill and Buck, 2012). In the last decade, much attention has been focused on the stronger binding ligands, particularly siderophores (Rue and Bruland, 1995; Wilhem, 1995; Butler, 1998; Hopkinson and Morel, 2009). Important classes of siderophores that have been identified from cultures of marine bacteria under Fe limited conditions include hydroxamates, catecholates and mixed functional group (β-hydroxy aspartate/catecholate) ligands (Reid et al., 1993; Wilhelm and Trick, 1994; Wilhelm et al., 1997; Granger and Price, 1999; Witter et al., 2000). It has been demonstrated that the presence of siderophores in the medium can stimulate the growth of diatoms (Hutchins et al., 1999; Soria-Dengg and Horstmann, 1995). There is scarce information about the interaction of iron with weak ligands such as saccharides, amino acids or phenolic compounds in the ocean. Saccharides and amino acids are abundant throughout the ocean water column (Benner, 2011; Hassler et al., 2011). Unlike siderophores, that are found in seawater at picomolar levels (Mawji et al., 2008), saccharides have been found in relatively high concentrations in surface waters, from nanomolar to micromolar (Panagiotopoulos and Sempere, 2005). A recent study has shown that saccharides increase the uptake of iron in natural communities of phytoplankton (Hassler et al., 2011). Phenols are also ligands excreted by diatoms (Rico et al., 2013). Like sugar, amino acids and polysaccharides, the phenolic compounds have functional groups, such as hydroxyl, together with carboxylic groups, that can form weak complexes with iron and modify the chemical speciation and bioavailability of this trace metal (Lodovici et al., 2001; Re et al., 1999; Sroka and Cisowski, 2003; Brown et al., 1998; Mira et al., 2002; Andjelković et al., 2006). Some polyphenols can also form strong complex with iron with stability constants, log K, ranging from 18 to 22 (Hynes and O'Coinceanainn, 2001; 2004), although the affinity of these compounds for Fe(III) is much lower than that of natural siderophores such as

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enterobactin or desferrioxamine which form hexadentated complexes (Elhabiri et al., 2007). Catechol is a frequent functional group found in the catecholate siderophores and in polyphenols excreted by the phytoplankton (Rico et al., 2013). The catechol forms complexes with Fe(III) and subsequently decomposes to form Fe(II) in a two step redox process in seawater that is favored at pH values lower than 8 (Santana-Casiano et al., 2010). Therefore, the role of ligands that can simultaneously act as chelating agents for Fe(III) and iron reducing agents through intermolecular redox reactions should be considered.

Iron in the marine environment exists in two different oxidation states, Fe(II) and Fe(III). In seawater, Fe(II) oxidizes to Fe(III), the thermodynamic stable form, through Eqs. (1)–(4) (Millero and Izaguirre, 1989). Fe(III) is less available for uptake of phytoplankton than Fe(II) because of its formation of ferric hydroxides and oxides and high stable ferric chelates, which are generally not directly bioavailable. In general, Fe(II) is much less strongly bound by organic ligands and does not form insoluble precipitates, and thus it is more bioavailable (Morel et al., 2008; Shaked et al., 2005; Shaked and Lis, 2012).

However, Fe(III) can be reduced to Fe(II) by some specific organic compounds, depending on physical–chemical conditions, particularly pH, light and biological activity (Santana-Casiano et al., 2000, 2010; Strlic et al., 2002; Rose and Waite, 2003; Steigenberger et al., 2010; Shaked and Lis, 2012), and then oxidized back through Fenton type reactions (Eqs. (1)–(4)). In the presence of organic ligands (L), Eqs. ((5)–(9)) may take place and the generation of $OH^{\cdot}$ from Eq. (3) may be strongly affected (Miller et al., 2013).

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^- \quad (1)$$

$$Fe(II) + O_2^{\overset{\breve{A}}{n}-} \rightarrow Fe(III) + H_2O_2 \quad (2)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^{\overset{\breve{A}}{n}} + OH^- \quad (3)$$

$$Fe(II) + OH^{\overset{\breve{A}}{n}} \rightarrow Fe(III) + OH^- \quad (4)$$

$$Fe(II) + L = Fe(II)L \quad (5)$$

$$Fe(II)L + O_2 \rightarrow Fe(III)L \quad (6)$$

$$Fe(III) + L = Fe(III)L \quad (7)$$

$$Fe(III) + L \rightarrow Fe(II) + L' \quad (8)$$

$$Fe(III)L \rightarrow Fe(II) + L'. \quad (9)$$

In surface seawater, a small fraction of iron occurs as thermodynamically unstable Fe(II) and the reason is largely unknown (Roy et al., 2008) although it has been observed that the presence of organic ligands can regulate the Fe(II) oxidation (Roy and Wells, 2011). Moreover, the superoxide radical plays an important role in the biologically mediated Fe(III) reduction pathway (Rose et al., 2008).

The aim of this study is to characterize the phenol compounds excreted by the diatom *Phaeodactylum tricornutum* (*P. tricornutum*) and their effect on the Fe(II) persistence in seawater. Two of the most abundant phenol exudates excreted by the diatom have been selected to study the possible reduction of Fe(III) to Fe(II), namely catechin and sinapic acid.

P. tricornutum has been selected as a representative diatom species because it occupies a variety of habitats and it is one of the most abundant photosynthetic organisms in the marine environment. The diatoms are responsible for close to 40% of marine primary productivity (Falkowski et al., 1998). The *P. tricornutum* cells present adaptations to low-iron environments which include strategies to lower the cellular iron requirements and to enhance iron uptake (Lommer et al., 2012). Information about the organic compounds liberated by these algal species is very limited and a stronger effort must be made in order to characterize the composition of organic compounds that affect the speciation and behavior of iron in the ocean.

2. Experimental

2.1. Chemicals

The stock solution of Fe(II) (4×10^{-4} M) was prepared using ammonium iron(II) sulfate hexahydrate (Sigma), acidified at a pH of 2 with Suprapur HCl (Sigma) in NaCl (0.7 M). All solutions were prepared with Milli-Q water and filtered by 0.1 μm. All chemicals used for the Fe(II) determination were trace analytical grade.

Methanol was high performance liquid chromatography (HPLC) grade (Panreac). Formic and acetic acids (Merck) were the analytical quality reagents. Polyphenol standard gallic acid, protocatechuic acid, chlorogenic acid, (−) epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+) catechin, sinapic acid, quercitrin, kaempferol, and apigenin were purchased from Sigma-Aldrich Chemie (Steinheim, Germany); rutin and gentisic and caffeic acids were supplied by Merck (Darmstadt, Germany).

The solid phase extraction (SPE) cartridges used were Chromabon with polar modified polystyrene divinylbenzene (500 mg) from Macherey-Nagel.

2.2. Culture of algae

P. tricornutum was the phytoplankton species selected in order to study the exudates generated by the algae. This diatom was supplied by the Spanish Bank of Algae (BEA) of Taliarte (Gran Canaria).

In order to obtain the exudates, the algae were cultured in seawater (filtered at 0.45 μm) by adding a modified f/2 nutrient media (Guillard, 1975). The seawater used in this study was collected off the coast of Gran Canaria. The seawater was enriched with nitrate, phosphate and silicate as for the f/2 media, but without added chelators, metals or vitamins to minimize any change in the natural speciation (Re et al., 1999). The stock solutions of nutrients were sodium nitrate (Sigma) (0.88 M), potassium hydrogen phosphate (Sigma) (0.03 M) and sodium silicate (Sigma) (0.14 M). The concentration of each nutrient was kept constant at 8.82×10^{-4} M, 2.93×10^{-5} M and 1.42×10^{-4} M for NO_3^-, HPO_4^{2-} and SiO_3^{2-}, respectively. Cells were counted daily using a light microscope (Microbiotest, Inc.) with a hemacytometer and by measuring the absorbance (640 nm) with a spectrophotometer (USB2000). The initial cell concentration was 10^7 cell/L. The temperature was kept constant at 24 °C under permanent illumination (24 h) in a culture chamber (Friocell, FC111). The cultures reached the stationary phase with 4.98×10^8 cell/L after 8 days. Before each experiment, the culture was filtered by gravity with a 1.2 μm filter in order to remove the cells and avoid cell rupture. The remaining solution, containing the exudates, was filtered by vacuum using a 0.1 μm filter. All the filters used in this study were previously washed with HCl solution (10%) and kept in Milli-Q water for two hours before use. The control seawater was seawater enriched with the same f/2 nutrient concentrations and filtered at 0.1 μm.

2.3. Preparation of exudate extract from seawater

Seawater and seawater enriched with diatom exudates (2.5 L) were passed through the pre-conditioned Chromabon Easy cartridges (successive elution with 3 mL of methanol and 3 mL deionized water) at a flow of 2.5 mL min^{-1} (800 mL per cartridge approximately, using 3 cartridges). In the elution procedure for each cartridge 2% acetic acid in a 5% methanol solution was used. The retained analytes were eluted using aqueous methanol solutions (5%, 10%, and 20% methanol, 2 mL

for each). Prior to the analyses, the fractions were evaporated to dryness in a rotary vacuum evaporator at 25 °C, 3500 rpm, dissolved in 500 μL of methanol, filtered through a 0.45 μm nylon syringe filter and injected directly into the HPLC system.

2.4. Determination of phenolic compounds

Chromatographic analysis was performed using a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostatted column compartment and a diode array detector connected to a ChemStation software. A reverse phase Pursuit XRs C18 (250 mm × 4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian) were used for the separation processes. A gradient system was used involving two mobile phases. Eluent A was water with 0.1% formic acid, and eluent B was methanol. The flow rate was 1.0 ml/min. 60 μl of exudates extract were injected (rheodyne injector). The temperature was maintained at 27 °C. The peaks of polyphenols were identified by comparing their retention times (RT) and by overlaying UV spectra with those of standard compounds.

To quantify the compounds sinapic acid, quercitrin, kaempferol and apigenin in the extracts the wavelength was fixed at 254 nm (López et al., 2013). The relative standard deviations (RSD) of ten replicate samples ranged from 7.40% to 10.33%. The accuracy, expressed as the recovery of the standard compounds, was in the range of 79.2%–102.6%. The limits of detection were found to be in the range of 8.12×10^{-2} nmol mL^{-1} to 0.47 nmol mL^{-1} and the limits of quantification were observed in the range of 0.27 nmol mL^{-1} to 1.58 nmol mL^{-1}.

The phenolic compounds gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin, syringic acid, chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid, rutin, myricetin, and quercetin were quantified using previously reported methods (López et al., 2011; Rico et al., 2013). The RSD values ranged from 2.1% to 6.4%. The recovery was found to be in the range of 87.3%–108%. The limits of detection were found to be in the range of 1.59×10^{-3} nmol mL^{-1} to 0.52 nmol mL^{-1} and the limits of quantification were observed in the range of 4.25×10^{-3} nmol mL^{-1} to 0.17 nmol mL^{-1}.

2.5. Fe(II) oxidation experiments

The Fe(II) oxidation rate was studied in the control seawater (SW_n) and in the seawater enriched with exudates (SW_{nE}) in air saturated conditions, by bubbling the solution with pure air for 1 h, prior to and during the experiments. The samples were stirred at 120 rpm with a teflon-coated magnetic stirrer. The pH (free scale and at 25 °C) was adjusted to the desired value and in order to keep the pH constant during the experiment to ±0.01, small additions of suprapur HCl 0.1 M were done using an automatic titration system (Titrino 719S, Metrohm). The studies were carried out in darkness in a glass thermostat vessel (250 mL) at a constant temperature of 25 ± 0.02 °C using an AG-2™ bath. The studies were done in triplicate and the average results are shown. The addition of the Fe(II) (25 nM) to the sample corresponds to the zero time of the reaction.

The Fe(II) concentration was determined by UV–vis spectroscopy using the ferrozine method (Violler et al., 2000). The ferrozine and Fe(II) formed a peak at 562 nm. Every 30 s, 10 mL of the sample was added to a 25 mL glass flask containing ferrozine (50 μL of 0.01 M), acetate buffer (2 mL, pH 5.5) and NaF (50 μL of 7.1×10^{-4} M) (González-Dávila et al., 2005).

A 5 m long waveguide capillary flow cell (World Precision Instruments™) was connected to the UV–vis detector USB2000 (Ocean Optics™) and used to measure Fe(II) at nanomolar concentrations. The light used was a halogen light source (HL-2000-FHSA from Mikropack). The capillary flow cell and the detector were connected using optical fiber. The spectra were recorded using the OOIBase32 software by Ocean Optics. The sample was introduced into the column using a peristaltic pump (EXPETEC Perimax 12) with a flux of 1 mL/min.

The Fe(II) oxidation rate equation in the presence of oxygen is defined by the equation (Santana-Casiano et al., 2005)

$$\frac{d[Fe(II)]}{dt} = -k_{app}[Fe(II)][O_2]. \quad (10)$$

Under air saturated conditions, the plot of ln[Fe(II)] versus time gave a straight line and from its slope, the pseudo-first order rate constant is obtained according to the equation:

$$\frac{d[Fe(II)]}{dt} = -k'[Fe(II)] \quad (11)$$

where $k' = k_{app}[O_2]$.

2.6. Fe(II) regeneration studies

A 200 nM concentration of Fe was used in 100 ml of solution (NaCl or seawater) in a 200 ml thermostatted vessel controlled to 25 ± 0.02 °C. The experiments were done in a cover cell in order to avoid light effects and photooxidation reactions. The solution was initially aerated with air for two hours under oxygen saturation conditions in order to fully oxidize the added Fe(II) solution. After that time, 10^{-3} mol L^{-1} ferrozine (FZ) was added. Fe(II) was not detected in the absence of the phenolic compounds. A blank was done for the solution containing the formed Fe(III) and the FZ. In order to keep a similar proportion between iron (Boye et al., 2006) and phenol compounds to that in seawater (Table 1), 1 μmol L^{-1} of each of the organic compounds (catechin or sinapic acid) was added to the solution and this was considered the time zero for the reaction. Some experiments were also done by adding Fe(III) directly instead of Fe(II) and no differences were observed. When both Fe(III) and catechin or sinapic acid were present in the solution, the Fe(II)–Ferrrozine complex was followed by UV–VIS spectroscopy at 562 nm. The measured sample was discharged after the analyses.

The FZ method for the Fe(II) analysis was tested in the presence of both catechin and sinapic acid at pH 8.0 and 7.5 in NaCl and seawater. When the catechin was added, the spectrum showed a slight change with a maximum peak of absorbance at 450 nm, but did not interfere in the Fe(II)–FZ complex signal. In the presence of sinapic acid no interference in the Fe(II)–FZ complex signal was observed. Studies for

Table 1
Concentrations of the eighteen individual phenolic compounds determined from seawater with nutrients at f/2 concentrations (SW_n) and with the presence of exudates from *Phaeodactylum tricornutum* after 8 days of culture (SW_{nE}).

Compound	SW_n nM/L	SW_{nE} nM/L	SW_{nE}–SW_n nM/L
Gallic acid	0	0.13	0.13
Protocatechuic acid	0.81	1.37	0.56
Catechin	1.34	6.91	5.57
Vanillic acid	0.22	1.14	0.92
Epicatechin	0	3.08	3.08
Syringic acid	0	1.88	1.88
Chlorogenic acid	0	1.07	1.07
Gentisic acid	0	2.84	2.84
Caffeic acid	0	0.67	0.67
Coumaric acid	0.39	0.87	0.48
Ferulic acid	0.46	1.24	0.78
Rutin	0.04	0.49	0.45
Myricetin	1.03	1.90	0.87
Quercetin	0	0	0
Sinapic acid	2.76	7.30	4.54
Quercitrin	0	3.58	3.58
Kaempferol	1.07	4.25	3.18
Apigenin	0	4.30	4.3
TOTAL	8.12	43.02	34.9

sinapic acid were also done at pH 7.5 in artificial seawater (Millero, 2006) and there was no interference.

3. Results

3.1. Oxidation of Fe(II) in the presence of phytoplankton exudates

The oxidation kinetic of 25 nM Fe(II) was studied in both seawater (SW_n) and seawater enriched with exudates (SW_{nE}), under oxygen saturated conditions, at two different pH values, 8.0 and 7.5. When the exudates were present in the solution, the oxidation rates of Fe(II) decreased at the two studied pH values, as is observed in Fig. 1, indicating that the presence of organic compounds excreted by the diatoms affected the rate of oxidation of Fe(II) in the solution.

At pH = 8.0, in the reference seawater, the apparent rate constant ($M^{-1}\ s^{-1}$) was log k_{app} (SW_n) = 1.24 ± 0.00 while in the presence of exudates log k_{app} (SW_{nE}) was 1.09 ± 0.02. The Fe(II) oxidation rate constant decreased by 29%. At pH = 7.5 the same behavior was observed, changing from log k_{app} (SW_n) = 0.70 ± 0.00 to log k_{app} (SW_{nE}) = 0.34 ± 0.02. At pH 7.5, the Fe(II) oxidation rate constant decreased by 56%. The decrease observed in the rate of oxidation of Fe(II) in the presence of exudates is due to the interaction of these organic compounds with iron. These exudates result from an undetermined and uncharacterized mixture of saccharides, amino acids, phenolic compounds and other organic compounds that may react with iron changing its speciation and affecting the redox chemistry.

3.2. Phenol identification and quantification

In order to elucidate the effects of the organic compounds present in the medium on the kinetics of oxidation of Fe(II), we focused this study on the identification, characterization and quantification of selected phenols exudated by the diatom. Eighteen polyphenols were considered and were resolved and identified in both extracts of SW_n and SW_{nE}. The total concentration of the polyphenols in each extract was quantified. In the seawater extract (SW_n) a total polyphenol concentration of 8 nM/L was determined, and 9 of the 18 analyzed polyphenols were found (Table 1). The vanillic acid, coumaric acid, ferulic acid, and rutin polyphenols were at concentrations below 0.5 nM/L. The most abundant polyphenols were the sinapic acid (2.8 nM/L), catechin (1.3 nM/L), myricetin (1.0 nM/L), kaempherol (1.0 nM/L) and protocatechuic acid (0.8 nM/L). The proportion of those individual compounds and dissolved Fe(II) in an oceanic seawater sample (0.1–0.2 nM, Boye et al., 2006) would range from 1:1 to 10:1.

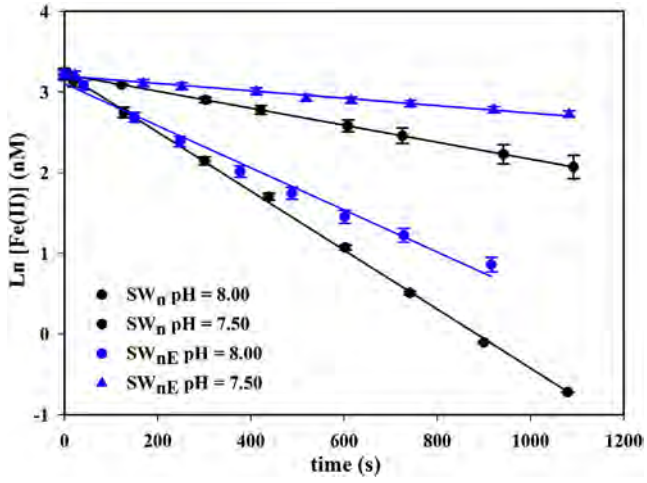

Fig. 1. Pseudo-first order plot for the oxidation kinetics of Fe(II) in reference seawater (SW_n) and in seawater enriched with exudates (SW_{nE}) at pH = 8.00 and pH = 7.50 at 25 °C.

In the extract of seawater with exudates (SW_{nE}), a total concentration of 43 nM/L was obtained. In this extract 17 of the 18 polyphenols analyzed were found, the most important being the sinapic acid (7.3 nM/L), catechin (6.9 nM/L), kaempherol (4.3 nM/L), quercitrin (3.6 nM/L) and epicatechin (3.1 nM/L). Quercentin was not present in either SW_n or SW_{nE}. However, the quercitrin that was not present in SW_n was found at a relatively high concentration in the SW_{nE}, as was the case for the apigenin and epicatechin (Table 1). In the seawater enriched with exudates, the proportion of each individual phenolic compound and dissolved Fe(II) increased to reach ratios between 1:1 and 40:1. Table 1 shows the concentration of the polyphenols released by *P. tricornutum* after 8 days of culture. Taking into account the ability of some phenolic compounds to reduce Fe(III) (Mira et al., 2002; Santana-Casiano et al., 2010), the two major phenols released by the *P. tricornutum* with different characteristics, catechin and sinapic acid (Fig. 2) were selected for further experimentation.

3.3. Catechin

Catechin is a flavonoid type compound with a catechol group in the B-ring, a resorcinol group in the A-ring and a hydroxyl group at position 3 in the C-ring (Fig. 2). The reduction of Fe(III) to Fe(II) in the presence of catechin (initial Fe(III):catechin ratio, 1:5) in 0.7 M NaCl-2 mM $NaHCO_3$ was followed by adding FZ to the Fe(III)-catechin solution at three different pH values 8.0, 7.5 and 6.0. The presence of the Fe(II) chelator FZ in the media acted as a sink for the released Fe(II), thereby preventing any back reactions and allowing the determination of the direct reduction process. Fig. 3 shows that Fe(II) was efficiently formed and this process was pH dependent. The reduction of Fe(III) followed a pseudo-first order kinetic with the rate constant (k' in s^{-1}) increasing as pH decreases from log k' = −5.35 ± 0.07 at pH = 8.00 to log k' = −3.68 ± 0.13 at pH 6.0 (Table 2).

In seawater, log k' changed from −6.15 ± 0.02 at pH = 8.00 to −3.79 ± 0.03 at pH 6.0. In seawater the Fe(III) reduction was lower than in NaCl–$NaHCO_3$ solution. In Table 2, values for log k' in NaCl–$NaHCO_3$ solutions with 0.05 M of Mg^{2+} and 0.01 M of Ca^{2+} are also included. The addition of seawater levels of Mg^{2+} and Ca^{2+} contributed to decrease the Fe(III) reduction rates.

3.4. Sinapic acid

Sinapic acid is a phenylpropanoid member, a cinnamic acid derivative, which possesses 3, 5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of the cinnamic acid (Fig. 2). The regeneration of Fe(II) from the reduction of Fe(III) in the presence of sinapic acid (initial Fe(III):sinapic acid ratio, 1:5) was studied at three different pH values, 8.0, 7.5 and 6.0 in 0.7 M NaCl–2 mM $NaHCO_3$ and in seawater. This process was also pH dependent (Fig. 3). Studies with both artificial seawater and NaCl solution with added Mg^{2+} were also done at pH 7.5 in order to elucidate the effect caused by this major ion in seawater.

In NaCl–$NaHCO_3$ solution the reduction of Fe(III) followed a pseudo-first order kinetic with rate constants increasing as pH decreased from log k' = −5.86 ± 0.08 at pH = 8.0 to log k' = −3.83 ± 0.02 at pH 6.0 (Table 3). In seawater, log k' changed from −6.57 ± 0.04 at pH 8.0 to −3.90 ± 0.10 at pH 6.0. In seawater, the regeneration of Fe(II) was also lower than in NaCl–$NaHCO_3$ solution due to the presence of the major ions. In Table 3, values for log k' in NaCl–$NaHCO_3$ solutions with 0.05 M Mg^{2+} added and artificial seawater are also included for pH 7.5. At this pH, the rate of Fe(III) reduction changed from log k' = −4.62 ± 0.07 in NaCl–$NaHCO_3$ solution to log k' = −6.10 ± 0.01 in NaCl–$NaHCO_3$ with added Mg^{2+}, and to log k' = −6.28 ± 0.08 in the artificial seawater. These values were quite similar to those found in seawater, log k' = −6.16 ± 0.00. No effects were observed when Ca^{2+}, K^+, F^- were added to the solution.

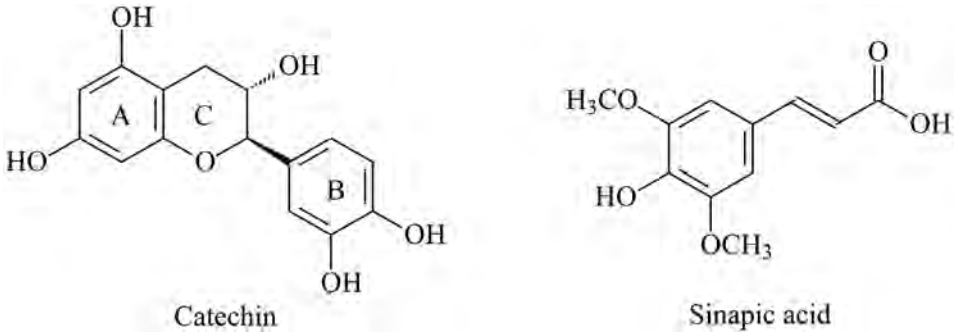

Fig. 2. Catechin and sinapic acid chemical structure.

4. Discussion

The presence of organic compounds in aqueous solutions is known to modify the oxidation rates of Fe(II) (Theis and Singer, 1974; Santana-Casiano et al., 2000; 2004; Rose and Waite, 2003). This study showed a decrease in the oxidation rate of Fe(II) due to the presence of the ligands exuded from the diatom *P. tricornutum*. This decrease could be explained by the formation of an Fe(II)–ligand complex that has a lower oxidation rate than the inorganic Fe(II), Eq. (6). Alternatively, the exudates could complex the Fe(III) formed from the oxidation of Fe(II), and facilitate the reduction of Fe(III) to Fe(II). The first mechanism is unlikely as Fe(II) only forms weak complexes with oxygen containing ligand groups such as OH^- and carboxyl groups, which are the ligand groups in the extracellular compounds studied here (Fig. 2). The second mechanism, the regeneration of Fe(II) from the reduction of Fe(III) in the presence of organic compounds excreted by the *P. tricornutum*, should be considered as the most effective process. The study of the behavior of Fe(III) in the presence of two model ligands, catechin and sinapic acid, able to reduce Fe(III) to Fe(II), was the focus of this investigation. For this purpose, the identification and quantification of the phenols excreted by the phytoplankton cells were considered.

The phenols identified in the exudates of the diatom *P. tricornutum* were simple phenols (gallic acid, protocatechuic acid, vanillic acid, syringic acid, gentisic acid), phenylpropanoids (caffeic acid, coumaric acid, ferulic acid, sinapic acid) and flavonoids. The structure and characteristic groups of these compounds have been fully described in the literature (Mira et al., 2002; Onofrejová et al., 2010) and it is known that they could play an important role in the chemistry of iron in seawater (Rico et al., 2013). The phenols that present a catechol group in the B-ring have the capacity to reduce Fe(III) to Fe(II). The capacity can be increased in some flavonoids with the simultaneous presence of both the catechol groups in the B-ring and the 3-hydroxyl group in the C-ring. The presence of the 2,3-double bond in conjugation with the 4-oxo group in the C-ring is also particularly important for Fe(III) reducing activity, as it has been demonstrated by the comparison of quercetrin and catechin (Mira et al., 2002).

It has been shown that the presence of catechin reduced Fe(III) to Fe(II) at the three selected pH values. The process was more efficient at low pH values in both NaCl–$NaHCO_3$ solution and seawater showing higher Fe(II) concentrations with increasing $[H^+]$, which suggests that $Fe(OH)_2^+$ is the most reactive species. However, in seawater the Fe(III) reduction rate was lower than in NaCl–$NaHCO_3$ solution. This is first, due to a change in the speciation of both iron and the added organic ligands and second, to the interaction of the major ions Mg^{2+} and, to a lesser extent, Ca^{2+} (Table 4) with the catechin and with the benzoquinone radical, as was demonstrated by Santana-Casiano et al. (2010) for the studies done with catechol. The catechin forms a Fe(III)–catechin complex (Mira et al., 2002), that is reduced by intermolecular redox reactions in which the Fe(III) is reduced to Fe(II). The phenol complex is oxidized first to the semiquinone, and then to the corresponding quinone after reacting with another Fe(III) species. In the presence of Mg^{2+}, this ion forms a complex with catechin that limits the free catechin concentration to form the Fe(III)–catechin complex, as can be seen in Table 4, where the Fe(II) formed in NaCl media with added Mg^{2+} is less than 10% of that formed in absence of Mg^{2+}. Moreover, in the presence of Mg^{2+} and Ca^{2+} the semiquinone intermediate forms complexes with these major ions in weakly alkaline aqueous solutions, blocking any further oxidation to the quinone (Nikolić et al., 1988). This is a pH dependent process, and the amount of Fe(II) formed from the Fe(III)–catechin complex would decrease to half of that formed in the absence of any competing ion. This study has also demonstrated that sinapic acid was able to regenerate Fe(II) in the experimental conditions considered, especially at low pH values. The sinapic acid does not form complexes with Fe(III) and the reduction of Fe(III) to Fe(II) takes place by a single reaction step with an electron transfer reaction

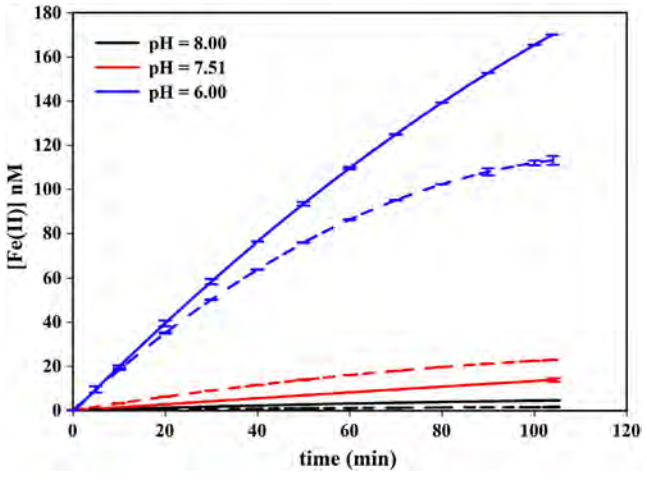

Fig. 3. Fe(II) regeneration from 200 nM Fe(III) in the presence of 1 μM catechin (continuous lines) and sinapic acid (dashed lines) as a function of pH.

Table 2
Pseudo-first order rate constant for Fe(III) reduction in the presence of catechin (1 μM) at constant temperature (25 °C) in different media and pH values.

Media	pH	$k'(s^{-1})$	log k'
NaCl 0.7 M; $NaHCO_3$ 2 mM	8.00	$4.46 \cdot 10^{-6}$	-5.35 ± 0.07
	7.51	$1.17 \cdot 10^{-5}$	-4.93 ± 0.01
	6.00	$2.10 \cdot 10^{-4}$	-3.68 ± 0.13
Seawater	8.00	$7.02 \cdot 10^{-7}$	-6.15 ± 0.02
	7.50	$2.86 \cdot 10^{-6}$	-5.54 ± 0.01
	6.00	$1.61 \cdot 10^{-4}$	-3.79 ± 0.03
NaCl 0.7 M; $NaHCO_3$ 2 mM and Mg^{2+} 0.05 M	8.00	Nd	Nd
	7.51	$9.45 \cdot 10^{-7}$	-6.02 ± 0.04
NaCl 0.7 M; $NaHCO_3$ 2 mM and Ca^{2+} 0.01 M	8.00	$2.25 \cdot 10^{-6}$	-5.65 ± 0.07
	7.51	$3.42 \cdot 10^{-6}$	-5.47 ± 0.02

Table 3
Pseudo-first order rate constant for Fe(III) reduction in the presence of sinapic acid (1 μM) at constant temperature (25 °C) in different media and pH values.

Media	pH	$k'(s^{-1})$	log k'
NaCl 0.7 M; $NaHCO_3$ 2 mM	8.00	$1.39 \cdot 10^{-6}$	-5.86 ± 0.087
	7.51	$2.39 \cdot 10^{-5}$	-4.62 ± 0.07
	6.00	$1.46 \cdot 10^{-4}$	-3.83 ± 0.02
Seawater	8.00	$2.67 \cdot 10^{-7}$	-6.57 ± 0.04
	7.50	$7.00 \cdot 10^{-7}$	-6.16 ± 0.00
	6.00	$1.25 \cdot 10^{-4}$	-3.90 ± 0.01
NaCl 0.7 M; $NaHCO_3$ 2 mM and Mg^{2+} 0.05 M	8.00	Nd	Nd
	7.51	$7.91 \cdot 10^{-7}$	-6.10 ± 0.01
Artificial seawater	7.50	$5.21 \cdot 10^{-7}$	-6.28 ± 0.08

between the sinapic acid and iron(III), resulting in the oxidation of sinapic acid (Hynes and O'Coinceanainn, 2004). The effect observed when Mg^{2+} is added to the NaCl solution, and in seawater solutions, is explained by the complexation of sinapic acid with Mg^{2+} that reduces the free sinapic concentration to be reduced in the presence of Fe(III) in a solution. Tables 4 and 5 compare the percentage of Fe(II) formed from the reduction of Fe(III) after 50 and 103 min in the presence of catechin and sinapic acid under the different media conditions. The major Fe(III) reduction is produced at low pH and in NaCl–$NaHCO_3$ solutions for both catechin and sinapic acid. For the same initial concentration of ligands, 1 μM, the Fe(II) regeneration is higher in the presence of catechin than sinapic acid. The reduction of Fe(III) to Fe(II) decreased in seawater, due the presence of the major divalent ions that interacted with the phenolic compounds. At pH 8.0 in seawater with 200 nM of iron and after 103 min, Fe(II) regeneration was 0.4% in the presence of catechin and 0.1% for sinapic acid. When pH decreases to 7.5, Fe(II) regeneration was 1.7% in the presence of catechin and 0.5% for sinapic acid. At pH 6 Fe(II) regeneration increased to 60.4% in the presence of catechin and 46.3% for sinapic acid.

The presence of phenols modified the behavior of iron in the medium and, when they are present, the reduction of Fe(III) to Fe(II) is favored in conditions where the pH (free scale and at 25 °C) is below 8.0 as observed at the depth of the chlorophyll maximum. According to the results presented in this work, in coastal upwelling regions and in a future scenario of ocean acidification, where pH is below 8.0 and the diatoms are expected to be the predominant species, the presence of this type of compounds can affect the iron redox chemistry.

Table 4
Fe(II) regenerated (%) from an initial Fe(III) concentration of 200 nM in the presence of 1 μM catechin.

Catechin 1 μM			
Media	pH	Time (min)	% Fe(II) regenerated
NaCl 0.7 M and 2 mM $NaHCO_3$	6.003	50	46.8
	6.003	103	84.6
	7.508	50	3.4
	7.508	103	6.8
	7.997	50	1.3
	7.997	103	2.2
Seawater	6.000	50	37.2
	6.000	103	60.4
	7.504	50	0.9
	7.504	103	1.7
	7.995	50	0.2
	7.995	103	0.4
NaCl 0.7 M, 2 mM $NaHCO_3$ and Mg^{2+} 0.05 M	7.508	50	0.3
	7.508	103	0.6
NaCl 0.7 M, 2 mM $NaHCO_3$ and Ca^{2+} 0.01 M	7.508	50	1.1
	7.508	103	2.3
	7.997	50	0.7
	7.997	103	1.2

Table 5
Fe(II) regenerated (%) from an initial Fe(III) concentration of 200 nM in the presence of 1 μM sinapic acid.

Sinapic acid 1 μM			
Media	pH	Time (min)	% Fe(II) regenerated
NaCl 0.7 M and 2 mM $NaHCO_3$	6.003	50	38.0
	6.003	103	56.5
	7.508	50	6.9
	7.508	103	11.4
	7.997	50	0.5
	7.997	103	0.7
Seawater	6.000	50	34.0
	6.000	103	46.3
	7.504	50	0.2
	7.504	103	0.4
	7.995	50	0.0
	7.995	103	0.1
NaCl 0.7 M, 2 mM $NaHCO_3$ and Mg^{2+} 0.05 M	7.508	50	0.2
	7.508	103	0.4
ASW	7.504	53	0.2
	7.504	103	0.3

5. Conclusions

Phenolic compounds such as catechin and sinapic acid are part of the organic compounds exudated by *P. tricornutum* that play an important role in both the decrease of the Fe(II) oxidation rate and the reduction of Fe(III) to Fe(II). Although the regeneration of Fe(II) in seawater is low compared to that in NaCl solution, the amount obtained is significant as the pH of the seawater decreases. This study shows how the ability of some organic compounds to favor the reduction of Fe(III) to Fe(II) is reduced due to the competitive effect of major ions such as Ca^{2+} and Mg^{2+} for the specific sites of complexation. A decrease in pH contributes to an increase in the amount of regenerated Fe(II), showing the acidification may contribute to an increase in the level of reduced iron in the environment.

Acknowledgments

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CHEMISTRY, MULTIDISCIPLINARY	157	47	Q2
OCEANOGRAPHY	61	9	Q1

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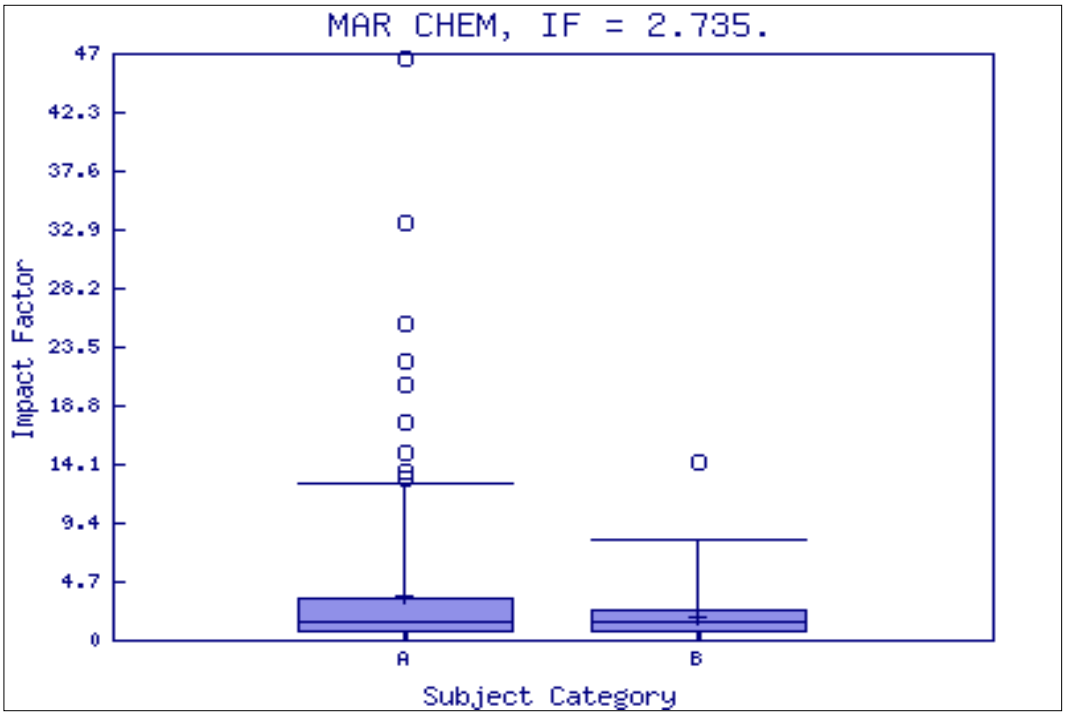
This is a box plot of the subject category or categories to which the journal has been assigned. It provides information about the distribution of journals based on Impact Factor values. It shows median, 25th and 75th percentiles, and the extreme values of the distribution.



Key

A - CHEMISTRY, MULTIDISCIPLINARY

B - OCEANOGRAPHY

4.3. *Dunaliella tertiolecta*. Perfil fenólico de la microalga verde *Dunaliella tertiolecta* cultivada en presencia de altas concentraciones de hierro y cobre.

El presente estudio se centra en determinar el perfil fenólico de exudados y de extractos de microalgas *Dunaliella tertiolecta* cultivadas en agua del mar en ausencia (control) y en presencia de Cu(II) (315 y 790 nmol L^{-1}) y Fe (III) (900 nmol L^{-1}) para identificar y cuantificar los compuestos fenólicos producidos bajo estas condiciones de estrés metálico. Se comprobó que la presencia de iones metálicos modifica el crecimiento celular, siendo la densidad celular 322% (respecto al control) en presencia de hierro, y 80% y 66% en presencia de 315 y 790 nmol L^{-1} de cobre, respectivamente. La concentración y el tipo de polifenoles detectados en los extractos preparados con las células y en los exudados están directamente relacionados con el metal y su concentración en los cultivos. El ácido gentísico, (+)-catequina y (−)-epicatequina fueron los compuestos fenólicos más abundantes detectados en los extractos de algas y mostraron elevada actividad antioxidante en la inhibición del radical DPPH.

La microalga excreta polifenoles para adaptarse a las condiciones ambientales. La cantidad total de los compuestos polifenólicos exudados por cada célula aumentó ligeramente con la adición de cobre al agua de cultivo, llegando a ser 66 amol $cell^{-1}$ a la máxima concentración de cobre estudiada (1,4 veces mayor que la cantidad excretada por cada célula en el

experimento de control, 47 amol cell^{-1}) (FIGURA 16).

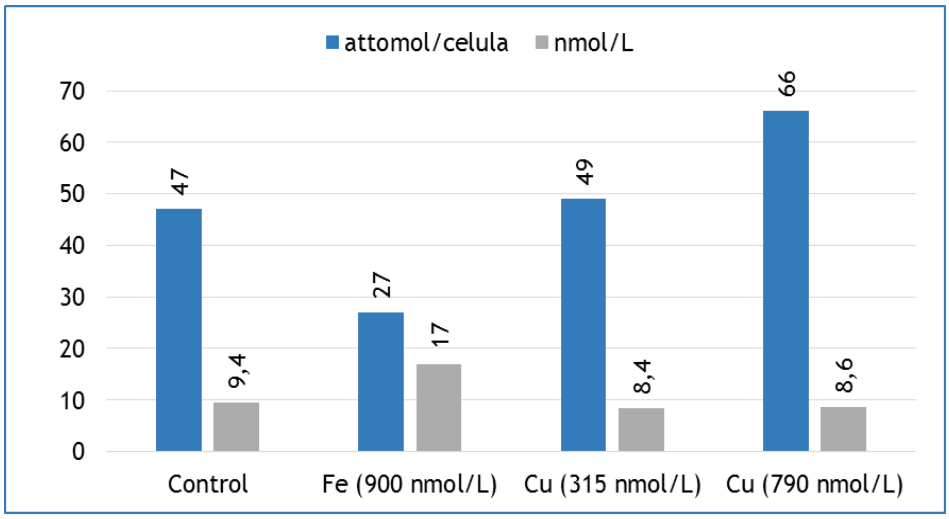


FIGURA 16.- Compuestos fenólicos totales excretados por las células

Sin embargo, no se produjo cambio significativo en la concentración total de polifenoles exudados por litro de agua de mar, que fue de 9,4 nmol L^{-1} en el control y 8,6 nmol L^{-1} en el experimento con mayor concentración de cobre (790 nmol L^{-1}), a pesar de que el número de células en este último cultivo es mucho más bajo (66%). Esto indica que las células expuestas a cobre deben excretar una mayor cantidad de polifenoles.

Las cantidades de los compuestos identificados en el interior de la célula disminuyeron en presencia de cobre, desde 281 amol cel^{-1} en el control a 160 amol cel^{-1} en las células cultivadas con agua de mar con cobre (790 nmol L^{-1}) es decir, 1,8 veces menor, lo que sugiere que la mayoría de los polifenoles son

excretados como mecanismo de defensa (FIGURA 17).

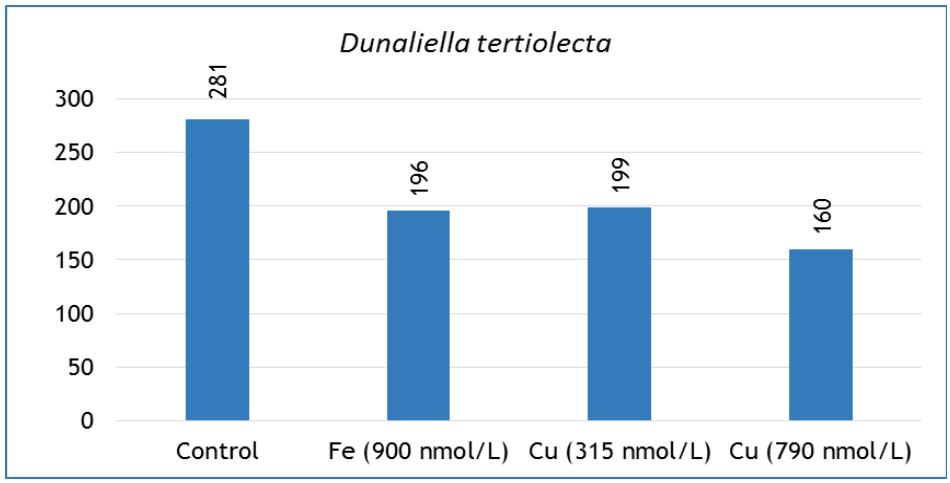


FIGURA 17.- Compuestos fenólicos totales en el interior de la célula (amol cel^{-1})

Cuando la microalga *D. tertiolecta* fue cultivada en presencia de hierro, la cantidad de polifenoles identificados en el interior de la célula disminuyó a 196 amol $cell^{-1}$, 1,4 veces menor que el valor observado en el control (281 amol $cell^{-1}$) (FIGURA 17) a pesar de la alta densidad celular, 322 % más alta que en el control. Para estas mismas condiciones, la cantidad total de polifenoles excretados por cada célula resultó de 27 amol $cell^{-1}$, menor, también, que la cantidad exudada por célula en el control (47 amol $cell^{-1}$). Sin embargo, la concentración de polifenoles en el agua de cultivo se duplicó, aumentando desde 9,4 nmol L^{-1} en el control a 17 nmol L^{-1} en el experimento de enriquecimiento del agua de cultivo con hierro, debido al elevado número de células(322% respecto al control) (FIGURA 16).

El compuesto más abundante en los extractos de *D. tertiolecta* fue el ácido gentísico, seguido de (+)-catequina y (-)-epicatequina, cuyas actividades antioxidantes para inhibir el radical DPPH son más altas que las observadas para la mayoría de los compuestos fenólicos analizados (FIGURA 12).

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Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress

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Abstract

Fourteen phenolic compounds were identified and quantified in extracts derived from in vitro culture of *Phaeodactylum tricornutum* diatom growing in natural seawater (control) and in natural seawater containing the following added copper (Cu) and iron (Fe) metals: Cu(II) (315 nmol L^{-1} and 790 nmol L^{-1}) and Fe(III) (900 nmol L^{-1}). The cell concentration was 471% for the last day of culture (expressed as a percentage of the control) for diatoms exposed to iron, while diatoms exposed to copper showed cell densities of 80% and 52.5% at concentrations of 315 nmol L^{-1} and 790 nmol L^{-1}, respectively. Each extract revealed the presence of 14 phenolic compounds, with the exceptions of gallic acid, which was detected only in the iron-enriched diatoms, and quercetin, which was not detected in the control diatom exudates. Significant differences in the phenolic profiles were found depending on the metal added to the culture media. These differences seem to be the response to the different needs of diatoms exposed to copper and iron concentrations. These results show that increasing levels of metal result in a general increase in levels of total phenolic contents in the cells of *P. tricornutum*. The great increase in phenolic compounds in the cells at the highest copper concentration was 2.4 times higher than in the control, which may reflect the involvement of these compounds in protection against copper toxicity. All the extracts revealed radical scavenging activity against the stable radical 1,1-diphenyl-2-picrylhydrazyl, with the most active extracts from the copper enrichments.

Phenolic compounds are an important group of natural products involved in responses to different kinds of biotic and abiotic stresses (Treutter 2006). The main chemical process where polyphenols are involved is in the scavenging of reactive oxygen species (Neudorffer et al. 2006; Bentes et al. 2011), but phenolic compounds from plants can also act as antioxidants by chelating metal ions (Mira et al. 2002). Therefore, plants under metallic stress conditions accumulate elevated amounts of phenolic compounds for protection and recovery from heavy-metal injury. There is considerable evidence with respect to the implications of phenolic compounds in plants growing in conditions of metal deficiency and stress (Michalak 2006). Phenolic compounds are frequently reported as the main components of root exudates in response to iron deficiency (Wei et al. 2007). Stimulation of phenolic metabolism in response to the toxicity produced by several heavy metals has also been found in chamomile (*Matricaria chamomilla* L.), wheat (*Triticum aestivum*), and in maize (*Zea mays*) (Díaz et al. 2001; Winkel-Shirley 2002; Kovacik and Backor 2007). Studies carried out in order to evaluate the effects of high soil concentrations of copper (Cu) and nickel on phenolics in Scots pine (*Pinus sylvestris* L.) showed that trees exposed to nickel had higher concentrations of condensed tannins than controls. However, concentrations of several other phenolic compounds decreased when seedlings were exposed to high levels of copper or to a combination of nickel and copper (Roitto et al. 2005). Loponen et al. (2001) reported both increased and decreased levels of individual phenolic compounds in mountain birch *Betula pubescens* ssp. *czerepanovii* leaves from forest sites polluted by copper and nickel. The important role of phenolic compounds has also been observed in mangroves (*A. corniculatum*) exposed to heavy metals where the total foliar phenolics were markedly enhanced (Guangqiu et al. 2007) and in waterlily (Nymphaeaceae), capable of accumulating heavy metals with no observed toxicity to the plant (Lavid et al. 2001). In addition, polyphenols and peroxidases seem to play a major role in heavy-metal accumulation and tolerance in *Nymphaea* plants, involving at least two mechanisms: direct chelation by polyphenols and binding and trapping of heavy-metal precipitates during the process of polyphenol polymerization by peroxidases.

In addition, planktonic algae are an important component of natural surface waters. They can regulate the speciation and bioavailability of trace metals through the production and release of organic ligands (Koukal et al. 2007). These organic ligands bind $> 99\%$ of the total iron (Fe) and copper in natural waters (Gledhill and van den Berg 1994; Wu and Luther 1995). Wells et al. (2005) demonstrated that diatoms of the genus *Pseudo-nitzschia* have an unusual capacity for adapting to iron limitation in synthetic growth medium through the production of the strong iron-complexing organic ligand, domoic acid. Few reports have focused on the analysis of bioactive phenolic acids, flavonoids, or similar polyphenols and their implications in algae growing under metal stress (Onofrejová et al. 2010; López et al. 2011). Recently, there have been reports of increases in the total phenolic content and flavonoids in submerged macrophyte *Vallisneria natans* exposed to lead (Pb) stress (Wang et al. 2011).

The aim of this study was to determine the differences in the phenolic profile of the diatom *Phaeodactylum tricornutum*

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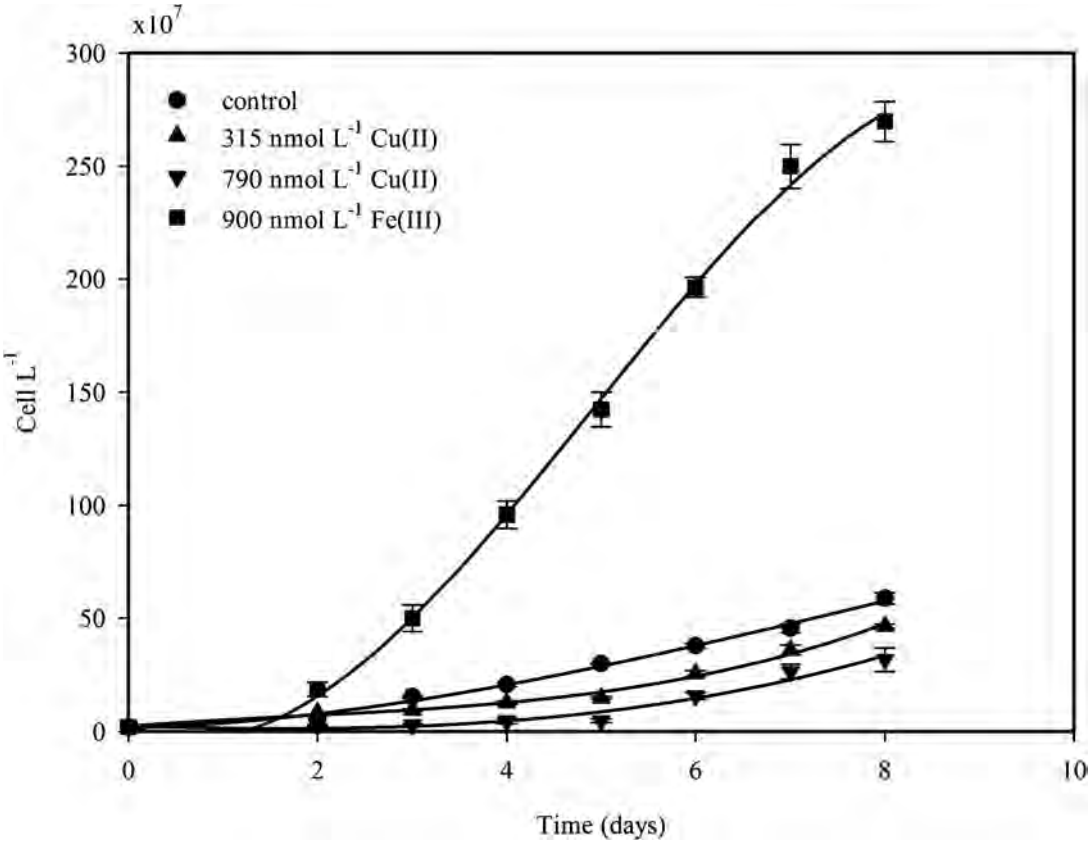

Fig. 1. Growth rates (cell division) of diatoms exposed to iron and copper.

harvested in natural seawater (control) and in natural seawater enriched with heavy metals: Cu(II) (315 nmol L^{-1} and 790 nmol L^{-1}) and Fe(III) (900 nmol L^{-1}). These two metals were chosen because of their opposite effects. It is well known that iron acts as fertilizer in the ocean and in special in high-nutrient low-chlorophyll areas, whereas copper, a nutrient at low concentration, becomes toxic at high concentrations (Stumm and Morgan 1996; Franklin et al. 2001). Iron is needed by most phytoplankton (Ho et al. 2003) and, due to its redox properties, is a component of many enzymes and electron transferring proteins in photosynthesis and respiration (Raven et al. 1999). Copper, with its two oxidation states, is also a valuable cellular constituent, performing an important role in respiration and in the photosynthetic apparatus in some marine diatoms (Peers and Price 2006). Copper replaces iron in some metabolic functions and is also involved in the high-affinity iron transport system of some iron-limited diatoms (Maldonado and Price 2001). Thus, due to iron limitation, copper is important for the growth of oceanic phytoplankton. At physiological and molecular level, this interaction is, however, not well studied. The differences in the phenolic profile may have their roots in the response of the diatoms when exposed to different metal conditions. The effect of those metal concentrations on the growth of the diatoms (cell division) was also studied. A solid-phase extraction method for isolation of the phenolic compounds was developed. Reverse phase high-performance liquid chromatography (RP-HPLC) was applied for the analysis and quantification of 14 phenolic compounds. The antioxidant activity of diatom extracts in scavenging of the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was also determined.

Methods

Chemicals—Methanol, hexane, and acetone were HPLC grade (Panreac). Milli-Q water (18MQ, Millipore) was used throughout the entire study. Formic and acetic acids (Merck) were analytical quality reagent. DPPH and polyphenol standard gallic acid, protocatechuic acid, chlorogenic acid, (−)-epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+)-catechin, sinapic acid, quercitrin, kaempferol, and apigenin were purchased from Sigma-Aldrich Chemie (Steinheim); rutin and gentisic and caffeic acids were supplied by Merck.

The SPE cartridges used were Chromabon with polar modified polystyrene divinylbenzene (500 mg) from Macherey-Nagel.

The stock solutions of nutrients were prepared using sodium nitrate (Sigma) (0.88 mol L^{-1}), potassium hydrogen phosphate (Sigma) (0.03 mol L^{-1}), and sodium silicate (Sigma) (0.14 mol L^{-1}). Both the copper and iron were used direct from the stock solution for atomic absorption spectroscopy (Fluka).

Algae cultivation and preparation—Axenic cultures of *P. tricornutum* were given support by the Spanish Bank of Algae at Taliarte (Gran Canaria, Spain). The main culture of

diatom was kept in a clean culture chamber (Friocell FC111) using f/2 media (Guillard 1975) at constant temperature (24°C) with permanent illumination at 8000 lux.

Experimental cultures were carried out in seawater with f/2 nutrients (Guillard 1975). Seawater was ultraviolet treated (off the coast of Gran Canaria) and filtered by 0.45 μm. The f/2 nutrients ($[NO_3^-]$ = 883 μmol L^{-1}, $[HPO_4^{2-}]$ = 29.3 μmol L^{-1}, $[SiO_3^{2-}]$ = 142 μmol L^{-1}) were added to the seawater. Under these conditions, *P. tricornutum*, with an initial cell density of 2×10^7 cells L^{-1}, reached the stationary phase after 8 d of growth (Fig. 1). This time of culture was used throughout to collect the samples in the same conditions under different conditions of metal concentrations.

The experimental cultures were produced under four different conditions: (1) at the reference culture, in seawater with f/2 nutrients. The effect of copper was tested (2) with 315 nmol L^{-1} of Cu(II) and (3) 790 nmol L^{-1} of Cu(II). The effect of iron (4) was studied with 900 nmol L^{-1} of Fe(III). All of the experiments were triplicated.

The cell concentration was counted daily using a light microscope (Microbiotest) with a hemocytometer and measuring the absorbance (640 nm) with a spectrophotometer (USB4000). Once the culture reached the stationary phase, the cell was collected by filtration in two steps: gravity (1.2 μm) and vacuum (0.1 μm). This treatment avoids rupturing cells. Both cells and enriched seawater were used in this study for the analysis of the phenolic compounds.

Preparation of P. tricornutum *diatom extracts for isolation and quantification of the phenolic compounds*—The algae were freeze-dried and 1 g of the dry material was extracted by stirring with methanol (25 mL) for 1 h. After centrifugation at 3500 revolutions per minute (rpm) for 30 min, the supernatant was collected and evaporated. The dry residue (100 mg) and 4 mL of acetone : hexane (1 : 4) were mixed and stirred for 10 min. The supernatant was discarded and the residue was mixed and homogenized with 5 mL of methanol using a vortex (10 min). After centrifugation at 3000 rpm, the supernatant was separated and the residue was extracted twice with 5 mL of methanol. All the methanolic fractions were collected and evaporated in a rotary vacuum evaporator (Eppendorf, Concentrator plus) at 30°C (6800 rpm). The residues were resolved in 5 mL of HCl (1 mol L^{-1}) and the samples were hydrolyzed at room temperature, 3800 rpm for 30 min. The hydrolysates were purified using solid-phase extraction (SPE).

Solid-phase extraction—The cartridge Chromabon Easy (Macherey-Nagel, 500 mg, particle size 93 μm) was preconditioned by successive elution with 3 mL of methanol and 3 mL of deionized water. The hydrolysates (5 mL) were passed through the cartridges at a flow of 2.5 mL min^{-1}. The cartridge was then rinsed using 2% acetic acid in a 5% methanol solution (Onofrejová et al. 2010). The retained analytes were subsequently eluted using aqueous methanol solutions (5%, 10%, and 20% methanol, 2 mL for each). Prior to the analyses, the fractions were evaporated to dryness in a rotary vacuum evaporator at 25°C, 3500 rpm, resolved in 500 μL of methanol, filtered through a 45 μm nylon syringe filter, and injected directly into the HPLC system.

Seawater enriched with diatom exudates (2.5 L) were passed through the conditioned cartridges (as described above) at a flow of 2.5 mL min^{-1} (~ 800 mL per cartridge, using three cartridges), following the same elution procedure described above for each cartridge. The fractions from the three cartridges were collected, evaporated as above, and the residue resolved in 500 μL of methanol, and then filtered to be injected into the HPLC system.

Quantification of the phenolic compounds by RP-HPLC—Chromatographic analysis was performed on a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostatted column compartment, and a diode array detector, connected to a ChemStation software. A reverse phase Pursuit XRs C18 (250 mm × 4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian) with a gradient system involving two mobile phases were used. The flow rate was 1.0 mL min^{-1} and the injection volume was 60 μL of crude extracts. The system operated at 27°C. The phenolic compounds gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin and syringic acid, chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid, rutin, myricetin, and quercetin were quantified in line with previously reported methods (López et al. 2011). In brief, the eluent A was Milli-Q water with 0.1% formic acid and eluent B was methanol. The elution conditions applied were 0–5 min, 20% B isocratic; 5–30 min, linear gradient from 20% to 60% B; 30–35 min, 60% B isocratic; 35–40 min, linear gradient from 60% to 20% B; and, finally, washing and reconditioning of the column. Each standard was individually tested to determine its retention times (RT) as follows: gallic acid (RT: 5.3 min), protocatechuic (RT: 10.0 min), catechin (RT: 12.7 min), chlorogenic acid (RT: 14.9 min), gentisic acid (RT: 17.1 min), vanillic acid (RT: 17.7 min), epicatechin (RT: 17.9 min), caffeic acid (17.9 min), syringic acid (RT: 18.9 min), coumaric acid (RT: 23.4 min), rutin (RT: 28.1 min), ferulic acid (RT: 24.3 min), myricetin (RT: 30.6 min), and quercetin (RT: 34.6 min) were well resolved. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, (+)-catechin, vanillic acid, (−)-epicatechin, and syringic acid), 324 nm (chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid, and ferulic acid), and 373 nm (rutin, myricetin, and quercetin) for quantification. Limits of detection (LODs) and limits of quantification (LOQs) were estimated from signal-to-noise ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10, respectively. The LODs were found to be in the range of 1.59×10^{-3} nmol mL^{-1} to 0.52 nmol mL^{-1} and the LOQs were observed in the range of 4.25×10^{-3} nmol mL^{-1} to 0.17 nmol mL^{-1}.

SPE accuracy and precision—The presence of polyphenols in the extracts was confirmed by comparison of the retention times and overlapping ultraviolet spectra with those of standard compounds. The precision of the SPE-RP-HPLC method was estimated by measuring the repeatability and the relative standard deviations of 10 replicate samples

Table 1. Solid-phase extraction recoveries and relative standard deviations (RSD) ($n = 10$).

Compound	Recovery* (%)	RSD (%)
Gallic acid	94±1	4.2
Protocatechuic acid	95.1±0.7	2.6
Catechin	94±1	2.6
Vanillic acid	108±1.0	3.9
Epicatechin	105.3±0.8	3.2
Syringic acid	96.8±0.8	3.1
Chlorogenic acid	93.6±0.5	2.9
Gentisic acid	91.1±0.5	2.1
Caffeic acid	106.5±0.8	6.4
Coumaric acid	97±1.0	5.6
Ferulic acid	93.4±0.8	3.9
Rutin	112±1	3.4
Myricetin	89.4±0.6	3.0
Quercetin	87.3±0.6	4.5

* Means ± standard deviation of 10 measurements.

containing the following concentrations: 146.9 μmol L^{-1} for (+)-catechin; 117.6 μmol L^{-1} for gallic acid, 129.8 μmol L^{-1} for protocatechuic acid, 118.9 μmol L^{-1} for vanillic acid, 100.9 μmol L^{-1} for syringic acid, 129.8 μmol L^{-1} for gentisic acid, 32.7 μmol L^{-1} for rutin, and 73.4 μmol L^{-1} for (−)-epicatechin; 41.3 μmol L^{-1} for chlorogenic acid, 91.4 μmol L^{-1} for *p*-coumaric acid, 77.2 μmol L^{-1} for ferulic acid, 47.1 μmol L^{-1} for myricetin, 55.5 μmol L^{-1} for caffeic acid, and 33.1 μmol L^{-1} for quercetin. Relative standard deviation (RSD) values ranged from 2.1% to 6.4%. The accuracy was expressed as the recovery of the standards and was found to be in the range of 87.3–107.5%. RSD values and recoveries are shown in Table 1.

Preparation of P. tricornutum *diatom extracts for DPPH assay*—The algae were freeze-dried and 1 g of the dry material was extracted by stirring with methanol (25 mL) for 1 h. After centrifugation at 3500 rpm for 30 min, the supernatant was collected and evaporated. The dry residue (100 mg) was extracted for 1 h at room temperature with 3 mL of methanol by mixing with a magnetic stirrer. Each extract was centrifuged at 7500 rpm for 20 min, and the supernatant collected and filtered through 0.45 μm filter paper.

Free radical scavenging activity on DPPH—The reducing ability of antioxidants on DPPH radical was evaluated by measuring the loss of DPPH color at 515 nm after reaction with the test extracts (Bondet et al. 1997). The sample solution (100 μL) was rapidly mixed with 1 mL of a solution of 0.1 mmol L^{-1} DPPH. After 25 min incubation time in the dark at ambient temperature (23°C), the decline in absorbance (Abs) was measured against a methanol blank. The inhibition percentage values, expressed in terms of radical scavenging activity (RSA), were calculated by the equation:

$$\mathrm{RSA} = 100 \times [1 - (\text{Abs in the preence of sample}) / (\text{Abs in the absence of sample})] \quad (1)$$

Results

Effects of copper and iron on the growth of P. tricornutum—The cultures of *P. tricornutum* were produced at three metal concentrations in order to stimulate the production of exudates. To this effect, the diatom was harvested in seawater with f/2 nutrients (control seawater) as the reference growth (Fig. 1) in close concordance with recent studies (Vasconcelos and Leal 2008; González et al. 2012). At these conditions, the cell density increased from 2×10^7 cells L^{-1} to 5.9×10^8 cells L^{-1} after 8 d of culture. The slope for the exponential phase was 8.9×10^7 cells L^{-1} d^{-1}. The growth rate during the 8 d of culture was 7.6×10^7 cells L^{-1} d^{-1}.

The growth of *P. tricornutum* was also carried out in the presence of 315 nmol L^{-1} of Cu(II) (Fig. 1). The cell density increased to 4.7×10^8 cells L^{-1}, with a growth rate of 2.6×10^7 cells L^{-1} d^{-1} over the 8 d of culture, meaning that the *P. tricornutum* cell density decreased 20% with respect to the reference culture. In addition, during the initial 5 d of culture, the growth curve showed a linear relationship with time, where the slope was 2.26×10^7 cells L^{-1} d^{-1}. Between the sixth and eighth days of culture, the cell density reached a slope of 10.4×10^7 cells L^{-1} d^{-1}, comparable to the reference culture in the absence of copper.

The third culture of *P. tricornutum* was carried out under conditions of 790 nmol L^{-1} of Cu(II) (Fig. 1). This concentration allows for the determination of the polyphenolic profile under high copper stress. The growth curve showed that the cell density increased to 3.1×10^8 cells L^{-1}. The cell concentration decreased 47.5% with respect to the reference culture. In this experiment, a similar growth pattern was found as in the study at 315 nmol L^{-1} of Cu(II). The growth slope was only 0.53×10^7 cells L^{-1} d^{-1} through to the fifth day of culture and increased to 8.01×10^7 cells L^{-1} d^{-1} over the following days.

The iron effect was studied at 900 nmol L^{-1} of Fe(III) added to the culture of *P. tricornutum.* Under these conditions, the cell density reached a maximum concentration of 2.70×10^9 cells L^{-1}, one order of magnitude higher than the reference culture. The growth slope in the exponential phase was 46.9×10^7 cells L^{-1} d^{-1}, and the growth rate was 5.4×10^8 cells L^{-1} d^{-1} for the 8 d of culture (the cell density increased 471% with respect to the reference culture).

Polyphenolic profiles—The polyphenolic profile of *P. tricornutum* was determined both in the extracts of cells collected from the cultures and in seawater enriched with exudates. Tables 2 and 3 show all the polyphenols quantified in this work.

The proposed polyphenols were identified in the extracts of cells, except for gallic acid, which was only detected in the iron enrichment experiment. In addition, in the seawater enriched with exudates, only quercetin was not detected.

The phenolic compound content in cell extracts of *P. tricornutum* exposed to copper was strongly affected by the metal concentration (Table 2). As compared to the control,

Table 2. Phenolic compounds contents in extracts of diatom *Phaeodactylum tricornutum* exposed to high iron and copper concentrations.

Phenolic compound	Control	[Fe(III)] 900 nmol L^{-1}	[Cu(II)] 315 nmol L^{-1}	[Cu(II)] 790 nmol L^{-1}
Gallic acid*	nd†	43±4	nd	nd
Protocatechuic acid*	46.9±0.4	65±4	59±3	144±10
Catechin*	236±3	277±1	208±4	670±42
Vanillic acid*	53.8±0.8	59±4	83±6	95±2
Epicatechin*	123±5	129±6	174±13	234±15
Syringic acid*	65±4	76±3	67±2	118±3
Chlorogenic acid*	50±2	47±2	62±4	106±2
Gentisic acid*	138±4	74±5	174±8	278±10
Caffeic acid*	35±1	35.1±0.5	50±2	75±1
Coumaric acid*	38.2±0.2	38.1±0.6	60±2	81±2
Ferulic acid*	37.0±0.9	60±5	79±9	122±10
Rutin*	12.9±0.3	60±3	20±2	45±4
Myricetin*	56.1±0.5	209±9	119±9	136±6
Quercetin*	5.3±0.3	36±2	3.1±0.4	9.5±0.6
Sum‡	897±22	1208±49	1158±64	2114±108

* nmol of phenolic compound per cell × 10^{10} ± standard deviation of two measurements.
† nd, not detected.
‡ nmol of total phenolic compounds per cell × 10^{10} ± standard deviation of two measurements.

the amount of most of the identified phenolic compounds increased slightly when the copper concentration was 315 nmol L^{-1} (growth rate 80%). Significantly, the greatest amount (around double with respect to the control) of all the identified phenolic compounds was found when the diatom *P. tricornutum* was exposed to copper concentration of 790 nmol L^{-1} (Table 2). Similar findings were given in the seawater samples enriched with exudates, with double the sum of all the identified phenolic compounds exuded per cell in the culture enriched with a copper concentration of 790 nmol L^{-1} as opposed to those in the control (Table 3). The concentration of polyphenols increased in the culture seawater enriched with copper at concentration 790 nmol L^{-1} to 39 nmol L^{-1}.

In the iron enrichment experiments, the amount of the quantified phenolic compounds per cell increased under these conditions of high cell growth (471%). The sum of all the identified compounds was 1208 nmol of polyphenols, 1.3 times higher than the control values of 897 nmol of polyphenols (Table 2). In these experiments, myricetin and (+)-catechin were the predominant phenolic compounds, and gallic acid was detected only in the diatoms exposed to iron. The contents of polyphenols such as rutin, myricetin, and quercetin increased by 365%, 273%, and 579%, respectively, whereas in the copper enrichment experiment ([Cu(II)] = 790 nmol L^{-1}) the increases were inferior (249%, 142%, and 79%, respectively). However, gentisic acid decreased in the presence of iron and increased when

Table 3. Phenolic compounds contents in seawater enriched with exudates of diatom *P. tricornutum* under high iron and copper concentrations.

Phenolic compound	Control	[Fe(III)] 900 nmol L^{-1}	[Cu(II)] 315 nmol L^{-1}	[Cu(II)] 790 nmol L^{-1}
Gallic acid*	2.3±0.1	10.3±0.5	0.58±0.07	4.8±0.5
Protocatechuic acid*	23.2±0.6	5.9±0.5	19.7±0.9	43±2
Catechin*	125±11	11.6±0.1	112±4	283±15
Vanillic acid*	19±1	4.3±0.3	18.3±0.9	36±2
Epicatechin*	55.9±0.8	11.7±0.0	66±7	107±3
Syringic acid*	32.0±0.1	7.8±0.4	24±2	58±2
Chlorogenic acid*	18.2±0.7	3.5±0.0	24±2	35.2±0.8
Gentisic acid*	48.3±0.7	6.8±0.2	61±5	93±7
Caffeic acid*	11.4±0.1	2.4±0.2	16.4±0.8	21.4±0.6
Coumaric acid*	14.8±0.3	2.9±0.2	19.6±0.7	27.3±0.6
Ferulic acid*	21.0±0.4	4.1±0.2	27±3	41±4
Rutin*	9.0±0.3	6.5±0.6	11.4±0.6	16.8±0.6
Miricetin*	32.4±0.9	21±2	59±3	60±2
Quercetin*	nd†	5.5±0.2	2.2±0.2	1.9±0.1
Sum*	413±17	104±5	461±30	828±40
Sum‡	24±1	28±2	30±2	39±2

* nmol of phenolic compound per cell × 10^{10} ± standard deviation of two measurements.
† nd, not detected.
‡ nmol L^{-1} ± standard deviation of two measurements.

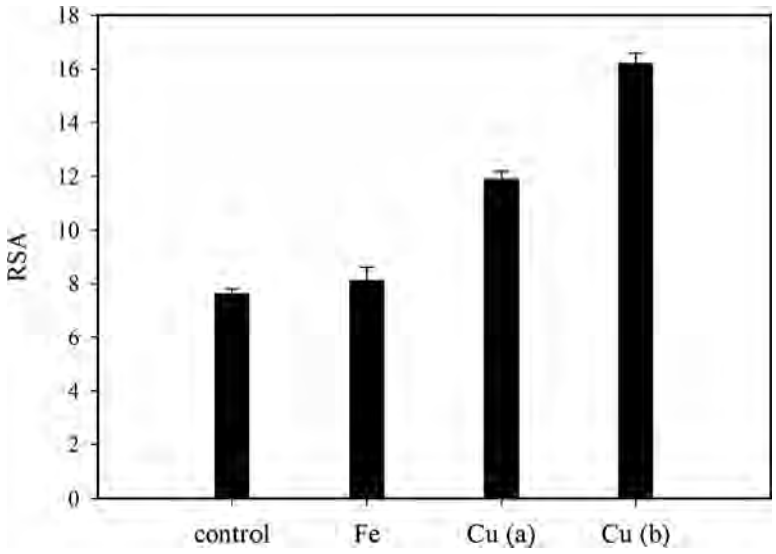

Fig. 2. Relative activity of the extracts derived from diatom *Phaeodactylum tricornutum* to scavenge the stable radical DPPH under different conditions: control seawater, [Fe(III)] = 900 nmol L^{-1}, Cu(a) was [Cu(II)] = 315 nmol L^{-1}, and Cu(b) was [Cu(II)] = 790 nmol L^{-1}. RSD values represented means of triplicate determinations ± standard deviation.

the diatom was exposed to copper, whereas the rest of the polyphenols showed major increases when the diatoms were exposed to a copper concentration of 790 nmol L^{-1}. Quantification of phenolic compounds in the seawater samples enriched with exudates from the iron enrichment experiments showed that the amount of single phenolic compounds exuded per cell declined dramatically as compared to the control, with the sum of all the identified compounds exuded per cell 4 times less than those in the control (Table 3). However, the concentration of the polyphenols in nmol L^{-1} increased in the culture seawater enriched with iron from 24 nmol L^{-1} (control) to 28 nmol L^{-1} due to the large number of cells.

Antioxidant activities of the diatom extracts—Figure 2 shows the relative antioxidant efficiency of the diatom extracts against the DPPH radical. Antioxidants suppressed the absorbance at 515 nm on a time scale dependent on the antioxidant activity of the extracts. As may be observed, the extracts derived from the diatom exposed to copper showed the highest radical scavenging activities (16.2% and 11.9%) followed by the extract derived from cells under iron enrichment conditions (8.1%) and the control (7.6%).

Discussion

Copper and iron stress affect growth and the phenolic contents of the marine diatom *P. tricornutum*, but not in the same way. The behavior of *P. tricornutum* under copper additions suggested that the diatoms metabolically produced exudates capable of complexing the copper present in the solution, thereby decreasing the toxic effect. Once the conditions were optimal, the diatom growth began. The effect of copper on diatom growth concords well with results published in previous reports with respect to the effect of copper toxicity on microalgae growth. Kagalou et al. (2002) reported that Cu(II) concentrations of 15.7 μmol L^{-1} had significant effects on the growth rate of the microalgae *Isochrysis galbana*, whereas lower concentrations (157 and 1570 nmol L^{-1}) produced less decline in algal growth. Franklin et al. (2002) concluded that the copper concentrations required to inhibit growth rate by 50% increased from 72.4 to 252 nmol L^{-1} for the tropical freshwater alga *Chlorella* sp. and from 104 to 268 nmol L^{-1} for the temperate species *Selenastrum capricornutum* as the initial cell density increased from 10^5 to 10^8 cells L^{-1}. The initial cell density is a very important parameter in toxicity tests carried out using microalgae. Moreno-Garrido et al. (2000) reported copper growth inhibition tests on four marine microalgal species including *P. tricornutum*. Diatoms were cultivated in artificial seawater enriched with a modification of f/2 medium, lacking ethylenediaminetetraacetic acid, which decreases the toxicity of heavy metals. In these conditions, Moreno-Garrido et al. (2000) observed EC_{50} values (representing the concentration of copper that caused a 50% reduction in the cell division rate compared with controls and calculated by semilogarithmic plotting of average inhibition data fits to straight line) for *P. tricornutum* of 154, 542, and 551 nmol L^{-1} for the initial cellular densities 10^6, 5×10^6, and 10^7 cells L^{-1}, respectively (after 72 h exposure to copper). Franklin et al. (2001) reported that copper had an inhibitory effect on the cell division rate of several algal species after 48 h and 72 h exposure to copper (initial cell density 2×10^7 to 4×10^7 cells L^{-1} and using filter-sterilized seawater supplemented with $[NO_3^-]$ = 242 μmol L^{-1} and $[PO_4^{3-}]$ = 15.8 μmol L^{-1} as a culture medium to maintain exponential growth over 72 h). In said study, the 48 h and 72 h EC_{50} values were 142 ± 47 and 158 ± 63 nmol L^{-1}, respectively, and complete growth inhibition was observed at 11.8 μmol L^{-1}. The cell light scatter properties of *P. tricornutum* depended on the cell size and intracellular granularity. These properties were found to be

a useful indicator of chronic copper toxicity to the marine alga *P. tricornutum*. Franklin et al. (2001) found that copper caused an increase in cell size after 24 h exposure, with 50% or more of the cells larger than the controls at 3.15 μmol L^{-1}, whereas similar increases in cell size were observed after 48 h and 72 h at 157.5 nmol L^{-1}, with an EC_{50} value of 126 $\pm$ 47 nmol L^{-1}. Similar changes in cell size and granularity were also reflected in changes in side-angle light scatter with an EC_{50} value of 189 nmol L^{-1} (after 48 h and 72 h exposure to copper). The diatoms *P. tricornutum* exposed to lower doses of copper in said study than those used by the present researchers at the same initial cell density, showed changes in cell size, granularity, and growth rate, considered to be indicators of copper toxicity.

P. tricornutum showed the highest growth rate at iron concentration 900 nmol L^{-1}. Iron is well known as a fertilizer and as an essential micronutrient participating in essential metabolism processes such as photosynthesis, respiration, and nitrogen fixation (Stumm and Morgan 1996). Morel et al. (2008) have demonstrated that unchelated Fe is highly available for uptake but that chelated Fe is necessary in order to explain the total uptake system. There is even evidence of direct internalization of siderophores by microorganisms using reductive mechanisms with varying degrees of efficiency (Hopkinson and Morel 2009). Our results align well with Kudo et al. (2000), who reported the combined effect of iron nutrition and temperature on growth (cell division) of the diatom *P. tricornutum*, concluding that the growth rate of cells cultivated with Fe(III) 2.0 μmol L^{-1} was twice as fast as the growth rate of diatoms cultivated with lower Fe(III) concentration (2.0 nmol L^{-1}) at 20°C.

In this study, increased phenolic compound content has been reported in *P. tricornutum* exposed to increasing doses of Cu(II). At the highest copper concentration (790 nmol L^{-1}), the phenolic content per cell was 2.4 times higher than in the control. In addition, the concentration of polyphenols increased in the culture seawater enriched with copper at concentration 790 nmol L^{-1} to 39 nmol L^{-1} (Table 3), despite the number of cells being much lower (52.5%), indicating that cells exposed to copper must excrete a larger amount of polyphenols. The diatom must make an extra metabolic effort in order to live in these levels of copper concentrations and must produce relevant amounts of polyphenols to slow down the toxicity of the copper in the solution, acting as a protective mechanism. This role has also been described for soil bacteria (*Pseudomonas aureofaciens*) (González et al. 2010) and plants that present mechanisms of detoxification, such as the exudation of phenols that act as chelate towards heavy metals to protect cells from induced oxidative damage (Jung et al. 2003). Increasing phenolic compounds may also contribute to enhancing the ability to cope with osmotic stress by binding metal ion to wall-associated polyphenols, countering metal toxicity at the cell surface (Suresh and Subramanyam 1998; Jung et al. 2003). Plant intracellular detoxification mechanisms may involve specific scavenging of reactive oxygen species through the action of enzymes and a wide array of metabolites, including phenolic components or direct chelate formation by polyphenols (Quideau et al. 2011). Wells et al. (2005) reported a synergistic link between the release of the iron-complexing ligand domoic acid and the limitation by iron and copper in toxigenic diatoms *Pseudo-nitzschia* spp. In said study, copper was found to be a key factor facilitating the success of these diatoms in iron-deficient waters, by inducing a high-affinity iron uptake. Iron limitation is the primary trigger for domoic acid production, which doubled under iron deficiency and increased by one order of magnitude under copper-limiting conditions. However, similar increases in domoic acid production were detected under conditions of toxic copper concentration, presumed to reduce copper toxicity.

The findings here evidenced increased phenolic compounds per cell under iron-rich media conditions ([Fe(III)] = 900 nmol L^{-1}) as compared to the control. However, the amounts of phenolic compounds exuded per cell decreased drastically, resulting four times lower than the control. This decline in excreted polyphenol concentrations per cell may have its explanation in the high cell densities in the culture that help to minimize the stress suffered per cell, making the metabolic effort needed to uptake iron to decrease.

P. tricornutum produced and exuded phenolic compounds mixtures with different phenolic profiles depending on the culture conditions. The extracts derived from the diatom exposed to copper showed the highest radical scavenging activities (16.2% and 11.9%). The phenolic profiles for these more active extracts were very similar. However, the diatoms exposed to iron showed different phenolic profiles that may vary the antioxidant potential. The antioxidant activity is not only dependent on the concentration of antioxidants, but also on the structure (Wright et al. 2001). The antioxidant activity is also dependent on the interaction among the antioxidants (phenolic compounds may act synergistically or antagonistically) (Jacobo-Velazquez and Cisneros-Zevallos 2009). Amoros et al. (1992) demonstrated the synergistic effect of binary flavone–flavonol combinations against herpes simplex virus type 1 in cell culture. Gao et al. (2011) reported that four phenolic compounds (vanillic acid, protocatechuic acid, ferulic acid, and caffeic acid) exerted additive and synergistic inhibition effects on the growth of *Microcystis aeruginosa* depending on the mix ratios. Tafesh et al. (2011) reported that hydroxytyrosol at 400 μg mL^{-1} caused growth inhibition of the four bacterial isolate gram-positive (*Streptococcus pyogenes* and *Staphylococcus aureus*) together with the gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*), whereas gallic acid at 200 and 400 μg mL^{-1} inhibited the growth of *S. aureus* and *S. pyogenes* strains, respectively (no growth inhibition was observed for the gram-negative bacteria). However, the combination of both compound gallic acid and hydroxytyrosol at lower concentrations (100 and 200 μg mL^{-1}, respectively) caused a complete inhibition of the four bacterial strains. In the present study, significant differences in the phenolic profiles were found in *P. tricornutum* diatoms growing under iron fertilization conditions and under copper stress. These differences seem to be the response to the different needs of the diatoms when grown under different stress conditions: as compared to the control, the amount of gentisic acid decreased when the culture seawater was enriched with iron

and increased when the seawater was enriched with copper; cells under iron enrichment conditions revealed a higher content of quercetin, myricetin, and rutin than the other diatom extracts and gave no change for (−)-epicatechin or chlorogenic acid, which increased in diatoms exposed to copper (790 nmol L^{-1}) (Table 2). As Gao et al. (2011) suggested, the profile of the phenolic compounds in a mixture and their mix ratios determine the joint action of the compounds, with either synergistic or additive effects. In addition, various different kinds of activities of the phenolic mixture may also depend on the mix ratios, where this mixture may be more active in complexing or reducing trace metals in solution. Therefore, further research is required to study the joint action of the phenolic compounds identified in cells exposed to multiple metal stresses and the influence of the mix ratio of said phenolic compounds on the kind and intensity of the mix activity.

Polyphenols have attracted a great deal of attention recently, mainly focused on their role in preventing diseases produced as a result of oxidative stress (Dillard and German 2000; Dai and Mumper 2010). Dietary antioxidants from plants are believed to help prevent aging and many degenerative diseases, such as cardiovascular complaints and cancers, as a result of their radical scavenging activity (Virgili et al. 2003; Quideau et al. 2011). Therefore, there is considerable interest in the field of preventive medicine in the development of natural antioxidants obtained from botanical sources. Seaweeds are considered to be a rich source of antioxidants, and recent studies have suggested using *microalgae* as "health" foods (Chacón-Lee and González-Mariño 2010). The results of this study confirm that *P. tricornutum* is a natural source of well-known antioxidant compounds (Silva et al. 2002) and afford more than sufficient arguments for researching the viability of the use of *P. tricornutum* diatom in the health and food industries in general, as well as in the pharmaceutical industry.

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LIMNOLOGY	20	2	Q1
OCEANOGRAPHY	59	5	Q1

Category Box Plot

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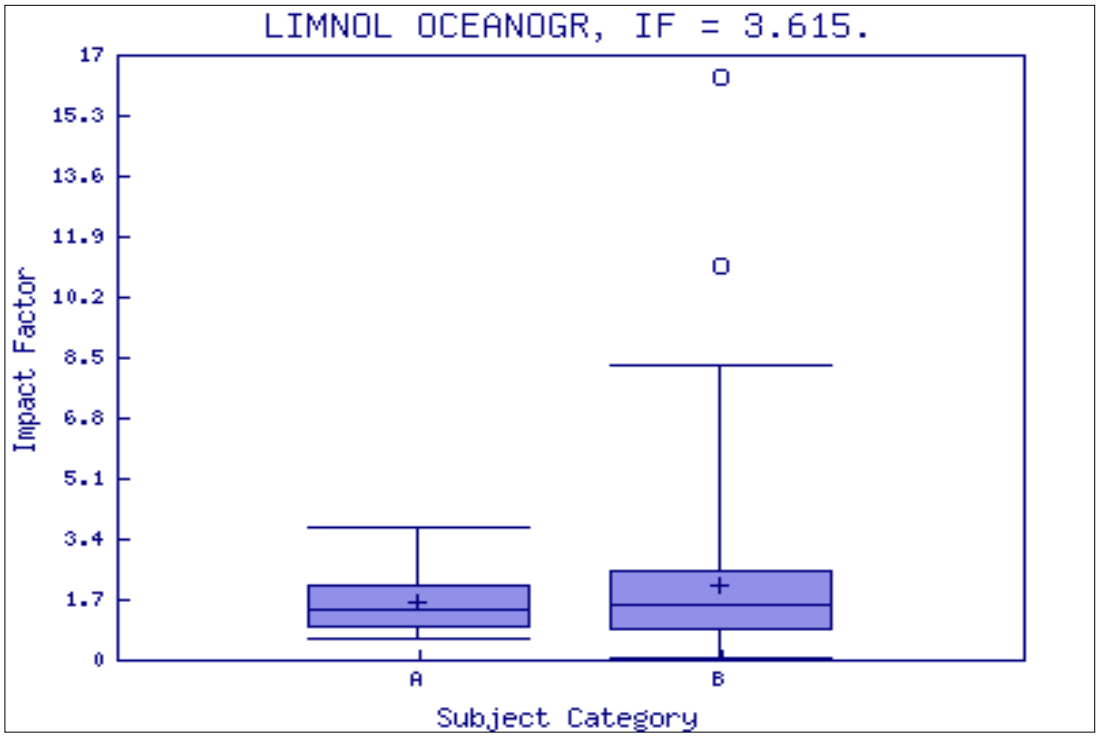
This is a box plot of the subject category or categories to which the journal has been assigned. It provides information about the distribution of journals based on Impact Factor values. It shows median, 25th and 75th percentiles, and the extreme values of the distribution.



Key

A - LIMNOLOGY
B - OCEANOGRAPHY

4.4. Diferencias entre las dos especies de microalgas

En la FIGURA 18 se muestran las densidades celulares alcanzadas por cada microalga en las diferentes condiciones de cultivo. Como se puede observar, la diatomea es menos tolerante a la presencia de cobre y, prácticamente, se llega a alcanzar la inhibición del crecimiento celular.

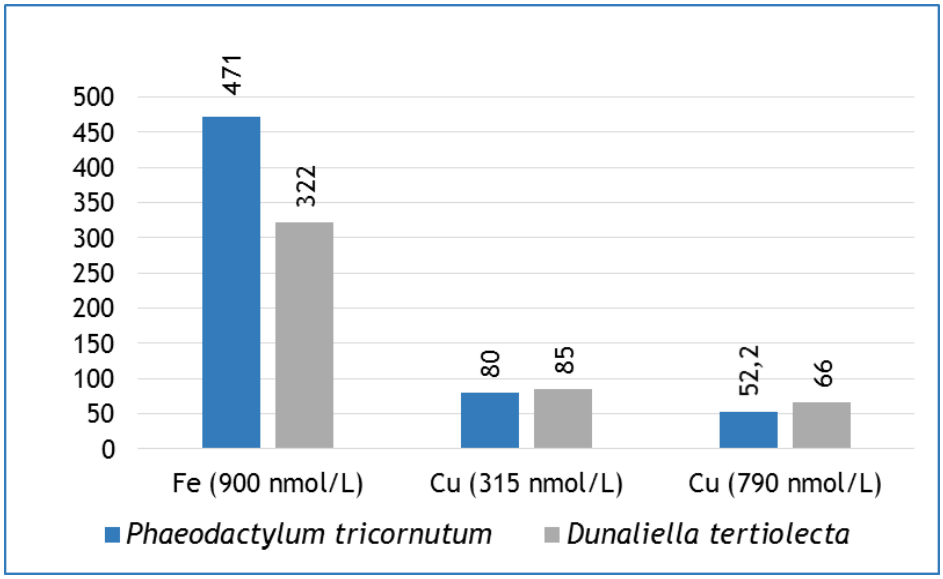


FIGURA 18.- Densidades celulares de los cultivos de microalgas en % con respecto al control

Las diferencias encontradas entre las dos especies de microalgas están de acuerdo con los resultados de los estudios de tolerancia llevados a cabo por Levy y colaboradores (2008), que han observado la adsorción e internalización de cobre de tres

especies de microalgas con diferentes tolerancias al cobre: *P. tricornutum*, *Tetraselmis sp* y *D. tertiolecta*.

La diatomea *P. tricornutum* se muestra particularmente sensible al cobre, con un IC50-72 h (concentración de cobre necesaria para inhibir la tasa de crecimiento un 50% después de 72 horas) de 126 nmol Cu(II) L^{-1}, muy inferior a la mostrada en nuestros trabajos, y en contraste con las algas verdes *Tetraselmis sp*. (IC50-72h de 740 nmol Cu(II) L^{-1}) y *D. tertiolecta* (IC50-72h de 8346 nmol Cu(II) L^{-1}). El cobre destruye la permeabilidad de la membrana celular de diatomeas *P. tricornutum*, lo que explica esta baja tolerancia observada por Levy y colaboradores (2008) y también en nuestros trabajos. A la concentración de 790 nmol L^{-1} de cobre, la diatomea presenta el doble de cobre en el interior de la célula que la microalga *D. tertiolecta* (TABLA 1).

Microalga	[Cu]IC50[a]	Cobre intracelular[b]	[Cu][c]
P. tricornutum	126	0,02	0,06
Tetraselmis sp	740	0,2	0,2
D. tertiolecta	8346	0,06	0,03

[a] Concentración de cobre necesaria para inhibir el crecimiento un 50% después de 72 horas en nmol L^{-1}
[b] Cobre intracelular para esas concentraciones IC50 en pg Cu $cell^{-1}$
[c] Cobre intracelular en pg Cu $cell^{-1}$ a la concentración de cobre externa de 790 nmol L^{-1}

TABLA 1.- Internalización de cobre y tolerancia de tres microalgas marinas [Levy y colaboradores, 2008]

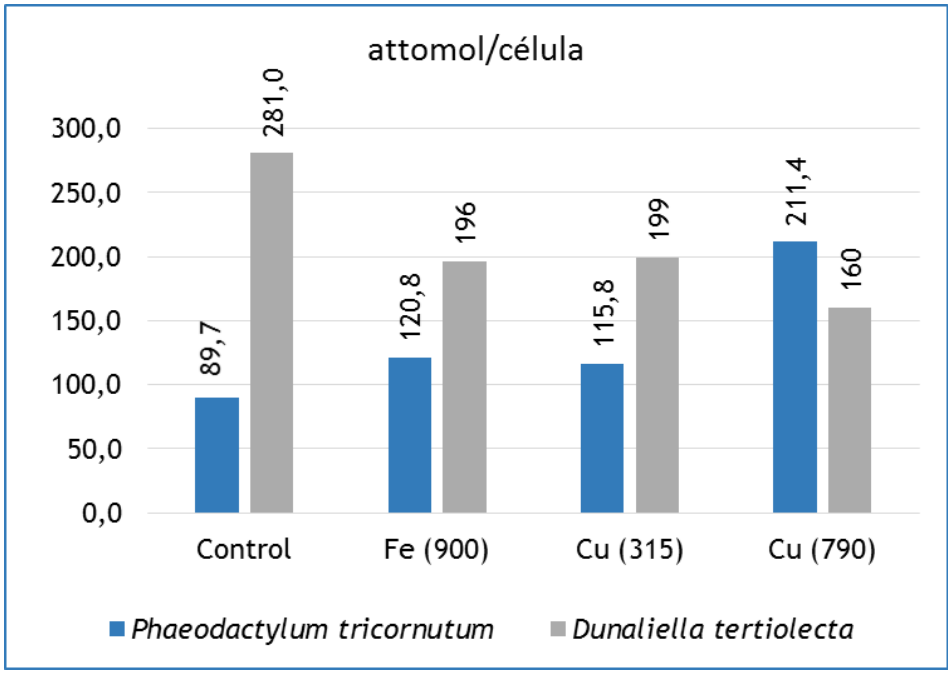


FIGURA 19.- Contenido total de compuestos fenólicos dentro de la célula

La diatomea no es capaz de excluir el cobre y debe producir cantidades relevantes de polifenoles para disminuir la toxicidad del metal tanto en el interior como en el exterior de la célula.

La microalga *D. tertiolecta* presenta menor tasa de internalización de cobre por lo que no es necesaria la producción de cantidades relevantes de polifenoles en el interior de la célula respecto al control como ocurre en la diatomea *P. tricornutum* (FIGURA 19). Además, Nikookar y colaboradores (2005) explicaron la alta tolerancia observada en la *D. tertiolecta* a través de otros mecanismos de detoxificación que implica ascorbato peroxidasa

(APX), que es una enzima involucrada en mecanismos de protección mediante la inhibición de especies reactivas de oxígeno.

La adsorción de metales en la superficie de la pared de la célula es un paso fundamental antes de la internalización y, por lo tanto, en la tolerancia de la célula al metal. González-Dávila y colaboradores (1995) realizaron la caracterización físico-química de la microalga *D. tertiolecta* cultivada en agua de mar enriquecida con cobre. Se encontraron tres grupos funcionales principales presentes en la superficie de las células: ácido carboxílico (pKa4,92±0,16), grupos fenólicos primarios (pKa10,06±0,09) y aminoácidos (pKa6,28±0,09). La presencia de grupos fenólicos sugiere mecanismos fisicoquímicos evolucionados por las células para contener la toxicidad metálica en la superficie de la célula impidiendo la entrada del cobre [Suresh y Subramanyan, 1998; Jung y colaboradores, 2003]. González Dávila y colaboradores (1995) también determinaron la constante de estabilidad para el complejo de Cu-exudado en solución, con un valor K_{cond} = 9,30±0,12.Esta constante corresponde con ligandos fuertemente unidos al cobre y está de acuerdo con la observaciones presentadas en nuestros trabajos, en los que ambas microalgas, *D. tertiolecta* y *P. tricornutum*, excretan mayor cantidad de polifenoles en el experimento realizado en presencia de 790 nmol L^{-1} de cobre que en el cultivo de referencia (FIGURA 20).

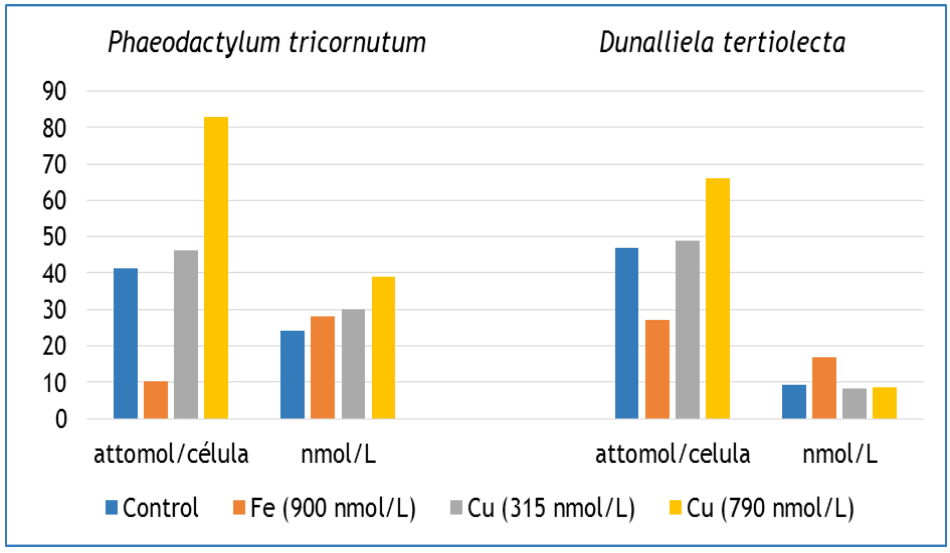


FIGURA 20. Compuestos fenólicos totales excretados por las células

4.5. Aplicación de la metodología desarrollada a extractos de distintos materiales vegetales terrestres y marinos

- López A (autora principal), Suárez de Tangil M, Vega-Orellana O, Ramírez AS, Rico M. Phenolic Constituents, Antioxidant and Preliminary Antimycoplasmic Activities of Leaf Skin and Flowers of Aloe vera (L.) Burm. f. (syn. A. barbadensis Mill.) from the Canary Islands (Spain). *Molecules* (2013) 18:4942-4954.

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Phenolic Constituents, Antioxidant and Preliminary Antimycoplasmic Activities of Leaf Skin and Flowers of *Aloe vera* (L.) Burm. f. (syn. *A. barbadensis* Mill.) from the Canary Islands (Spain)

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Abstract: The methanol extracts of leaf skins and flowers of *Aloe vera* from the Canary Islands were analyzed for their phenolic profiles and screened for their antioxidant and antimycoplasmic activities. The use of reversed phase high performance liquid chromatography (RP-HPLC) allowed the identification of 18 phenolic constituents. Leaf skin extracts were characterized by the abundance of catechin, sinapic acid and quercitrin. Gentisic acid, epicatechin and quercitrin were the most prominent phenolic compounds of the flowers. The *in vitro* antioxidant activities determined by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric antioxidant reducing power (FRAP) assays revealed that both extracts exhibited antioxidant activity, being the leaf skin extract the most active fraction. The leaf skin extract was also found to be active against the microbial strains tested. Therefore, *A. vera* extracts from leaf skin and flowers can be considered as good natural antioxidant sources.

Keywords: *Aloe vera*; leaf skin; phenolic constituents; antioxidant activity; antimycoplasmic activity

1. Introduction

The botanical name of *Aloe vera* is *Aloe barbadensis* Miller. It belongs to the Asphodelaceae (Liliaceae) family, and is a shrubby or arborescent, perennial, xerophytic, succulent pea-green colored plant [1]. The origins of these plants are the dry regions of Africa, Asia, and Southern Europe, especially in the Mediterranean regions. In the Canary Islands, *Aloe vera* plants naturally grow anywhere and everywhere and there is a considerable generally shared belief in the beneficial action of the gel among the population, estimated to be one of the few botanical medications in widespread domestic use. In folk medicine, the brown juice has been used traditionally for its purgative effects, and the fresh leaf gel in cosmetics and nutraceutical formulations [2]. Among the purported benefits of *Aloe vera* not supported by experimental or clinical data are the following: treatment of acne, haemorrhoids, psoriasis, anemia, glaucoma, petit ulcer, tuberculosis, blindness, seborrhoeic dermatitis, and fungal infections [2]. Several reports have demonstrated the antioxidant, antinociceptive and anti-inflammatory activities of the aloe species [3,4]. In addition, recent studies have shown the anti-cancer effect of aloe-emodin, an anthraquinone compound present in the leaves of *Aloe vera* [5]. Studies of the *in vitro* antimicrobial properties of the ethanolic extract of *Aloe vera* leaf gel revealed that it was active against most of the studied pathogenic bacteria and fungi, even at very low doses [6]. The list of different illnesses and conditions aided by the use of *Aloe vera* is indeed impressive, covering everything from burns and slight infections to extremely serious medical conditions. Several reviews have focused on the main scientific discoveries on *Aloe vera* reported over the last three decades [2,7–12]. These reviews deal with the botany, the chemical properties, the gel stabilization technique, the biological functions, and the current uses and applications of *Aloe vera* (mainly focusing on the exudate and gel of the *Aloe vera* leaves).

Plant polyphenols have been implicated in diverse functional roles, including plant resistance against microbial pathogens and animal herbivores such as insects (antibiotic and antifeeding actions), protection against solar radiation, besides reproduction, nutrition, and growth [13]. Phenolic compounds have also been reported to prevent diseases resulting from oxidative stress [14–16].

Screening of the phytochemical (qualitative and quantitative) analysis of the *Aloe vera* leaf (leaf skin and gel) showed that almost all of the chemical constituents are present: tannin, phlobatannins, saponin, flavonoids, steroids, terpenoids, and cardiac glycosides anthroquinones, which are used for medicinal purposes [17]. Phenolic compounds are the second major substances found in *Aloe vera*. The main active constituent of the *Aloe vera* plant extract is aloine, an anthraquinone heteroside [18]. Several papers have also been published that focus on the identification of the main phenolic compounds from the gel and leaf exudate of *Aloe*. Okamura *et al.* developed a procedure for determination of aloesin, 2'-*O*-feruloylaloesin, aloeresin A, barbaloin, isobarbaloin, aloenin, aloe-emodin, 8-C-glucosyl-7-*O*-methyl-(*S*)-aloesol, isoaloeresin D and aloeresin E, which are phenolic constituents of aloe [19]. Thirteen phenolic compounds from *Aloe barbadensis (syn. A. vera)* and *A. arborescens* were identified and quantified: aloesin, 8-*C*-glucosyl-7-*O*-methyl-(*S*)-aloesol, neoaloesin A, 8-*O*-methyl-7-hydroxyaloin A and B, 10-hydroxyaloin A, isoaloeresin D, aloin A and B, aloeresin E and aloe-emodin from *A. barbadensis;* and aloenin, aloenin B, 10-hydroxyaloin A, aloin A and B, and aloe-emodin from *A. arborescens* [20]. A mixture of phenolic compounds, mainly anthrones (aloenin, aloenin B, isobarbaloin, barbaloin and other aloin derivatives from *Aloe*

secundiflora (Aloeaceae) has been determined from the leaf exudate [21]. So far, little attention has been paid to the flowers or the leaf skin of the *Aloe*. Previous studies have suggested using *Aloe vera* flowers for phytotherapeutical purposes due to the presence of some polyphenols [22].

The aim of this study was to determine the differences in the phenolic profile of the methanol extracts derived from the leaf skin and flowers of *Aloe vera* (L.) Burm. f. (syn. *A. barbadensis* Mill.) from the Canary Islands to investigate the potential of the flowers and the leaf skin for uses in the health food industry, as well as in pharmaceuticals. As a result eighteen phenolic components were identified and quantified by reverse phase-high performance liquid chromatography (RP-HPLC). The antioxidant activities of the extracts were studied, as well as the preliminary *in vitro* susceptibility of some mycoplasma strains. Mollicutes, trivially known as mycoplasmas, are phylogenetically related to the Gram-positive branch of the eubacteria and can be divided into five phylogenetic groupings, including the *anaeroplasma, asteroleplasma, spiroplasma, pneumoniae*, and *hominis* groups [23]. Mycoplasmas are commensals or parasites on vertebrate, insect, and plant hosts, representing many significant pathogens in human and veterinary medicine. They are bacteria characterized by their lack of a cell wall and for their small genomes and highly structural and functional simplicity. Besides, they do not synthesize nucleotides or amino acids, express an unusual form of RNA polymerase, and certain species produce atypical ribosomes. All these characteristics make them naturally resistant to many antibiotics, reducing treatment options to tetracyclines, macrolides, and fluoroquinolones. Therefore, they represent magnificent targets for anti-microbe testing [24].

2. Results and Discussion

2.1. Determination of the Phenolic Profile by HPLC

The presence of polyphenols in the extracts was confirmed by comparing retention times (RT) and overlapping UV spectra with those of standard compounds. The phenolic compounds sinapic acid (RT: 6.9 min), quercitrin (RT: 7.6 min), kaempferol (RT: 10.8 min) and apigenin (RT: 11.3 min) were well resolved. Limits of detection (LOD) and limits of quantification (LOQ) were estimated from signal-to noise-ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10 respectively [25]. The LODs were found to be in the range of 0.032–0.127 $\mu g \cdot mL^{-1}$ and the LOQs were observed in the range of 0.106–0.355 $\mu g \cdot mL^{-1}$ (Table 1). This indicated that the proposed method offers adequate sensitivity for the quantification of polyphenols. Reproducibility, expressed as the relative standard deviation (RSD), was obtained by analysing six replicate samples containing 20 $\mu g \cdot mL^{-1}$ of each of the four compounds. The accuracy was expressed as the recovery of standard compounds added to the pre-analysed sample [26]. The results are summarized in Table 1. The phenolic compounds gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin and syringic acid, chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid, rutin, myricetin, and quercetin were quantified according to a previously reported method [27].

Table 1. Method validation data for the quantitative determination of four phenolic compounds using RP-HPLC.

Compounds	Regression equation (r)	LOD [a] μg·mL^{-1}	LOQ [a] μg·mL^{-1}	Recovery [b] (%)	RSD [c] (%)
Sinapic acid	y = 34493x − 25074 (0.9988)	0.10661	0.3554	118 ± 3	2.57
Quercitrin	y = 88302x − 20416 (0.9976)	0.03640	0.1235	116 ± 4	3.69
Kaempferol	y = 15436x − 28177 (0.9998)	0.09775	0.3258	105 ± 8	7.61
Apigenin	y = 20306x − 67494 (0.9993)	0.03196	0.1065	99 ± 3	2.66

[a] Detection limits are calculated as signal to noise ratio of ten times; [b] Means ± standard deviation of three measurements; [c] Reproducibility was obtained by analyzing six replicate samples containing 20 μg·mL^{-1} for every standard.

The proposed polyphenols were identified in the extracts, except for gallic acid, which was only detected in the flower extract. In addition, quercetin was only detected in the leaf skin extract. The results here evidenced that catechin, sinapic acid, gentisic acid and epicatechin were the most abundant compounds of those under study and their mix ratio changed depending on the source of the extract (aloe leaf skin or flowers). Catechin and sinapic acid were most abundant in the leaf skin extract and gentisic acid and epicatechin were predominant in the flower extract (Table 2).

Table 2. The polyphenol contents in aloe extracts presented as average values ± standard deviation of two measurements.

Phenolic compound	Leaf skin [a]	Flowers [a]
Sinapic acid	54 ± 3	15.0 ± 0.6
Quercitrin	23 ± 1	31.9 ± 0.5
Kaempferol	4.03 ± 0.03	2.86 ± 0.01
Apigenin	3.3 ± 0.4	3.03 ± 0.00
Gallic acid	nd [b]	12.6 ± 0.2
Protocatechuic	1.1 ± 0.0	0.57 ± 0.02
Catechin	95 ± 3	7.6 ± 0.2
Vanillic acid	2.30 ± 0.04	0.8 ± 0.1
Epicatechin	16.2 ± 0.7	58.0 ± 0.1
Syringic acid	4.9 ± 0.5	5.0 ± 0.3
Chlorogenic acid	7.8 ± 0.2	2.8 ± 0.2
Gentisic acid	6.0 ± 0.3	101 ± 2
Caffeic acid	4.9 ± 0.1	9.3 ± 0.1
Coumaric acid	0.8 ± 0.0	7.6 ± 0.4
Ferulic acid	7.9 ± 0.4	3.1 ± 0.1
Rutin	22.3 ± 2	11.6 ± 0.2
Miricetin	19.6 ± 0.7	1.76 ± 0.02
Quercetin	34.4 ± 2	nd [b]
Sum	307.5	274.5

[a] mg per 100 gram of freeze-dried aloe material; [b] nd indicates not detected.

The chemistry of the aloe plant has been studied for many years from a number of viewpoints [3–12]. Interest has centered on the parenchyma gel and its well-known therapeutic properties. Previous studies regarding the content of polyphenols in the leaf skin were not found. However, various phenolic compounds such as chlorogenic, caffeic, *p*-coumaric and ferulic acids were detected in the *Aloe* flowers [22]. In the present study, we detected and quantified eighteen phenolic compounds (Table 2), confirming that the *Aloe* leaf skin and flowers are natural sources of well-known antioxidant compounds [28].

2.2. Radical Scavenging Activity (RSA) on DPPH and Ferric Reducing Antioxidant Power (FRAP)

The reducing ability of antioxidants on the DPPH radical was evaluated by measuring the loss of DPPH color at 515 nm after reaction with the test samples. The leaf skin extract was more active to DPPH than the flower extract (Table 3). The FRAP assay was used to study the ability of the antioxidants in these extracts to reduce the ferric iron to a ferrous form. The redox reaction is carried out at pH 3.6. At more acidic conditions than the physiological pH, the reducing capacity may be suppressed due to protonation on the phenolics. However, the same behaviour as was observed in the DPPH assay was observed instead (the extract of the leaf skin was more active than the flower extract) (Table 3).

Table 3. Antioxidant and antimycoplasmic activities of the aloe extracts.

Extract	RSA [a,b]	FRAP [a,c]	Antimycoplasmal activity [a,d]			
			M. mycoides capri	*M. agalactiae*	*Acholeplasma laidlawii*	*M. gallisepticum*
Leaf skin	58.8 ± 0.4	2.4 ± 0.1	239 ± 51 [b]	CCM	2253 ± 123	1466 ± 213
Flowers	53 ± 2	1.7 ± 0.0	-	-	-	-

[a] Values represented mean ± standard deviation of three measurements; [b] % inhibition; [c] mmol of Fe (III) reduced to Fe(II); CCM: changes in the colony morphology around the disc - indicates no growth inhibition; [d] in micrometers.

In general, the literature reports that there is a relation between the content of phenolic compounds and the antioxidant properties [29]. Our results confirm the correlation between the phenolic content (calculated as the sum of the identified polyphenols) and the antioxidant activity (the extract with higher phenolic content gave the highest activities), allowing us to conclude that phenolic constituents are mainly responsible for the observed antioxidant activity in the extracts.

2.3. Antimycoplasmic Activity

The four mycoplasmas were selected as representatives of four of the five mycoplasma phylogenetic groups [23]. The group without representation is the *anaeroplasma* group, composed of anaerobic commensals found in ovine and bovine rumen. Also the three mycoplasmas included in the genus *Mycoplasma* are ruminant and poultry pathogens, while the mycoplasma from the genus *Acholeplasma* is a frequent contaminant of eukaryotic cell cultures. The results of the *in vitro* susceptibility test are given in Table 3. The concentration of the extracts is slightly higher than the ones used by Al-Momani *et al.* [30]. Antimycoplasmic activity was only found in the leaf skin extract (from simple changes in the colony morphology (CCM) as occurred in the case of *Mycoplasma agalactiae (M. agalactiae)* or in greater inhibition zones (*Acholeplasma laidlawii* and *Mycoplasma*

gallisepticum)). Further studies have to be undertaken to validate and extend these observations over a wider selection of bacteria and fungi in order to determine minimum inhibitory concentrations (MIC) of these extracts.

The extract from the flowers did not show antimycoplasmic activity against the bacterial strains tested in the present study, but might express strong properties against other test organism or other kind of properties depending on its composition in phenolic compounds. Rodríguez Vaquero *et al.* reported that bacterial species exhibited different sensitivities towards the different concentrations of pure phenolic compounds [31]. *Escherichia coli* was the most sensitive bacterium and *Flavobacterium sp*. was resistant against all phenolic compounds tested. The activity of the extracts is not only dependent on the concentration of the phenolic compounds but also on the structure and nature of the compounds [32]. Several reports have suggested that the biological activity of the extracts is also dependent on the interaction among the phenolic compounds [33,34]. Tafesh *et al.* [35] reported that hydroxytyrosol at 400 μg·mL^{-1} caused growth inhibition of the four bacterial isolate gram-positive (*Streptococcus pyogenes and Staphylococcus aureus*) together with the gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*), while gallic acid at 200 and 400 μg mL^{-1} inhibited the growth of the *S. aureus* and *S. pyogenes* strains, respectively (no growth inhibition was observed for the gram-negative bacteria). However, the combination of both compound gallic acid and hydroxytyrosol at lower concentrations (100 and 200 μg·mL^{-1}, respectively) caused a complete inhibition of the four bacterial strains. Gao *et al.* [36] reported that four phenolic compounds of those under study (vanillic acid, protocatechuic acid, ferulic acid and caffeic acid) exerted additive and synergistic inhibition effects on the growth of *Microcystis aeruginosa* depending on the mix ratios. In said study, the authors concluded that the profile of the phenolic compounds in a mixture and their mix ratios determine the joint action of the compounds, with either synergistic, antagonistic and/or additive effects. The efficacy of a combination of different phenolic compounds structures might be greater than that of other combinations on a kind of activity. Therefore, various different kinds of activities of a phenolic mixture may also depend on the mix ratios, where the mixture may be more active in contributing to UV-B protection or warding off microbial infection or protecting the plants from herbivores. The differences in the phenolic profile in the present work may be the result of the involvement of these compounds in different functional roles in the flowers from in the leaf skin. Further research is required to study the joint action of the phenolic compounds identified in the extracts and the influence of the mix ratio of said phenolic compounds on the kind and intensity of the mix activity.

3. Experimental

3.1. Chemicals

Methanol (of HPLC grade), ferric chloride ($FeCl_3 \cdot 6H_2O$), ferrous sulphate ($FeSO_4 \cdot 7H_2O$) and glacial acetic acid were obtained from Panreac (Barcelona, Spain) with formic acid and sodium acetate provided by Merck (Darmstadt, Germany) of analytical quality. The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) were from Sigma-Aldrich Chemie (Steinheim, Germany).

The antimicrobial activity of the plant extracts was tested using susceptibility test disks (Oxoid, CT0998B, Ø = 5 mm). Polyphenol standards of gallic acid, protocatechuic acid, chlorogenic acid, (−) epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+) catechin, sinapic acid, quercitrin, kaempferol and apigenin were purchased from Sigma-Aldrich Chemie; rutin and gentisic and caffeic acids were supplied by Merck (Hohenbrunn, Germany).

3.2. Mycoplasmas

Four types of strains of mollicutes: *Mycoplasma mycoides* subsp. Capri (Y-goat) (*M. mycoides* Capri), *Mycoplasma agalactiae* (PG2) (*M. agalactiae*), *Acholeplasma* (A.) *laidlawii* (PG8) (*A. laidlawii*) and *Mycoplasma gallisepticum* (PG31) (*M. gallisepticum*) were used in this study with the former two cultivated in PH medium [37] and the latter two in SP4-II medium [38]. The mycoplasmas were cultured under aerobic conditions at 37 °C.

3.3. Plant Material

The *Aloe vera* leaves and flowers were collected fresh in February 2010. The plant was identified in the Herbarium of the Viera y Clavijo Botanical Gardens in Gran Canaria where a voucher specimen was deposited (LPA: 27058-27060). A voucher specimen was deposited in the Herbarium of the Viera y Clavijo Botanical Gardens in Gran Canaria (LPA: 27058-27060). Soon after collection, the skin of the leaves and the flowers were separated, shaken and frozen. The leaf skin was separated and cleaned with a knife. The frozen samples were then freeze-dried and pulverized into powder using a blender (Moulinex, 600 W, Ecully Cedex, France) and were subsequently kept in the dark at −20 °C under nitrogen.

3.4. Preparation of Aloe Extracts

Freeze-dried plant material (10.0 g of leaf skin and 10.0 g of flowers separately) was extracted with solvent (175 mL) in a Soxhlet extractor for 3 h. The extraction was repeated four times, using the same plant material, but different solvents: hexane, acetone, ethanol and methanol were used consecutively. Several studies have reported that high levels of polyphenols may be associated with the use of polar solvents in the extraction [27,39]. Therefore, hexane, acetone and ethanol extracts were discarded. After extraction, 10 mL of methanol extract was reserved for the antioxidant activity assays.

To prepare the samples for the antimycoplasmic activity determination, the methanol extract was evaporated under reduced pressure to give semi-solid residues. In order to avoid problems related to the use of plant extracts, their solubility and the use of solubilising agents [40], the only product used for solubilising the residues from methanol extracts was ultra-pure sterile water. Working concentrations were 111 mg·mL^{-1} for flower residues and 112 mg·mL^{-1} for the leaf skin residues.

3.5. Free Radical Scavenging Activity on DPPH

The reducing ability of the antioxidants on the DPPH radical was evaluated by measuring the loss of 1,1-diphenyl-2-picrylhydrazyl (DPPH) colour at 515 nm after reaction with test extracts [41]. The sample solution (100 µL of extracts) was rapidly mixed with 1 mL of a solution of 0.2 mM DPPH. After 30 min incubation in darkness at ambient temperature (23 °C), the reduction of the DPPH radical

was measured by monitoring the decline in absorbance (Abs) against a methanol blank at 515 nm using a Shimadzu 1700 UV-Vis spectrophotometer. The percentage inhibition was calculated by application of the equation: RSA = 100 (1 − Abs in the presence of sample/Abs in the absence of sample).

3.6. Ferric Reducing Antioxidant Power Assay (FRAP Assay)

Reducing power was determined according to [42]. This method is based on the reduction of Fe^{3+} to Fe^{2+}, which is recorded by measuring the formation of a blue coloured Fe^{2+}-tripyridyltriazine compound from the colourless oxidized Fe^{3+} form via the action of electron-donating antioxidants. The FRAP reagent consists of 300 mM acetate buffer (3.1 g sodium acetate + 16 mL glacial acetic acid, made up to 1 litre with distilled water; pH = 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1.

The extract (50 μL) was added to 1.5 mL freshly prepared and pre-warmed (37 °C) FRAP reagent. The mixture was incubated at 37 °C for 10 min and the absorbance was measured against a reagent blank (1.5 mL FRAP reagent + 50 μL distilled water) at 593 nm. A standard curve of Fe(II) was constructed over the concentration range of 0.1 mM to 2.0 mM. The results were determined by the regression equation of the curve (Abs = 0.00221[Fe(II)] + 0.02464, r = 0.9998) and expressed as μmol ferric ions reduced to ferrous form.

3.7. Determination of the Phenolic Profile by RP-HPLC

To prepare the samples for the HPLC quantification, the freeze-dried plant material (50 mg) and 2 mL of methanol were mixed and homogenized using a vortex for 30 s. The mixture was stirred in a rotator (SB 3, Stuart, Staffordshire, UK) for 60 min at room temperature in darkness. After centrifugation at 7,000 ×*g* for 20 min at 4 °C, the supernatant was collected and evaporated. The dry residue and 0.5 mL of water were mixed and filtered through a 45 μm nylon syringe filter prior to injection.

Chromatographic analysis was performed on a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostat column compartment and a diode array detector (DAD), connected to ChemStation software. The separation was performed with a reverse-phase Pursuit XRs C18 (250 mm × 4.6 mm, 5 micrometers (μm)) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian, Barcelona, Spain). A gradient system, involving two mobile phases, was used. Eluent A was water with 0.1% formic acid and eluent B, methanol. The flow rate was 1.0 mL/min, and the injection volume was 60 μL of crude extracts (rheodyne injector). The system operated at 27 °C. The elution conditions applied were: 0–4 min, linear gradient from 20% to 30% B; 4–10 min, 30% B isocratic; 10–13 min, linear gradient from 30% to 50% B; 13–15 min, linear gradient from 50% to 80% B and finally, washing and re-conditioning of the column. Monitoring was set at 254 nm for quantification.

Method 1: to quantify the compounds sinapic acid, quercitrin, kaempferol and apigenin in the extracts, five different concentrations of the analytes were injected in triplicate. The calibration curves were constructed by plotting the peak areas *versus* the concentration of each analyte. The linearity was assessed by linear regression analysis, which was calculated by the least squares method. Each point on the calibration plot was the mean of two area measurements. All correlation coefficients were over 0.9976 (Table 2). The wavelength was fixed at 254 nm for quantification. The selectivity of the method was determined by analysis of standard compounds and samples. The peaks of polyphenols

were identified by comparing their retention times (RT) and overlaying of UV spectra with those of standard compounds.

Method 2: the phenolic compounds gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin and syringic acid, chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid, rutin, myricetin, and quercetin were quantified in line with a previously reported method [27]. Briefly, eluent A was Milli-Q water with 0.1% formic acid and eluent B was methanol. The elution conditions applied were: 0–5 min, 20% B isocratic; 5–30 min, linear gradient from 20% to 60% B; 30–35 min, 60% B isocratic; 35–40, linear gradient from 60% to 20% B and finally, washing and reconditioning of the column. Each standard was individually tested to determine its retention times (RT) as follows: gallic acid (RT: 5.3 min), protocatechuic (RT: 10.0 min), catechin (RT: 12.7 min), chlorogenic acid (RT: 14.9 min), gentisic acid (RT: 17.1 min), vanillic acid (RT: 17.7 min), epicatechin (RT: 17.9 min), caffeic acid (17.9 min), syringic acid (RT: 18.9 min), coumaric acid (RT: 23.4 min), rutin (RT: 28.1 min), ferulic acid (RT: 24.3 min), myricetin (RT: 30.6 min), and quercetin (RT: 34.6 min) were well resolved. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, (+) catechin, vanillic acid, (−) epicatechin and syringic acid), 324 nm (chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid), and 373 nm (rutin, myricetin, and quercetin) for quantification. The limits of detection (LOD) and limits of quantification (LOQ) were estimated from the signal-to noise-ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10, respectively. The LODs were found to be in the range of 0.0003–0.1230 $\mu g \cdot mL^{-1}$ and the LOQs were observed in the range of 0.0008–0.4100 $\mu g \cdot mL^{-1}$. This indicated that the proposed method was suitably sensitive for the quantification of polyphenols. The linearity was assessed by linear regression analysis, which was calculated by the least square method. Each point on the calibration plot was the mean from two area measurements. All the correlation coefficients were no less than 0.9982. Reproducibility, expressed as the relative standard deviation (RSD), was obtained by analyzing six replicate sample RSDs values ranging from 1.91% to 5.81%. The accuracy was expressed as the recovery of standard compounds added to the pre-analyzed sample. The recovery was found to be in the range of 87.97%–115.79%.

3.8. Evaluation of the Antimycoplasmic Activity

The antimycoplasmic activity of all plant extracts was determined using a modified disc diffusion method as described for growth inhibition tests elsewhere [43]. A total of 25 µL of each extract was tested in the disc diffusion assay against four strains of mycoplasmas. The plates were subsequently incubated and examined daily for colonies (1–2 days) under the conditions described above. Organisms were considered resistant when their growth was not inhibited by the 25 µL extract-impregnated wafers (5-mm sterilized filter paper discs). The presence of a zone of inhibition, as well as any changes in the colony morphology or in the colony concentration, was considered to be indicative of antimycoplasmic activity. The inhibition zones were measured in µm using an optical microscope Olympus CKX41 (Olympus, Hamburg, Germany), with a digital camera ProgRes C12 plus (Jenoptik, Jena, Germany) inserted, and using ProgRes® Image Capture Software for the measurements. Each antimycoplasmal assay was performed at least in triplicate and inhibition zones were measured at least three times per well, at perpendicular angles. Mean values and standard

deviations (SD) were registered and calculated as mean ± SD to the effects of this study. Filter discs impregnated with 25 μL of Tilmicosin (0.4 μg/mL) were used as positive control for antimicrobial activity and impregnated with 25 μL of distilled sterilised water were used as negative controls.

4. Conclusions

Phenolic compounds are active principles of medicinal plants and exhibit pharmacological effects that contribute towards human health. The presence of polyphenols in these extracts and their antioxidant and antimycoplasmic activities offer motivating results that suggest the potential feasibility of using *Aloe vera* leaf skin and flowers in the health food and general food industries, or as an ingredient in other products, as well as their possible applications in the pharmaceutical industry.

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Conflict of Interest

The authors declare no conflict of interest.

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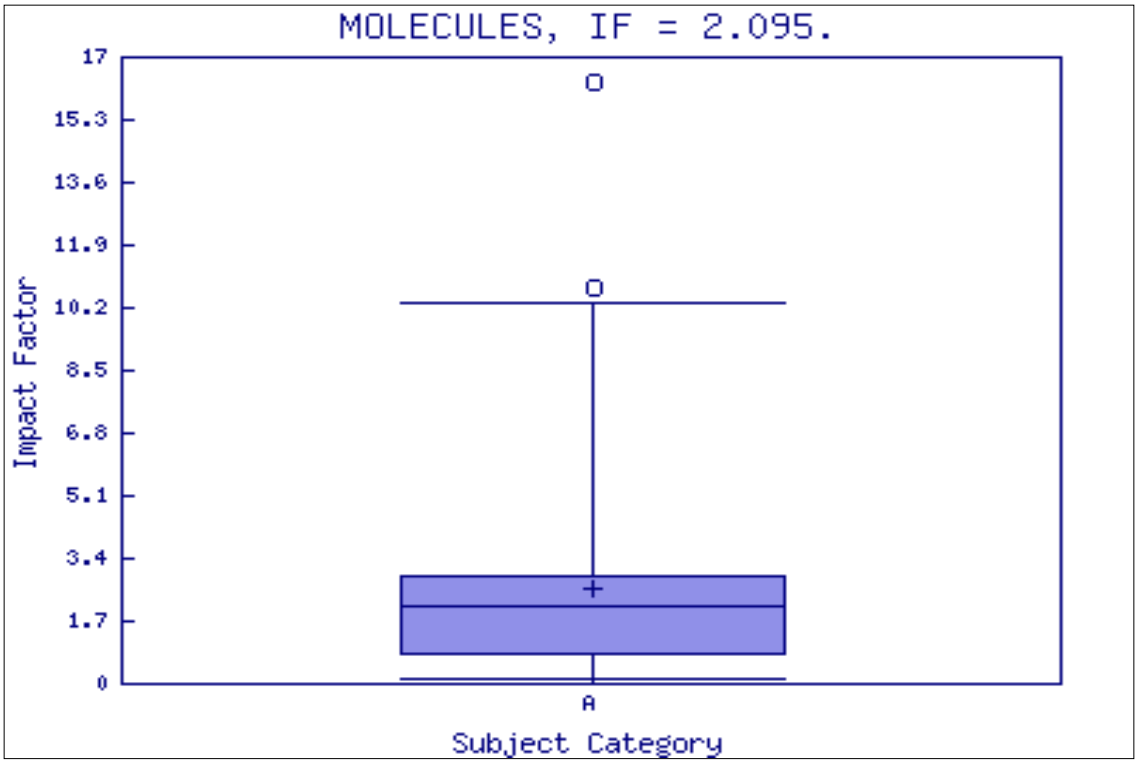

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Phenolic compounds, antioxidant activity and ultrastructural study from *Protea* hybrid 'Susara'

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ABSTRACT

In the present study we investigated the chemical composition and the phenolic contents of the ethanolic extract from aerial parts of *Protea* hybrid 'Susara', its antioxidant activity and also the structure and ultrastructure of the leaves and stems by light microscopy (LM) and transmission electron microscopy (TEM). Phytochemical research led to the isolation of twelve compounds including: (6-ethoxy-3,5-dihydroxy-4-oxotetrahydro-2H-pyran-2-yl)methyl 4-hydroxybenzoate (**1**), 4-ethoxy-2,3-dihydroxy-4-oxobutyl 4-hydroxybenzoate (**3**), 2-ethoxy-4-(hydroxymethyl)phenol (**4**) and 1,5-anhydro-D-glucitol (**5**). Moreover, fourteen phenolic compounds were identified and quantified by RP-HPLC, and the predominant was catechin, followed by gallic and syringic acids. Compound **4** showed the highest radical scavenging activity (98%) with a half-time ($t_{1/2}$) of 10 s. In TEM, the phenolic compounds were osmiophilic, appearing within the cell lumen of different tissues as isolated droplets of different sizes and fine granular material. Aerial parts presented thick cuticles and heavily lignified tissues with strong reinforcements of sclerenchyma, all indicating scleromorphic features. Our finding suggested that some agriculture waste biomass could be converted into high-added value products with a potential use within the food preservation, pharmaceutical, cosmetic and therapeutic industries.

1. Introduction

The angiosperm family Proteaceae is generally restricted to Gondwanic continental blocks and fragments mainly at the southern hemisphere. It constitutes about 80 genera and 1700 species that mainly include woody perennial shrubs or trees with compound inflorescences of tetramerous flowers that produce achenes, follicles or drupaceous fruits. The principal feature is the large and often very showy inflorescences, consisting of many small flowers densely packed into a compact head or spike (Weston, 2007). This family has a diverse leaf morphology (Johnson and Briggs, 1975) and anatomy (Carpenter, 1994) showing a range of specialized scleromorphic anatomical structures (Dillon, 2002).

The globalization of ornamental plants has been increasing during the last decade, the largest flower market in Europe Flora Holland reported that for 2010 their turnover was more than US$ 6.5 billion; 7% higher than the previous year, which is promising for the cut flower trade (Kras, 2011). One of the most noteworthy cultivars of the genus *Protea* is *P.* 'Susara' (*P. magnifica* × *P. susanne*), which has been introduced successfully in Portugal and Spain (Canary Islands) (Rodríguez-Pérez et al., 2009). The Proteaceae family plays an important role in the floristic trade and several aspects have been investigated that includes propagation, cultivation methods, post-harvest physiology, combating pests and diseases, etc. (Reinten et al., 2011). However, the study from phytochemical view of *Protea* genus are few; polyphenolic compounds (Perold and Carlton, 1989; Perold et al., 1979; Verotta et al., 1999), and carbohydrates (Boeyens et al., 1983) are the typical leaf constituents.

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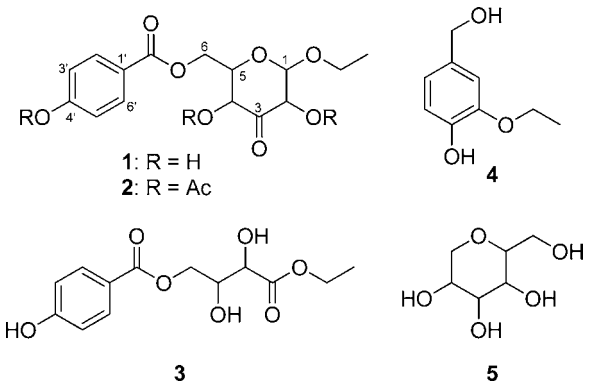

Fig. 1. Structure of compounds **1**–**5**.

The angiosperma dietary antioxidants from plants are believed to help prevent aging and many degenerative diseases, such as cardiovascular complaints and cancers, as a result of their radical scavenging activity (Quideau et al., 2011). Therefore, there is considerable interest in the field of preventive medicine in the development of natural antioxidants obtained from botanical sources. On the other hand, the most widely used synthetic food preservatives: butylated hydroxytoluene (BHT, E321) and butylated hydroxyanisole (BHA, E320) have been revised because of serious concerns about their carcinogenic potential (Ito et al., 1986; Kahl and Kappus, 1993). Toxic effects of BHA and BHT often occur only after high dosage and long-term treatment. In animals, BHA induces tumors of the fore stomach (EFSA, 2011), which are dose dependent, whereas BHT induces liver tumors in long-term experiments (EFSA, 2012). Because there is no indication of genotoxicity of BHA and BHT, all published findings agree with the fact that BHA and BHT are potential tumor promoters. Therefore, there is great interest in finding new and safe antioxidants from natural sources to replace the synthetics used in food preservation. Previous studies revealed that the addition of vegetal preparations to buffalo ghee was more effective in prolonging its stability than addition of BHA at the permitted level in ghee (Puravankara et al., 2000). On the other hand, Macaroni products incorporated with freeze-dried mango peel powder exhibited improved antioxidant properties. The content of polyphenols increased from 0.46 to 1.80 mg g^{-1} and carotenoid content increased from 5 to 84 μg g^{-1} with 7.5%. Incorporation of mango peel powder at this level did not affect its cooking, textural and sensory properties (Ajila et al., 2010).

The present work describes the isolation and structural elucidation of the constituents of the ethanolic extract of the aerial parts (see Section 2 and Fig. 1) of cultivars of the genus *Protea*, *P.* hybrid 'Susara' (Rodriguez-Pérez, 2007), along with the antioxidant activity of five of the isolated compounds. The antioxidant activity of the crude extract was also investigated and compared with those of the synthetic antioxidants BHT and BHA and the natural antioxidant α-tocopherol with regard to its potential uses. Furthermore, the morphological and ultrastructural studies show features occurring in leaves and stems of these plants and correlated with the presence of certain compounds in the cells of different tissues.

2. Materials and methods

2.1. Plant material

The plants of *Protea* hybrid 'Susara' (*P. magnifica* × *P. susanne*) (# 0312) were cultivated in experimental agricultural fields located at 564 m above sea level, belonging to the School of Agricultural Engineering (ETSIA), University of La Laguna, in northern Tenerife, Canary Islands, Spain. The cultivars were derived from vegetative propagation to assure their characteristics. The plant material was collected in March, 2012

2.2. Experimental procedures and chemicals

Optical rotations were recorded in a Perkin-Elmer model 343 polarimeter. Infrared (IR) spectra were recorded using a Bruker model IFS-55 spectrophotometer. Nuclear Magnetic Resonance (NMR): ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 and AMX-500 spectrometer with standard pulse sequences operating at 400 and 500 MHz in ^{1}H and 100 and 125 MHz in ^{13}C NMR respectively. $CDCl_3$ was used as solvent. Electron Impact Mass Spectrometer (EIMS) were taken on a Micromass model Autospec (70 eV) spectrometer. High Resolution Electrospray Ionization Mass Spectrometry (HRESIMS) was performed with a LCT Premier XE Micromass Waters spectrometer in positive ionization mode (Waters Corporation, USA). Column chromatography (CC) was carried out on silica gel 60 (230–400 mesh), and preparative TLC on silica gel 60 $PF_{254+366}$ plates (20 cm × 20 cm, 1 mm thickness) from Merck (Darmstadt, Germany). Methanol was of HPLC grade from Panreac (Barcelona, Spain). The Milli-Q water (18MQ, Millipore Milli-Q purification system, Billerica, MA, USA) was always used in this study. Formic and acetic acids and sodium acetate were purchased from Merck (Darmstadt, Germany) were analytical quality reagents. The chemical 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tri (2-pyridyl)-1,3,5-triazine (TPTZ) and polyphenol standards gallic acid, protocatechuic acid, chlorogenic acid, (−) epicatechin, quercetin, myricetin, ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, (+) catechin, α-tocopherol, butylated hydroxytoluene and butylated hydroxyanisol were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); rutin and gentisic and caffeic acids were supplied by Merck (Darmstadt, Germany). Ferric chloride ($FeCl_3{\cdot}6H_2O$), ferrous sulphate ($FeSO_4{\cdot}7H_2O$) and glacial acetic acid were obtained from Panreac (Barcelona, Spain).

2.3. Extraction and isolation

The aerial parts of *Protea* 'Susara' (456 g) were exhaustively extracted with 95% EtOH in a Soxhlet apparatus for 72 h. The solvent was concentrated under reduced pressure, and the 80 g extract was subsequently fractioned by silica gel column chromatography (CC) using hexane and ethyl acetate (EtOAc) mixtures of increasing polarity. The fractions eluted with hexane–EtOAc (3:2) were submitted to a sephadex LH-20 column using (hexane–dichloromethane–methanol, 1:1:1) to give stigmasterol (22 mg), β-sitosterol (16 mg), and *p*-hydroxy ethylbenzoate (8 mg). The fractions eluted with hexane–EtOAc (1:1) gave *p*-hydroxy acetophenone (12 mg), 2-ethoxy-4-(hydroxymethyl)phenol (**4**) (8 mg); *p*-hydroxy benzoic acid (100 mg) and kaempferol-3-methylether (22 mg). These compounds were isolated with CC using dicloromethane–acetone mixtures of increasing polarity and by successive preparative TLCs (benzene–EtOAc, 9.9:0.1, 2 elutions); dichloromethane–hexane 1:1, 3 elutions). The fractions eluted with hexane–EtOAc (2:3) were submitted to silica gel column using (dicloromethane–acetone, 1:1) to furnish (6-ethoxy-3,5-dihydroxy-4-oxotetrahydro-2H-pyran-2-yl)methyl 4-hydroxybenzoate (**1**) (32 mg), 4-ethoxy-2,3-dihydroxy-4-oxobutyl 4-hydroxybenzoate **3** (5 mg) and caffeic acid (18 mg). The fractions eluted with EtOAc gave, after recrystallization with methanol, hydroquinone (0.5 g) and 1,5-anhydro-D-glucitol (4.5 g).

*2.3.1. Compound (**1**): (6-ethoxy-3,5-dihydroxy-4-oxotetrahydro-2H-pyran-2-yl)methyl 4-hydroxybenzoate*

The following is a description of the chemical properties of compound (**1**). Amorphous solid; $[\alpha]_D^{25}$ +92 (0.003, Py); IR (KBr) ν_{max} 3382, 2877, 2925, 1736, 1701, 1630, 1606, 1517, 1445, 1374, 1276, 1165, 1117, 1070 cm^{-1}; ^1H NMR (500 MHz, MeOD-$CDCl_3$): δ = 7.84 (2H, d, J = 7.4 Hz, H-2′, H-6′), 6.78 (2H, d, J = 7.4 Hz, H-3′, H-5′), 5.15 (1H, d, J = 4.4 Hz, H-1), 4.60 (1H, dd, J = 2.4, 12.0 Hz, H-6a), 4.50 (1H, dd, J = 5.0, 12.0 Hz, H-6b), 4.38 (1H, dd, J = 1.2, 4.4 Hz, H-2), 4.24 (1H, dd, J = 1.6, 10.0 Hz, H-4), 3.92 (1H, ddd, J = 5.2, 7.4, 10.0 Hz, H-5), 3.49 (1H, dq, J = 6.8, 9.0 Hz, O-C$\underline{H_2}$CH$_3$), 3.68 (1H, dq, J = 6.8, 9.0 Hz, O-C$\underline{H_2}$CH$_3$), 1.12 (3H, t, J = 6.8, Hz, O-CH$_2$C$\underline{H_3}$); ^{13}C NMR (125 MHz, MeOD-$CDCl_3$): δ = 205.4 (C, C-3), 166.6 (C, $\underline{C}$OBz), 161.6 (C, C-4′), 131.8 (CH, C-2′, C-6′), 120.8 (C, C-1′), 115.2 (CH, C-3′, -5′), 100.8 (CH, C-1), 74.7 (CH, C-2), 73.2 (CH, C-5), 72.7 (CH, C-4), 64.3 (CH$_2$, O-$\underline{CH_2}$CH$_3$), 63.4 (CH$_2$, C-6), 14.5 (CH$_3$, O-CH$_2$$\underline{CH_3}$); HRESIMS (pos): 349.0898 $C_{15}H_{18}O_8Na$ (calcd. 349.0899).

*2.3.2. Acetylation of compound (**1**)*

A solution of **1** (8 mg) in a mixture of acetic anhydride (2 mL) and pyridine (1 mL) was allowed to stand at room temp. overnight. Pyridine was removed with a rotary evaporator and the remaining product was purified by TLC silica gel eluting with $CHCl_3$ to afford the acetate **2** (9.0 mg). Amorphous solid; $[\alpha]_D^{25}$ +81 (0.005, $CHCl_3$); IR (KBr) ν_{max} 2929, 2855, 1747, 1727, 1604, 1506, 1432, 1373, 1274, 1232, 1204, 1161, 1113, 1072, 1037, 1015, 913 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 8.00 (2H, d, J = 6.5 Hz, H-2′, H-6′), 7.13 (2H, d, J = 6.5 Hz, H-3′, H-5′), 5.36 (1H, d br, J = 4.2 Hz, H-2), 5.33 (1H, d br, J = 10.3 Hz, H-4), 5.24 (1H, d, J = 4.2 Hz, H-1), 4.58 (1H, dd, J = 2.8, 12.2 Hz, H-6a), 4.41 (1H, dd, J = 4.5, 12.2 Hz, H-6b), 4–30 (1H, ddd, J = 4.5, 6.8, 10.2 Hz, H-5), 3.72 (1H, dq, J = 7.0, 9.9 Hz, O-C$\underline{H_2}$CH$_3$), 3.55 (1H, dq, J = 7.0, 9.9 Hz, O-C$\underline{H_2}$CH$_3$), 2.25 (3H, s, OAc), 2.13 (3H, s, OAc), 2.10 (3H, s, OAc), 1.17 (3H, t, J = 6.8, Hz, O-CH$_2$C$\underline{H_3}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 193.1 (C, C-3), 169.5 (C, OAc), 168.9 (C, OAc), 168.8 (C, OAc), 165.3 (C, $\underline{C}$OBz), 154.7 (C, C-4′), 131.3 (CH, C-2′, C-6′), 127.0 (C, C-1′), 121.8 (CH, C-3′, -5′), 98.6 (CH, C-1), 74.7 (CH, C-2), 72.5 (CH, C-4), 69.7 (CH, C-5), 64.6 (CH$_2$, O-$\underline{CH_2}$CH$_3$), 62.9 (CH$_2$, C-6), 21.2 (CH$_3$, OAc), 20.4 (CH$_3$, OAc), 20.3 (CH$_3$, OAc), 14.7 (CH$_3$, O-CH$_2$$\underline{CH_3}$); HRESIMS (pos): 475.1207 $C_{21}H_{24}O_{11}Na$ (calcd. 475.1216).

*2.3.3. Compound (**3**): 4-ethoxy-2,3-dihydroxy-4-oxobutyl 4-hydroxybenzoate*

The following is a description of the chemical properties of compound (**3**). Amorphous solid; $[\alpha]_D^{25}$ +15 (0.0006, $CHCl_3$); IR (KBr) ν_{max} 3382, 2921, 2851, 1731, 1714, 1608, 1591, 1515, 1463, 1274, 1235, 1165 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.84 (2H, d, J = 8.7 Hz, H-2, H-6), 6.78 (2H, d, J = 8.7 Hz, H-3, H-5), 4.37 (2H, t br, J = 6.5 Hz, H$_2$-1′), 4.29 (1H, d, J = 4.0 Hz, H-3′), 4.21 (1H, dd, J = 6.0, 11.0 Hz, H-2′), 4.19–4.06 (2H, m, O-C$\underline{H_2}$CH$_3$), 1.23 (3H, t, J = 6.8, Hz, O-CH$_2$C$\underline{H_3}$); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.4 (C, C-4′), 166.7 (C, $\underline{C}$OBz), 161.8 (C, C-4), 131.9 (CH, C-2, C-6), 121.0 (C, C-1), 115.1 (CH, C-3, -5), 72.1 (CH, C-3′), 71.1 (CH, C-2′), 64.1 (CH, C-1′), 61.8 (CH$_2$, O-$\underline{CH_2}$CH$_3$), 13.9 (CH$_3$, O-CH$_2$$\underline{CH_3}$); HRESIMS (pos): 307.0792 $C_{13}H_{16}O_7Na$ (calcd. 307.0794).

2.4. Preparation of samples for antioxidant assays

The ethanolic extract residue was resolved in methanol by stirring at room temperature. The solutions were prepared at concentration 0.5 mg mL^{-1}. After centrifugation at 7000 rpm for 10 min, the supernatant was collected and stored at 4 °C. The pure compounds BHA, BHT, α-tocopherol, **1**, **2**, **3**, **4**, and **5** were made up to a concentration of 5 mg mL^{-1} (in methanol).

2.4.1. Free radical scavenging activity on DPPH

The reducing ability of antioxidants toward DPPH radical was evaluated by measuring the loss of DPPH color at 515 nm after reaction with test extracts (Bondet et al., 1997). The sample solution (30 μL) was rapidly mixed with 1 mL of a 0.1 mM DPPH solution. After 22 min incubation time in dark at ambient temperature (23 °C), the decline in absorbance against a methanol blank was recorded. The inhibition percentage values were calculated by equation: radical scavenging activity (RSA) = 100 (1 – Abs in the presence of sample/Abs in the absence of sample).

2.4.2. Ferric reducing antioxidant power assay (FRAP)

Reducing power was determined according to Benzie and Strain (1996). This method is based on the reduction of Fe^{3+} to Fe^{2+}, which is recorded by measuring the formation of a blue colored Fe^{2+}–tripyridyltriazine compound from the oxidized colorless Fe^{3+} form by electron donating antioxidants. The FRAP reagent consists of 300 mM acetate buffer (3.1 g sodium acetate + 16 mL glacial acetic acid, made up to 1 l with distilled water; pH = 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1.

Extract (10 μL) was added to 1.0 mL freshly prepared and pre-warmed (37 °C) FRAP reagent. The mixture was incubated at 37 °C for 10 min and the absorbance was measured against a reagent blank (1.0 mL FRAP reagent + 10 μL distilled water) at 593 nm. A standard curve of Fe^{2+} was constructed over the concentration range of 0.2–3 μM. The results were determined by the regression equation of the curve (y = 0.00221x + 0.0264, r = 0.9998) and expressed as μmol ferric ions reduced to ferrous form.

2.5. Determination of the phenolic profile by reverse phase High Performance Liquid Chromatography (RP-HPLC)

All HPLC analyses were performed on a Varian ProStar 210 system, equipped with a reverse phase (RP) Pursuit XRs C18 (250 mm × 4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian, Barcelona). A Waters LC Module I equipped with a UV detector 486 utilizing the Millennium 32 Chromatography Manager software (Waters, USA). Crude extract (2.5 mg) and 5 mL of methanol were mixed and homogenized using a vortex for 30 s. The mixture was stirred in a rotary shaker. After centrifugation at 7000 × g for 20 min at 4 °C, the supernatant was collected and filtered through a 45 μm nylon syringe filter prior to injection. Chromatographic analysis was performed on a Varian ProStar 210 system, equipped with a vacuum degasser, a binary pump, a thermostat column compartment and a diode array detector (DAD), connected to ChemStation software. The separation was performed with a reverse-phase Pursuit XRs C18 (250 mm × 4.6 mm, 5 μm) column and a Pursuit XRs C18 (10 mm × 4.6 mm, 5 μm) guard column (Varian). A gradient system, involving two mobile phases, was used. The flow rate was 1.0 mL/min, and the injection volume was 20 μL (rheodyne injector). The system operated at 27 °C. The phenolic compounds gallic acid, protocatechuic acid, catechin, vanillic acid, epicatechin and syringic acid, chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid, rutin, myricetin, and quercetin were quantified in line with a previously reported method (López et al., 2011; Rico et al., 2013). Briefly, eluent A was Milli-Q water with 0.1% formic acid and eluent B was methanol. The elution conditions applied were: 0–5 min, 20% B isocratic; 5–30 min, linear gradient from 20% to 60% B; 30–35 min, 60% B isocratic; 35–40 min, linear gradient from 60% to 20% B and finally, washing and reconditioning of the column. Each standard was individually tested to determine its retention times (RT) as follows: gallic acid (RT: 5.3 min), protocatechuic acid (RT: 10.0 min), catechin (RT: 12.7 min), chlorogenic acid (RT: 14.9 min), gentisic acid (RT: 17.1 min), vanillic acid (RT: 17.7 min), epicatechin (RT: 17.9 min), caffeic acid (17.9 min),

syringic acid (RT: 18.9 min), coumaric acid (RT: 23.4 min), rutin (RT: 28.1 min), ferulic acid (RT: 24.3 min), myricetin (RT: 30.6 min), and quercetin (RT: 34.6 min) were well resolved. Simultaneous monitoring was set at 270 nm (gallic acid, protocatechuic acid, (+) catechin, vanillic acid, (−) epicatechin and syringic acid), 324 nm (chlorogenic acid, gentisic acid, caffeic acid, *p*-coumaric acid and ferulic acid), and 373 nm (rutin, myricetin, and quercetin) for quantification. The limits of detection (LOD) and limits of quantification (LOQ) were estimated from the signal-to noise-ratio of the individual peaks, assuming a minimum detectable signal-to-noise level of 3 and 10, respectively. The LODs were found to be in the range of 0.0003–0.1230 μg mL^{-1} and the LOQs were observed in the range of 0.0008–0.4100 μg mL^{-1}. This indicated that the proposed method was suitably sensitive for the quantification of polyphenols. The linearity was assessed by linear regression analysis, which was calculated by the least square method. Each point on the calibration plot was the mean from two area measurements. All the correlation coefficients were no less than 0.9982. Reproducibility, expressed as the relative standard deviation (RSD), was obtained by analyzing six replicate sample RSDs values ranging from 1.91% to 5.81%. The accuracy was expressed as the recovery of standard compounds added to the pre-analyzed sample. The recovery was found to be in the range of 87.97–115.79%.

2.6. Methods for morphological and ultrastructural studies

The aerial vegetative parts, leaves and stems, were excised and processed for light microscopy (LM) and transmission electron microscopy (TEM) using standard protocols.

2.6.1. Light microscope (LM)

The material was fixed in FAA solution (formaldehyde 90%, absolute ethanol 5%, acetic acid 5%) for 24 h, transferred to 70% ethanol, dehydrated in an ethanol series (Johansen, 1940) and embedded in paraplast plus. Transverse sections (10–20 μm) were made using a microtome, mounted on slides and stained with safranin and fast green (Johansen, 1940). Semithin sections (1 μm) of resin-embedded material were obtained with glass knives and stained with toluidine blue. Observations were made with a LM Leica DM4000B using a Leica QWin computer image apprehension system.

2.6.2. Transmission electron microscopy (TEM)

For TEM observations, leaf and stem were cut into pieces 2 mm in length and fixed for 2 h in 3% glutaraldehyde and postfixed in 1% OsO_4, both in phosphate buffer (PB) 0.1 M. The samples were dehydrated in an alcohol series, transferred to acetone, and embedded in Spurr' resin (Spurr, 1969). Semi-thin (1 μm) and ultra-thin sections (70–90 nm) were cut using a Reichert-Jung ultramicrotome. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were studied using a Zeiss 902 or JEOL JEM-1010 microscope (Electron Microscopy Center, Madrid Complutense University, MCU).

3. Results

3.1. Identification of secondary metabolites

The constituents of this extract were purified by column chromatography (SiO_2, Sephadex LH-20) and preparative TLC. Thus, twelve compounds were isolated including (6-ethoxy-3,5-dihydroxy-4-oxotetrahydro-2H-pyran-2-yl)methyl 4-hydroxybenzoate (**1**), and 4-ethoxy-2,3-dihydroxy-4-oxobutyl 4-hydroxybenzoate (**3**), which are new in the literature together with the two sterols: stigmasterol, and β-sitosterol (Mock et al., 1973); six phenol derivatives: 2-ethoxy-4-(hydroxymethyl)phenol

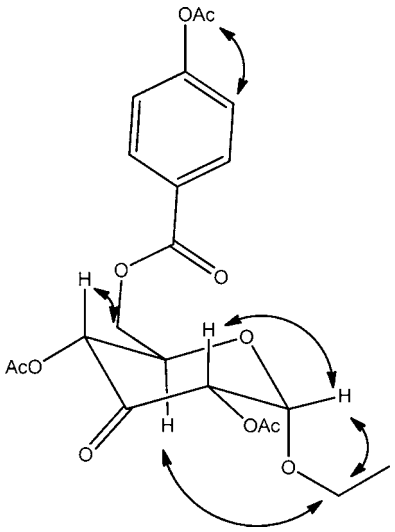

Fig. 2. Key NOESY correlations of **2**.

(**4**) (Collet et al., 1981), *p*-hydroxy ethylbenzoate (Schaefers and Herrmann, 1982), *p*-hydroxy acetophenone, *p*-hydroxy benzoic acid, hydroquinone and caffeic acid (van Rheede-van Oudtshoorn, 1963); a flavonoid: kaempferol-3-methylether (Gonzalez et al., 1995); and a deoxysugar: 1,5-anhydro-D-glucitol (**5**) (Boeyens et al., 1983). The structures of the known compounds were confirmed by comparison of their spectroscopic data (MS, ^1H and ^{13}C NMR) with literature references.

The HREIMS spectrum of **1** showed a molecular ion at *m/z* 349.0898 corresponding to the molecular formula $C_{15}H_{18}O_8Na$ (calcd. 349.0899). ^1H NMR spectra showed that compound **1** consisted of p-hydroxy benzoyl, an α-ketoglucopyranosyl and an ethoxy group residues. The assignments of ^1H and ^{13}C NMR are shown in materials and methods. The α configuration at the glucosyl bond was confirmed by the *J* value (4.4 Hz) at the anomeric position. The HMBC correlation of methylene of the ethoxyl group at 64.8 ppm to the glucopyranosyl anomeric proton at 5.15 ppm and the correlation of the benzyl carbonyl at 166.6 ppm to the proton at 4.50 and 4.60 ppm of the C-6 sugar revealed that the position of the linkage was the anomeric with the ethoxyl group and the C-6 with the *p*-hydroxy benzoyl group. The position of the keto group in the sugar was determined by COSY experiment; thus were observed CHCH and CH_2CHCH patterns, corresponding at H-1 and H-2; and H_2-6, H-5 and H-4 conjugations respectively. HMBC of the carbonyl signal at 205.4 ppm with the signals at H-1 and H-4, establish the position of the carbonyl group at C-3 in the sugar. The presence of three hydroxyl groups was confirmed by acetylation of **1** with acetic anhydride and pyridine yielding the triacetyl derivative **2**. The relative stereochemistry of **2** was determined by a NOESY experiment (Fig. 2) where the correlations were observed between the methylene belonging to the ethoxyl group and H-5; H-1 and H-2; H-4 and H_2-6, corroborating a α-ketoglucopyranosyl. Therefore, the structure of **1** was determined to be (6-ethoxy-3,5-dihydroxy-4-oxotetrahydro-2H-pyran-2-yl)methyl 4-hydroxybenzoate.

The ^{13}C NMR spectrum (see materials and methods) of the compound **3**, gave a total of 13 separate carbon resonances (one methyl, two methylenes, six methines, and four quaternary carbons), in agreement with the molecular formula $C_{13}H_{16}O_7$. The presence of two carbonyl groups was showed at 166.7 and 172.4 ppm and corresponded to the *p*-hydroxy benzoyl group and an ester group, respectively. The relationships between the proton signals in **3** were established from its spectrum, which disclosed the following connectivities: H_2-1′ to H-2′ and H-3′; and the methyl signal at 1.23 ppm with the methylene signal at 4.13 ppm corresponding

Table 1
Antioxidant activity of methanol solutions containing *Protea* 'Susara' crude extract and pure compounds at concentration of 0.5 mg mL^{-1}.

Assay	Crude extract	α-Tocopherol	BHA	BHT	1	2	3	4	5
DPPH[a]	41±2	84.7 ± 0.5	83.4 ± 0.3	33.3 ± 0.9	24.0 ± 0.3	8.02 ± 0.7	7.1 ± 0.2	98.0 ± 0.1	3.9 ± 0.9
$t_{1/2\,(seconds)}$	–	35	253	–	–	–	–	10	–
FRAP[b]	4.4 ± 0.1	6.0 ± 0.1	18.8 ± 0.5	3.1 ± 0.1	2.8 ± 0.1	1.4 ± 0.1	1.9 ± 0.0	19.8 ± 0.3	1.4 ± 0.1

[a] % inhibition ± standard deviation of three measurements.
[b] (μmol of Fe(III) reduced to Fe(II) ± standard deviation of three measurements) × 10.

to an ethoxyl group. The HMBC correlation of the ester carbon at 172.4 ppm to the methylenic protons at 4.09–4.16 ppm and the correlation of the benzyl carbonyl at 166.7 ppm to the protons at 4.37 ppm in C-1′ revealed the position of the linkage in the molecule. Thus, **3** was deduced to be 4-ethoxy-2,3-dihydroxy-4-oxobutyl 4-hydroxybenzoate.

3.2. Antioxidant activity

Table 1 shows the relative antioxidant efficiency of the crude extract against the DPPH radical and the ability of the antioxidants in the extract to reduce ferric iron to the ferrous form. As may be observed, the antioxidant activity of the crude extract of *Protea* 'Susara' was higher than that of the synthetic antioxidant BHT and lower than those of α-tocopherol and BHA at the same concentration (0.5 mg mL^{-1}). Of the five isolated compounds examined, **4** exhibited the highest antioxidant capacity (98%), higher than those of the traditional antioxidant standards α-tocopherol, BHA and BHT (84.7%, 83.4% and 33.3%, respectively). The compound **4** also showed the lowest half-life ($t_{1/2}$ = 10 s) defined as the time required to reach 50% residual color at the specified concentration of antioxidant, followed by α-tocopherol (35 s) and BHA (253 s) (Table 1). Similar results were found for the ferric reducing antioxidant assay where compound **4** was found to be the most active.

3.3. Determination of the phenolic profile by RP-HPLC

The presence of polyphenols in the extract was confirmed by comparing retention times (RT) and overlapping UV spectra with those of standard compounds. The extract showed the presence of all the fourteen RP-HPLC examined standards. Catechin was by far the predominant polyphenol followed by gallic and syringic acids (Table 2).

Table 2
Polyphenol content in *Protea* 'Susara' crude extract.

Phenolic compound	Phenolic content[a]	Phenolic content[b]
Gallic acid	326 ± 36	58 ± 6
Protocatechuic acid	33 ± 2	5.9 ± 0.4
Catechin	612 ± 17	110 ± 3
Vanillic acid	44 ± 4	7.8 ± 0.7
Epicatechin	221 ± 21	39 ± 4
Syringic acid	256 ± 6	46 ± 1
Chlorogenic acid	96 ± 12	17 ± 2
Gentisic acid	75 ± 7	13 ± 1
Caffeic acid	37 ± 2	6.6 ± 0.4
Coumaric acid	28 ± 1	5.1 ± 0.2
Ferulic acid	44 ± 1	7.9 ± 0.2
Rutin	123 ± 13	22 ± 2
Myricetin	149 ± 10	27 ± 2
Quercetin	47.3 ± 0.3	8.5 ± 0.1
Sum	2044 ± 132	374 ± 24

[a] Means mg per 100 g of dry ethanol residue ± standard deviation of two measurements.
[b] Means mg per 100 g of dry plant ± standard deviation of two measurements.

3.4. Morphological and ultrastructural features

Leaf. In cross-section the isolateral leaf showed a uni-layered epidermis, with slightly isodiametric cells, protected by a smooth and thick cuticle (Fig. 3A–C). It includes epidermal cells with thick and electron-dense tangential cell wall along with simple and enlarged trichomes on both sides. The leaves are amphistomatic (Fig. 3A) with kidney-shaped guard cells, that are slightly sunken and a large sub-stomatic chamber (Fig. 3B and C). The guard cells had a thick cuticle and the chloroplasts had poorly developed inner membranes. Both epidermises in paradermal view had paracitic stomata. The mesophyll had a distinct palisade and spongy parenchyma on both surfaces (Fig. 3A). The leaf had enlarged palisade parenchyma cells, chloroplasts surrounding the central vacuole with poorly developed tilacoidal system and occasional starch granules (Fig. 3D), phenolic droplets and fine granular material in the cell lumen (Fig. 3E–H) and plasmodesmata in the cell wall (Fig. 3E). Spongy parenchyma cells were round and showed different sizes, with thin cell walls compared to palisade parenchyma, chloroplasts and phenolic droplets in the lumen. Among the spongy parenchyma cells we observed stone cells of thick walls, devoid of (living) protoplast (Fig. 3A and B). The central ground tissue had sub-epidermal collenchyme on both surfaces. Collenchyme cells showed thickened corners, chloroplasts and phenolic droplets. The central-primary and secondary vascular bundles were surrounded by sclerenchyma in a cap-shaped manner (Fig. 3A). Sclerenchyma cells exhibited marked thickened and lignified walls with narrow central lumen. Both types of bundles showed collateral arrangement, with adaxial phloem (Fig. 3A) and the main vein with one or two rows of ray parenchyma with phenolic deposits in its lumen (Fig. 3I). Xylem tissue was wide in the main bundle (Fig. 3I). Xylem elements had different diameters and secondary cell wall thickening. Parenchyma cells associated to vascular bundles exhibited chloroplasts with lipid droplets in the stroma, mitochondria and sparse phenolic droplets (Fig. 3J). Vascular cambium consisted of 4–5 rows of cuboidal thin-walled cells (Fig. 3I). Phloem tissue showed thin-walled cells with chloroplasts and starch in the stroma. Phloem parenchyma cells were filled with fine granular material and phenols, which sometimes occupied the entire lumen (Fig. 3K). *Stem*. In cross-section the stem exhibited concentric tissue arrangement (Fig. 4A). It includes a uni-layered epidermis of papillose cells protected by a smooth and thick cuticle (Fig. 4B and C). Phenolic deposits were absent in the cell lumen. It also consists of glandular peltate trichomes, with unicellular stalk and head (Fig. 4B) and a sub-epidermal collenchyme consisting of 2–3 layers of thick walled cells at the corners (Fig. 4B–D). Their cytoplasm contained phenolic deposits as sparse droplets (Fig. 4D). Lenticular chloroplasts appeared surrounding the vacuole, close to the cell membrane and showed a poorly developed membrane system. Parenchymatic cortex consisted of a variable number (15–20) of cell layers and scarce intercellular spaces (Fig. 4A and B). Parenchyma cells showed plasmodesmata in the cell wall and phenol droplets in its cytoplasm (Fig. 4E). Among parenchymatic cortex cells appeared stone cells, alone or in groups (1, 2–8) of different number with thickened walls which at times occlude the entire lumen (Fig. 4B and F). Other stone cells contained phenolic

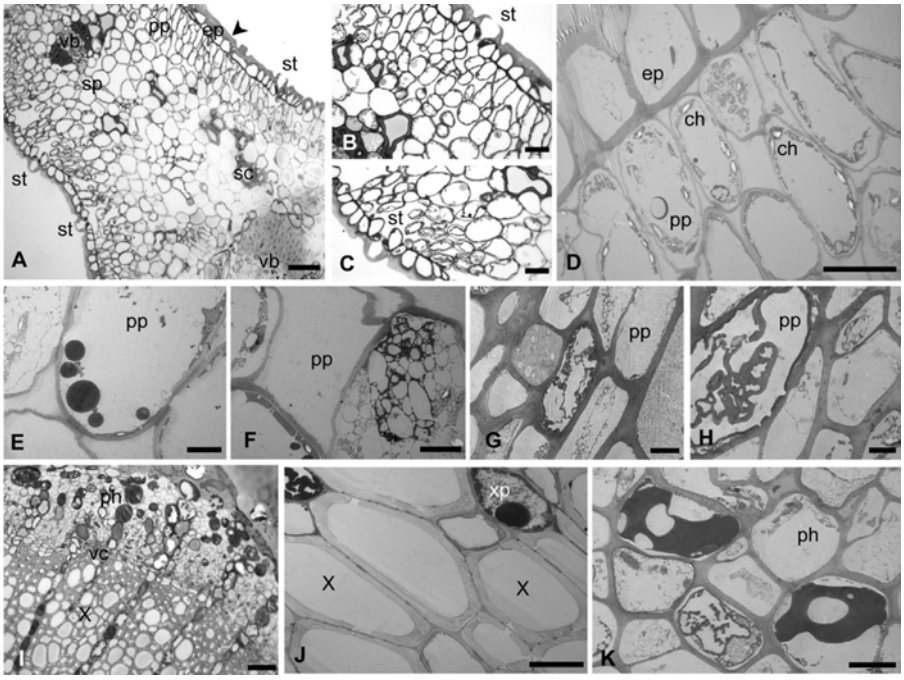

Fig. 3. Light and electron microscopy images of leaf anatomy in *Protea* 'Susara'. Leaf cross-section. (A) Unilayered epidermis with smooth cuticle (arrowhead) and stomata in adaxial and abaxial sides, mesophyll with palisade and spongy parenchyma and vascular bundles with sclerenchyma. (B) Adaxial epidermal cells and slightly sunken stomata. Guard cells with marked cuticle. Parenchymatic cells with phenolic inclusions. (C) Abaxial epidermal cells and slightly sunken stomata with large substomatal chamber. Guard cells with marked cuticle. Parenchymatic cells bearing phenolic inclusions. (D) Epidermal cells with thick outer tangential wall. Palisade parenchyma cells with chloroplasts and starch. (E–H) Parenchymatic cells with phenolic deposits, (E) parenchymatic cell walls show plasmodesmata. (I) Main vascular bundle. Collateral arrangement, centrifuge phloem and centripetal xylem with respect to the vascular cambium. Phenolic deposits in the vascular parenchyma. (J) Detail of xylem elements with thickened walls and xylem parenchyma with phenolic deposits. (K) Detail of phloem elements with phenolic deposits which sometimes occupy the cell lumen. (A)–(C) and (I) (scale bar = 25 μm); (D) (scale bar = 20 μm); (J) (scale bar = 10 μm); (E), (F) and (K) (scale bar = 5 μm); (G) and (H) (scale bar = 2 μm). ep, epidermis; pp, palisade parenchyma; sp, sponge parenchyma; sc, sclerenchyma; st, stomata; vb, vascular bundle; ch, chloroplast; ph, phloem; vc, vascular cambium; X, xylem; Xp, xylem parenchyma.

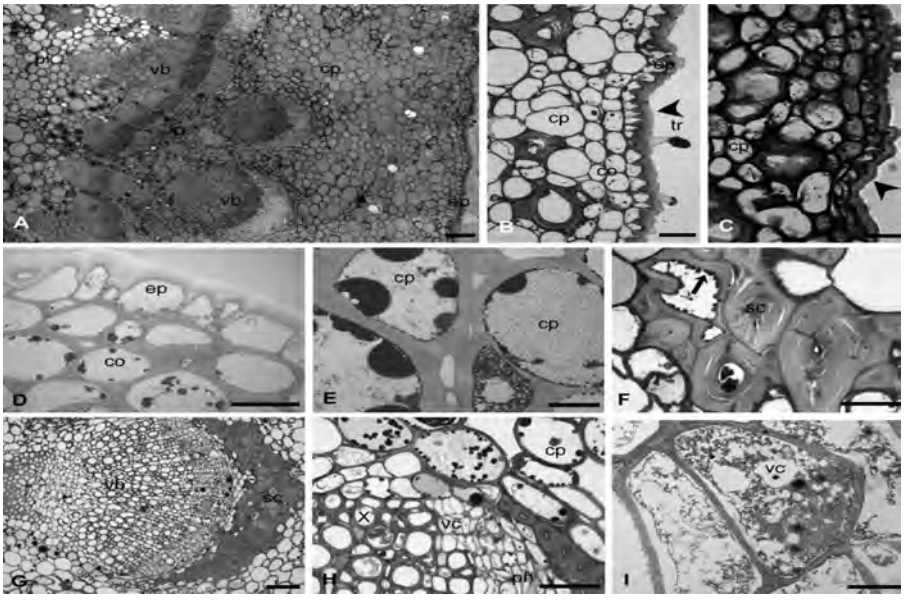

Fig. 4. Light and electron microscopy images of stem anatomy in *Protea* 'Susara'. Stem cross-section. (A) Unilayered epidermis with smooth cuticle, cortical parenchyma with stone cells and vascular bundles with cap-shaped sclerenchyma. (B) Epidermal cells with thick cuticle (arrowhead) and glandular trichomes. Subepidermal angular collenchyma and stone cells in the cortex. (C) Epidermis with safranin-stained cuticle (arrowhead). (D) Epidermal cells with thick outer tangential wall and angular collenchyma cells showing thickened wall and phenolic deposits arranged as sparse droplets. (E) Cortical parenchyma cells with phenolic deposits as sparse droplets and fine granules. (F) Stone cells with markedly thickened walls occluding the cell lumen and occasional phenolic deposits beneath the cell wall (arrow). (G) Collateral vascular bundle with perivascular sclerenchyma fibers. (H) Centrifuge phloem and centripetal xylem with respect to the vascular cambium. Phenolic deposits in parenchyma surrounding vascular bundles. (I) Detail of vascular cambium cells filled with fine granular material. (A)–(C), (F), (G) and (H) (scale bar = 25 μm); (D) (scale bar = 20 μm); (E) (scale bar = 5 μm); (I) (scale bar = 2 μm). cp, cortical parenchyma; ep, epidermis; ip, interfascicular parenchyma; pi, pith; vb, vascular bundle; co, collenchyma; sc, sclerenchyma; tr, trichome; ph, phloem; vc, vascular cambium; X, xylem.

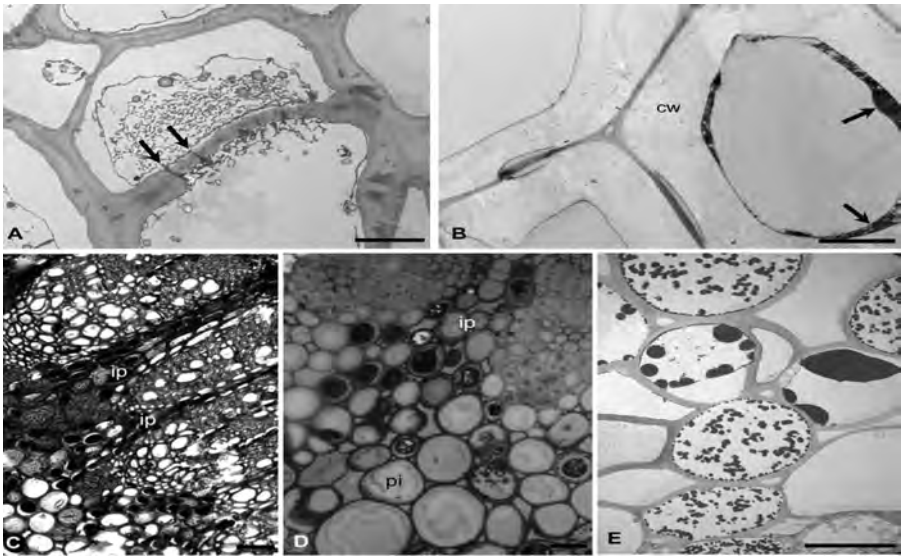

Fig. 5. Light and electron microscopy images of stem anatomy in *Protea* 'Susara'. Stem cross-section. (A) Detail of phloem elements with plasmodesmata in the cell wall (arrows), membranous debris and lipid inclusions. (B) Detail of thickened xylem wall and phenolic deposits beneath the cell wall (arrows). (C) Stem in transversal section stained with safranin-fast green. Interfascicular parenchyma and pith with phenolic compounds. (D) Large medullar parenchyma cells bearing osmiophilic deposits. (E) Detail of phenolic deposits into pith cells. (C) and (D) (scale bar = 25 μm); (E) (scale bar = 20 μm); (A) and (B) (scale bar = 2 μm). cw, cell wall; ip, interfascicular parenchyma; pi, pith.

deposits apposed to the plasma membrane (Fig. 4F). Perivascular sclerenchyma fibers showed markedly thick cell walls (Fig. 4A and G). Collateral vascular bundles with secondary growth surround an extensive parenchymatic pith and were separated by 4–5 rows of interfascicular parenchyma (Figs. 4A and 5C). The centrifuge phloem is in respect to the vascular cambium and xylem, in the inner cambium (Fig. 4G–H). The 4–5 layers of vascular cambium were evident (Fig. 4H). Cambium cells showed thin walls and granular osmiophilic material (Fig. 4I). Phloem walls contained plasmodesmata and cytoplams with granular material and lipid inclusions (Fig. 5A). Phloem parenchyma cells showed phenolic droplets. There were xylem elements of various sizes, with thickened walls and occasional phenolic deposits closely apposed to the tangential inner wall (Figs. 4G–H and 5B). The central pith contained round-shaped cells with scarce intercellular spaces and phenolic deposits in the lumen (Fig. 5C–E), similar to the interfascicular parenchymal cells (Fig. 5C and D).

4. Discussion

The presence of the isolated compounds in *Protea* 'Susara' are in agreement with literature (Boeyens et al., 1983; Perold and Carlton, 1989; Perold et al., 1979; Verotta et al., 1999). Although the compounds **1** and **3** are new, they can be considered artifacts presumably generated in the extraction of the plant with ethanol, through a direct nucleophilic substitution of most label hydroxyl group at high temperatures, besides we could not isolate their biogenetic precursors. The high content of the 1,5-anhydro-D-glucitol around 5.6% of the total amount of the extract make attractive the culture of *P.* 'Susara, not only as a crop, also as source of this important sugar (Kato et al., 2013). The plant extract exerted more potent RSA and FRAP values than the synthetic antioxidant BHT (RSA: 41% and 33.3% inhibition rate, respectively; FRAP values: 4.4 and 3.1 μmol of Fe(III) reduced to Fe(II), respectively). This latter is a pure compound and the extract is a complex mixture with active components being present at lower levels. Moreover, the crude extracts tend to have more interfering substances that may interact with the antioxidants decreasing the effectiveness of the crude extract. Ganesan et al. (2008) reported that several solvent fractions obtained from the crude methanol extract exhibited higher antioxidant activities as compared to the crude extract evaluated for antioxidant activity of three selected Indian red seaweeds. Among the pure compounds tested, **4** showed the highest activity (RSA: 98.0% and FRAP: 19.8), even much higher than those of the synthetic antioxidants BHA and BHT (RSA: 83.4% and 33.3, respectively; FRAP values: 18.8 and 3.1, respectively). These results are in full agreement with earlier reports where the highest antioxidant potency has been found for orthodiphenols such as compound **4** (Rico et al., 2013). The antioxidant activity of polyphenols and their derivatives depend on the number and position of hydroxyl groups bound to the aromatic ring, the binding sites and mutual position of hydroxyl groups in the aromatic ring, and the type of substituent (Rico et al., 2013; Rice-Evans et al., 1996). The antioxidant activities found in this study indicated that **4**, as well as the extract, are ideal for use in the health food industry, mainly as preservatives. In addition, compound **4** may be considered to have diverse potential therapeutic uses.

Polyphenols have attracted a great deal of attention recently, mainly focused on their role in preventing diseases produced as a result of oxidative stress (Dai and Mumper, 2010). The results here evidenced that catechin, syringic and gallic acids and epicatechin were the most abundant compounds of those under RP-HPLC study. Rababaha et al. (2011) determined the phenolic constituents of 10 different common edible plant species found in the Mediterranean region using HPLC. As a general rule, Rababaha et al. (2011) reported lower amounts of phenolic compounds for each analyzed plant species as compared to our results. In said study, the highest amount of gallic acid, syringic acid and epicatechin were 37.8, 3.3 and 16.2 mg per 100 g of dry plant respectively, much lower values than ours in the present work (Table 2). Therefore, *Protea* 'Susara' may be considered as a natural source of well-known antioxidant compounds (Cuvelier et al., 1992; Silva et al., 2002).

In plants, these phenolic compounds might contribute as photoprotective aid due to their antioxidant properties. They absorb radiation between 280 and 360 nm, and reduce the deleterious effect of the ultraviolet radiation on different cellular components (Caldwell et al., 1983; Landry et al., 1995; Rozema et al., 2002). Additionally, the cuticles protect the internal tissues acting as barriers against UV (Carpenter et al., 2007), pathogens and herbivores (Kunst and Samuels, 2009). Besides, the scleromorphic

characteristics should be related to maximizing leaf protection against solar radiation (Jordan et al., 2005).

In conclusion, the presence of polyphenols in the extract and their antioxidant activity offer motivating results that suggest the potential feasibility of using *Protea* 'Susara' in the health food and general food industries or as an ingredient in other products, as well as their possible applications in the pharmaceutical industry.

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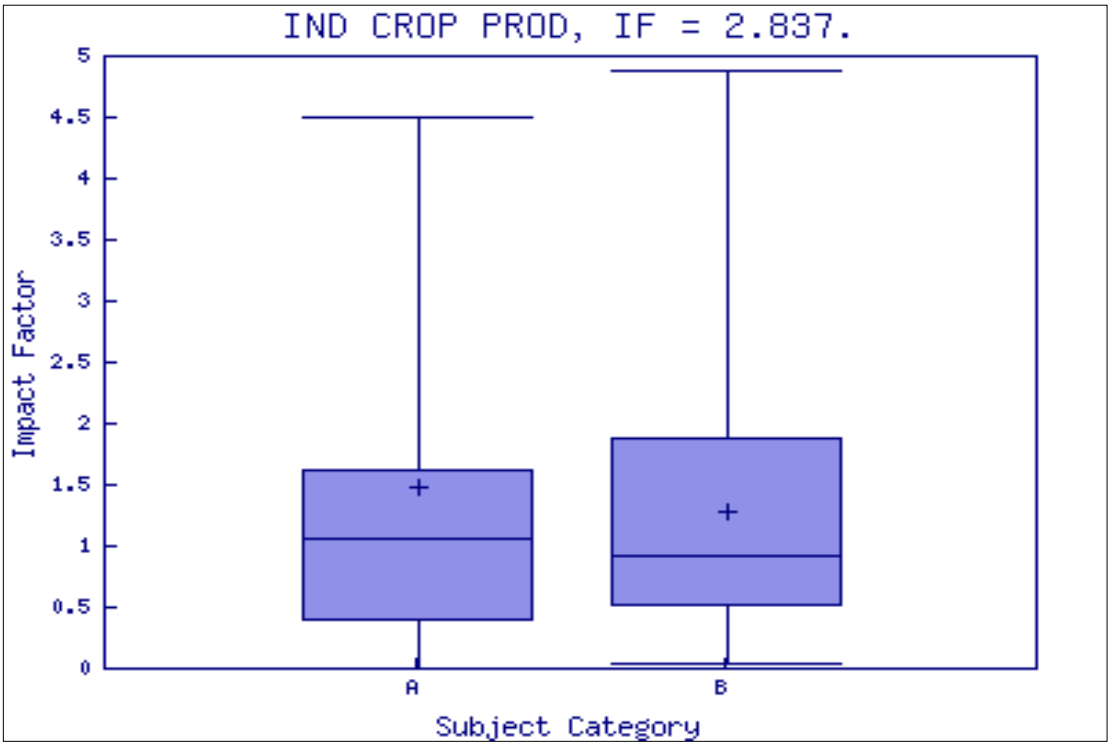

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Article

Secondary Metabolites from Two Species of *Tolpis* and Their Biological Activities

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Abstract: Phytochemical research of two *Tolpis* species, *T. proustii* and *T. lagopoda*, led to the isolation of three new compounds: 30-chloro-3β-acetoxy-22α-hydroxyl-20(21)-taraxastene (**1**), 3β,22α-diacetoxy-30-ethoxy-20(21)-taraxastene (**2**) and 3β,28-dihydroxy-11α-hydroperoxy-12-ursene (**3**). The structures of the new compounds were elucidated by means of extensive IR, NMR, and MS data and by comparison of data reported in the literature. The *in vitro* antioxidant activities of the extracts were assessed by the DPPH and ABTS scavenging methods. The cytotoxicity of several known compounds and its derivatives was also assessed against human myeloid leukemia K-562 and K-562/ADR cell lines.

Keywords: Asteraceae; *Tolpis proustii*; *Tolpis lagopoda*; ursane-triterpenes; taraxastane-triterpenes

1. Introduction

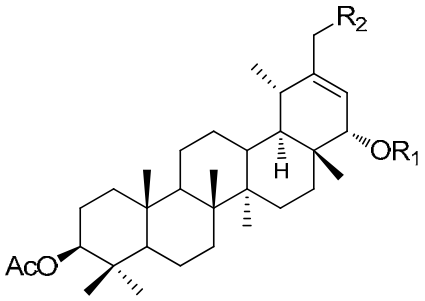
The *Tolpis* genus (Asteraceae: Cichorioideae, Cichoriinae) consists of some 20 species distributed throughout Europe, North Africa, Canary Islands, Cape Verde and Asia, this genus being represented in the Canary Islands by around ten species [1].

Although a wide-ranging study of different species of *Tolpis* from the Canary Islands and from other Macaronesian archipelagos has been carried out from the botanical point of view [2,3], only one phytochemical report has previously appeared on the isolation and structural elucidation of aromatic compounds, triterpenes, and sterols from *T. webbi and T. spp* [4].

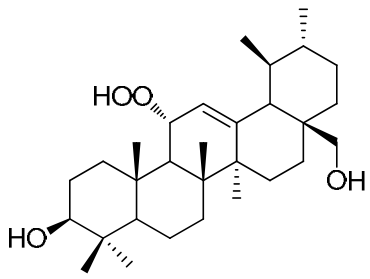
As a part of our continuing search for novel, plant-derived biological agents and our systematic investigation of the composition of Canarian endemic plants, the present work describes the isolation and structural elucidation of the constituents of the ethanolic extracts of the aerial parts of *T. proustii* Pitard in Pitard and Proust and *T. lagopoda* C.Sm. ex Buch. The constituents of these extracts were purified by CC, MPLC and preparative TLC. The structures of the known compounds were confirmed by comparison of their spectroscopic data with those reported in the literature.

From *T. proustii* seventeen compounds were isolated including two taraxan-triterpenoid 30-chloro-3β-acetoxy-22α-hydroxyl-20(21)-taraxastene **1**, and 3β,22α-diacetoxy-30-ethoxy-20(21)-taraxastene **2** a new ursan-triterpenoid 3β,28-dihydroxy-11α-hydroperoxy-12-ursene **3**. From *T. lagopoda*, eight compounds were isolated including a 3β,22α-diacetoxy-30-ethoxy-20(21)-taraxastene **2** (Figure 1).

Figure 1. Compounds **1**–**3** isolated of the *T. proustii* and *T. lagopoda*.



1 R_1=H ; R_2 = Cl
1a R_1=Ac; R_2 = Cl
2 R_1=Ac; R_2 = OEt



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The high level of solar radiation and the high temperature prevailing in the region of the Canary Islands (opposite the northwest coast of Africa) force plants to develop defence mechanisms against ultraviolet radiation and excessive production of free radicals through the accumulation of antioxidant metabolites [5]. This prompted us to evaluate the antioxidant activity of the crude extract of *T. proustii* and *T. lagopoda*, and that of some of the isolated compounds. Furthermore, one of the most important mechanisms by which tumor cells resist to cytotoxic effects of a variety of chemotherapeutic drugs is overexpression of the human multidrug resistance (MDR1) gene and its product, P-glycoprotein [6]. Here we have evaluated the effects of natural compounds and derivatives on the growth of the human leukemia K-562 and the P-glycoprotein-overexpressing K-562/ADR cell lines.

2. Results and Discussion

2.1. Structure Elucidation of Compounds **1**–**3**

The positive EIMS spectrum of compound **1** showed a molecular ion at *m/z* 518/520 (3:1) in agreement with the presence of a chlorine atom in the structure, and with the formula $C_{32}H_{51}O_3Cl$ for this compound. In the same spectrum, the ions at *m/z* 500/502 $[M\text{-}H_2O]^+$, 458/460 $[M\text{-}CH_3COOH]^+$ suggested that this compound contained a hydroxyl and an acetyl group, respectively. Confirmed by the IR spectrum with absorptions of a hydroxyl 3446 cm^{-1} and a carbonyl 1730 cm^{-1} group. Its HREIMS experiment indicated the molecular formula $C_{32}H_{51}O_3{}^{37}Cl$ (calcd. for $[M]^+$ 520.3497; found 520.3497) and $C_{32}H_{51}O_3{}^{35}Cl$ (calcd. for $[M]^+$ 518.3527; found 518.3517). All spectral data suggested that **1** was a pentacyclic triterpene with a trisubstituted double bond in the E ring [7] with a 20(21)-taraxastane structure.

The ^1H and ^{13}C-NMR (Table 1) spectra of **1** showed the presence of the oxygenated methine proton at δ_H 3.35 (1H, d, J = 6.6 Hz) and an unusual chloro atom at C-30 at δ_C 47.6 (Table 1). The structure elucidation and NMR assignments were therefore based primarily on the results of COSY, HSQC, HMBC, and NOESY experiments (Figure 2).

Table 1. ^1H- and ^{13}C-NMR data for compounds **1**, **1a** and **2** [a].

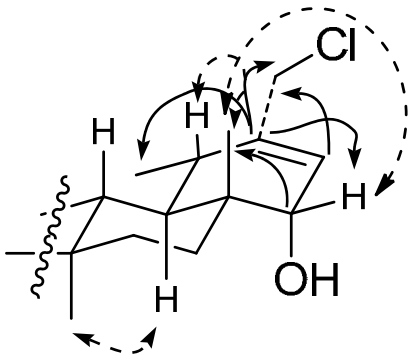
	1		**1a**		**2**	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.65 *	38.5	1.65 *	38.7	1.60 *	38.5
2	1.50 *	23.7	1.54 *	21.7	1.55 *	23.7
3	4.41 dd	81.0	4.43 dd	81.1	4.43 dd	81.0
	(6.1,10.8)		(6.4,10.0)		(5.1 10.4)	
4	-	38.3	-	38.0	-	37.8
5	0.75 *	55.4	0.74 *	55.6	0.75 *	55.4
6	1.45 *	18.2	1.44 *	18.0	1.30 *	18.2
	1.35 *		1.34 *			
7	1.35 *	34.2	1.34 *	34.4	1.35 *	34.2
8	-	41.2	-	41.3	-	41.1
9	1.25 *	50.3	1.26 *	50.6	1.25 *	50.4
10	-	37.1	-	37.3	-	37.1
11	1.20 *	21.6	1.21 *	21.4	1.20 *	21.6
12	1.60 *	27.6	1.60 *	27.7	1.58 *	27.5
	1.20 *					
13	0.95 *	38.6	0.95 *		1.66 *	38.6
14	-	42.3	-	42.4	-	42.3
15	1.72 *	26.7	1.71 *	29.9	1.50 *	26.6
	1.05ddd		1.02 *			
	(2.5,4.0,13.05)					
16	0.95 *	29.7	0.95 *	29.9	1.60 *	29.9
	1.85 dt (9.0,13.0)					
17	-	37.8	-	37.4	-	37.2
18	1.45 *	40.5	1.44 *	41.5	1.45 *	41.5

Table 1. *Cont.*

	1		1a		2	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
19	2.00 t (7.0)	31.6	2.03 tbr (7.0)	31.7	1.79 q (6.6)	32.3
20	-	144.9	-	146.5	-	147.7
21	5.89 d (6.5)	126.3	5.87 d (6.4)	123.1	5.75 d (6.3)	119.2
22	3.35 dbr (6.6)	73.3	4.51 d (6.4)	75.3	4.55 d (6.3)	75.3
23	0.78 s	28.0	0.79 s	28.1	0.81 s	28.0
24	0.77 s	16.6	0.78 s	16.7	0.80 s	16.5
25	0.82 s	16.4	0.82 s	16.7	0.81 s	16.4
26	0.99 s	16.1	0.98 s	16.2	0.96 s	16.1
27	0.93 s	14.7	0.91 s	14.7	0.92 s	14.6
28	0.63 s	17.8	0.70 s	17.9	0.70 s	18.2
29	0.99 d (6.5)	22.2	0.97 d (7.6)	22.1	0.96 d (6.6)	22.6
30	4.15 d (11.2)	47.6	4.14 d (11.2)	47.5	3.96 d (12.6)	72.3
	3.89 d (11.2)		3.89 d (11.6)		3.72 d (12.6)	
OH	3.14 m					
OAc	1.97 s	21.4	1.97 s	21.3	1.97 s	21.3
		171.4	1.98 s	21.3	1.98 s	21.3
				171.1		171.1
				171.1		171.1
OEt					1.13 t (6.9)	15.2
					3.35 m	65.9

[a] δ in ppm and *J* (in Hz) are in parentheses. Recorded in $CDCl_3$ at 400 MHz and 125 MHz for ^1H and ^{13}C-NMR, respectively. * overlapped.

Figure 2. Selected correlations of **1**. Double-ended arrows indicate NOESY, and single arrows indicate HMBC (C to H) correlations.



The most important HMBC and NOESY correlations are shown in Figure 1. Treatment of **1** with Ac_2O-pyridine gave a diacetyl derivative **1a** for which its HREIMS experiment indicated the molecular formula $C_{34}H_{53}O_4{}^{37}Cl$ (calcd. for $[M]^+$ 562.3603; found 562.3624) and $C_{34}H_{54}O_4{}^{35}Cl$ (calcd. for $[M]^+$ 560.3654; found 560.3644). The data implied the presence of a double bond between C-20 and C-21, the chloro atom at C-30, and the acetyl group at C-3 and hydroxyl group at C-22. H-22 was assigned in β-orientation on the basis of the coupling constant with the vinylic proton at C-21 and cross-peak in the NOESY experiment with the CH_3-28. From the above findings, the structure of

30-chloro-3β-acetoxy-22α-hydroxyl-20(21)-taraxastene was assigned to **1**, and it was named chlorotolpidiol. To the best of our knowledge, this is the first example of a pentacyclic triterpene of the taraxastane-ursane series with a chloro functionality.

Compound **2** was obtained as a colourless amorphous solid and its molecular formula was determined by a HRESIMS experiment as $C_{36}H_{58}O_5$ (calcd. for $[M+Na]^+$ 593.4182; found 593.4176). The IR spectrum revealed the absorption bands for a carbonyl group (1732 cm^{-1}) and double bond (2872 cm^{-1}). The ^{1}H-NMR spectrum (Table 1) exhibited six singlet methyl groups at δ_H 0.70, 0.80, 0.81 (6H), 0.92 and 0.96, a secondary methyl group at δ_H 0.96 (3H, d, *J* = 6.6 Hz) attributed to C-29, a vinyl proton at δ_H 5.75 (1H, d, *J* = 6.3 Hz), two acetyl groups at δ_H 1.97 s and 1.98 s, two oxymethine signals at δ_H 4.43 (1H, dd, *J* = 5.1, 10.4 Hz) and 4.55 (1H, d, *J* = 6.3 Hz), an oxymethylene signal at δ_H 3.96 (1H, d, *J* = 12.6 Hz) and 3.72 (1H, d, *J* = 12.6 Hz) and an ethoxy group at δ_H 3.35 (2H, m) and 1.13 (3H, t, *J* = 6.9 Hz). The extra acetyl signal at C-22 was observed, since the oxygenated methine proton of **1** at δ_H 3.35 (1H, d, *J* = 6.6 Hz) was displaced to δ_H 4.55 (1H, d, *J* = 6.3 Hz) in **2**. Moreover, the halogenated group was replaced by a ethoxyl group at C-30 since the carbon in **2** was displaced to low field at δ_C 72.3 (Table 1). The structure of **2** was determined by a combination of COSY, DEPT, HSQC, HMBC, and NOESY experiments. Based on the above data, the new compound tolpidiol A **2** was identified as 3β,22α-diacetoxy-30-ethoxy-20(21)-taraxastene.

Compound **3** was purified as its diacetate **3a** by treatment with acetic anhydride (Ac_2O) in pyridine, **3a** was isolated as a colourless amorphous solid and its HRESIMS experiment indicated the molecular formula $C_{34}H_{54}O_6$ (calcd. for $[M+Na]^+$ 581.3818; found 581.3801). The IR spectrum revealed the absorption bands for carbonyl 1728 cm^{-1}, and hydroxyl 3391 cm^{-1} groups, the presence of these groups was confirmed by the ^{1}H and ^{13}C-NMR spectra (Table 2). The ^{1}H-NMR spectrum of **3a** showed signals for five tertiary methyl groups at δ_H 0.81(6H, br, s), 1.00, 1.02 and 1.11, and two secondary methyl groups at δ_H 0.86 (3H, d, *J* = 6.4 Hz) and δ_H 0.88 (3H, d, *J* = 7.3 Hz) suggesting a pentacyclic triterpene with an ursane skeleton. An olefinic proton at δ_H 5.30 (1H, d, *J* = 3.1 Hz) was assigned to H-12, two oxygenated methines at δ_H 4.45 (1H, dd, *J* = 3.0, 9.6 Hz) and 4.46 (1H, dd, *J* = 5.0, 9.5 Hz) corresponding to H-3 and H-11 respectively, the latter showing vicinal correlation in the COSY experiment with the olefinic proton H-12, while the proton H-9 δ_H 1.81 (1H, d, *J* = 9.5 Hz) indicated the presence of a hydroperoxide at C-11. The presence of an oxygenated methylene was confirmed by the signals at δ_H 3.56 (1H, d, *J* = 11.0 Hz) and 3.93 (1H, d, *J* = 11.0 Hz). The ^{13}C-NMR (Table 2) and DEPT data indicated the presence of two ester carbonyl groups, nine methyl carbons, nine aliphatic methylenes, two olefinic carbons, and seven methine carbons. Thus, the position of acetyl groups in compound **3a** was assigned by a HMBC correlation between the signal at δ_C 171.0 and that at δ_H 4.45; and the signals at δ_C 171.3 and δ_H 3.93. The coupling constant between H-9 and H-11 (*J* = 9.5 Hz) established the α-orientation of the hydroperoxide at C-11. The EI-MS data of **3a** showed direct loss of H_2O *m/z* 540 and H_2O_2 *m/z* 524, confirming the presence of the hydroperoxide.

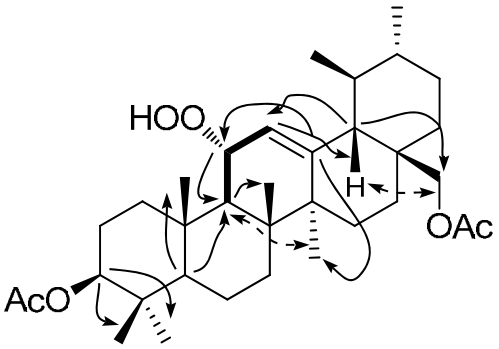
The structure elucidation and NMR assignments were based primarily on the results of HSQC, HMBC, and COSY experiments which allowed the complete assignment of all H- and C-atoms, and the NOESY (Figure 3) data provided the configuration of compound **3a**. Therefore, the structure of **3** was established as 3β,28-dihydroxy-11α-hydroperoxy-12-ursene. To the best of our knowledge, compound **3** is a novel triterpenoid, which we named tolpidiol B.

Table 2. ^{1}H and ^{13}C-NMR data for compound **3a** [a].

Position	δH	δC
1	0.85 m 2.08 dt (3.5, 7.0)	39.4
2	1.58 *	23.7
3	4.45 dd (3.0,9.6)	80.6
4	-	38.0
5	0.88 m	55.3
6	1.45 * 1.31 *	18.1
7	1.45 m 1.25 m	33.3
8	-	43.2
9	1.81 d (9.5)	48.8
10	-	37.8
11	4.46 dd (5.0, 9.5)	81.6
12	5.30 d (3.1)	125.8
13	-	144.5
14	-	42.0
15	1.60 * 0.90 m	26.2
16	1.16 * 1.92 td (3.5,9.0)	23.3
17	-	36.9
18	1.45 m	53.7
19	1.35 m	39.0
20	1.28 m	39.3
21	1.40 m	30.4
22	1.31 m 1.52 dt (3.0, 6.5)	35.5
23	0.81 s	28.2
24	0.81 s	16.7
25	1.02 s	16.8
26	1.00 s	18.0
27	1.11 s	22.2
28	3.56 d (11.0) 3.93 d (11.0)	71.0
29	0.86 d (6.4)	17.4
30	0.88 d (7.3)	21.3
OAc	1.97 s 1.97 s	21.0 21.3 171.0 171.3

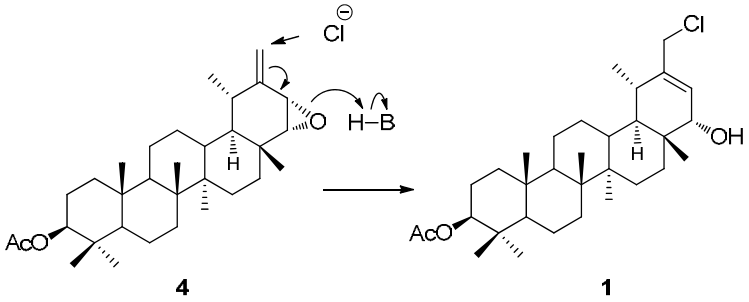
[a] δ in ppm and *J* (in Hz) are in parentheses. Recorded in $CDCl_3$ at 400 MHz and 125 MHz for ^{1}H- and ^{13}C-NMR, respectively. * overlapped.

Figure 3. Selected correlations of **3a**. Bond bolded indicate COSY, Double-ended arrows indicate NOESY, and single arrows indicate HMBC (C to H) correlations.



Compound **1** could derive from the known triterpene acetyl-ptiloepoxide **4** [8,9] which was identified by us from *T. proustii* as an inseparable mixture. Triterpenes containing an epoxide at the $\Delta^{21\text{-}22}$ position are known and have been isolated before from a *Tolpis* species [4]. Based on this, we envisioned the formation of compound **1** by chlorination of the double bond, followed by isomerization and β opening of the epoxide, and protonation, maintaining the α-orientation at the C-22 observed in the precursor compound acetyl-ptiloepoxide (Figure 4).

Figure 4. Tentative forming process of compound **1**.



Although the number is relatively small, several halogenated triterpenes and other higher terpenes have been described mainly from marine sources [10]. However, the presence of chlorinated triterpenoids in terrestrial plants is very rare and just few cases have been reported [11–14]. Initially, compound **1** seemed to be an artifact of the isolation process, but Chen *et al.* [14] proved that to obtain chlorinated compounds, a chlorine source such as $CHCl_3$ with HCl is necessary. During the isolation process, no chlorinated solvents were used (see Experimental). In the chromatographic separation, dichloromethane was used in the preparative TLC, which was not enough to interact with the possible precursor (ptiloepoxide) of **1**. Compound **2** was isolated as a presumed artifact; this compound was probably obtained from **1**, by a nucleophilic substitution reaction due to the use of hot EtOH during the extraction process.

Additionally, from *T. proustii* 15 known compounds were isolated, including aromatic compounds: scopoletin [15] aesculetin [15] and apigenin [16]; the diterpene phytene-1,2-diol [17] and the triterpenoids stigmasterol [18], ergosterol peroxide [19], ursolic acid [20], lupan-20(29)-ene-3β,30-diol [21], 21α-hydroxytaraxasterol [22], 11-oxo-β-amyrin [23], 3β-acetoxy-urs-12-ene-1β,11α-diol [24], 21α,22α-epoxy-20α-hydroxy-20(30)-dihydrotaraxasterol [4], 3β-acetoxy-21α,22α-epoxytaraxastan-20α-ol [25], 22-oxo-20-taraxasten-3β-ol [9], β-amyrin [26]. From *T. lagopoda* seven known

compounds were isolated, including aromatic compounds: 2,4′-dihydroxy-4-methoxybenzophenone [4] and triterpenoids: stigmasterol [18], ergosterol peroxide [19], a mixture of 7-oxo-β-sitosterol and 7-oxo-stigmasterol [26,27], ursolic acid [20], and α-amyrin [28]. Their structures were confirmed by comparison of their spectral data with those reported in the literature.

2.2. Antioxidant Activities

Natural antioxidants that are present in plants are responsible for inhibiting or preventing the deleterious consequences of oxidative stress. In Table 3 the relative antioxidant efficiency of both *Tolpis* extracts against the DPPH radical is shown. Antioxidants suppress the absorbance at 515 nm on a time scale dependent on the antioxidant activity of extracts. The RSA of the crude extract of *T. proustii* (59.6%) was higher than that of *T. lagopoda* (41.4%). FRAP assay was used to study the ability of the antioxidants in the extracts to reduce ferric iron to the ferrous form. The same behaviour as for the DPPH assay was observed, *T. lagopoda* being less active than *T. proustii* (4.1 and 18.1 μmol of Fe(III) reduced to Fe(II) per gram of dry plant respectively) (Table 3). On the other hand, the free radical scavenging and ferric reducing power assays revealed that aesculetin (isolated from *T. proustii*) showed the highest antioxidant activities as compared with those of α-tocopherol and BHA (Table 4). Aesculetin gave a RSA value of 100% with a $t_{1/2}$ (time required for 50% scavenging of DPPH radical in the specified concentration of antioxidant) of 22.5 seconds, while BHA and α-tocopherol showed RSA of 21.9 and 17.7% respectively after 20 min. Aesculetin (at concentration 0.1 mg mL^{-1}) showed also higher antioxidant activities than both extracts (at concentration 10 mg mL^{-1}), because the extracts are complex mixtures that include active components at lower levels. Moreover, the crude extracts tend to have more interfering substances that may interact with the antioxidants, decreasing their effectiveness. The antioxidant activities found in this study indicated that aesculetin, as well as both extracts, are ideal for use in the health food industry. Because of the high content of aesculetin found in the *T. proustii* extract (566.8 mg), this extract may be considered to be a natural source of aesculetin with diverse potential therapeutic uses.

Table 3. Antioxidant activity of crude extracts derived from *T. proustii* and *T. lagopoda*.

Assays	***T. proustii***	***T. lagopoda***
RSA [a]	59.6 ± 0.4	41.4 ± 0.1
FRAP [b]	18.1 ± 0.4	4.1 ± 0.2
FRAP [c]	93 ± 2	41 ± 1

[a] % inhibition ± standard deviation of three measurements. [b] μmol of Fe(III) reduced to Fe(II) per gram of dry plant ± standard deviation of three measurements. [c] μmol of Fe(III) reduced to Fe(II) per gram of ethanolic residue ± standard deviation of three measurements.

Table 4. Antioxidant activity of aesculetin, α-tocopherol and butylated hydroxyanisol (BHA).

Assays	**Aesculetin 0.1 mg mL^{-1}**	**α-Tocopherol 0.1 mg mL^{-1}**	**BHA 0.1 mg mL^{-1}**
DPPH [a]	100 ± 0	17.7 ± 0.1	21.9 ± 0.6
FRAP [b]	9.4 ± 0.7	0.97 ± 0.03	3.13 ± 0.05

[a] % inhibition ± standard deviation of three measurements. [b] μmol of Fe(III) reduced to Fe(II) per mg of compound ± standard deviation of three measurements.

2.3. Cytotoxic Activity

K-562 and K-562/ADR cells which are sensitive or resistant to doxorubicin, respectively, were incubated with the compounds shown in Table 5 to evaluate their potential cytotoxicity. After 72 h, cell survival was determined by the MTT assay and the IC_{50} values are summarized in Table 5. Among the different compounds ursolic acid and 22-oxo-20-taraxasten-3β-ol exhibit the strongest effects in mitochondrial reduction of tetrazolium salts to formazan, while the ursolic derivatives and the 1,2-diacetoxyphytene exhibit the weakest effects. Furthermore, K-562 and K-562/ADR cells exhibit comparable sensitivity to compounds ursolic acid and 22-oxo-20-taraxasten-3β-ol (Table 5). These results suggest that the overexpression of the drug efflux protein, P-glycoprotein does not confer resistance against these compounds.

Table 5. Effects of some compounds and derivatives isolated from *T. proustii* and *T. lagopoda* on the growth of the human leukemia cell lines.

Compound	IC_{50} (μM)	
	K562	K562/ADR
Ursolic acid	40.6 ± 3.6	49.2 ± 3.1
Ursolic acid methyl ester	59.3 ± 15.5	64.0 ± 14.5
Acetyl ursolic acid	99.5 ± 20.5	>100
Acetyl ursolic acid methyl ester	>100	>100
Aesculetin	63.2 ± 3.2	77.0 ± 5.1
Aesculetin acetyl	68.6 ± 17.1	70.3 ± 19.2
Aesculetin diacetyl	62.3 ± 6.6	59.5 ± 4.5
11-Oxo-β-amyrin	>100	>100
22-Oxo-20-taraxasten-3β-ol	30.0 ± 10.0	43.0 ± 7.0
1,2-Diacetoxyphytene	>100	>100

Cells were cultured for 72 h and the IC_{50} values were calculated as described in the Experimental section. The data shown represent the means ± SEM of 3–5 independent experiments with three determinations in each.

3. Experimental

3.1. General Experimental Procedures

Optical rotations: Perkin-Elmer *model 343* polarimeter. IR Spectra: Bruker model *IFS-55* spectrophotometer. ^{1}H and ^{13}C-NMR spectra: Bruker model AMX-500 and AMX-400 spectrometers with standard pulse sequences, operating at 500 and 400 MHz for ^{1}H-, and 125 MHz for ^{13}C-NMR, $CDCl_3$ was used as solvent and TMS as internal standard. EI–MS: *Micromass* model *Autospec* (70 eV) spectrometer. The constituents of the ethanolic extracts were separated by gravity column chromatography, medium pressure liquid chromatography (MPLC) and preparative TLC. Column chromatography (CC): silica gel SiO_2; (70–230 mesh, Merck), column fractions were monitored by TLC (silica gel *60* F_{254}), Medium pressure column chromatography (MPLC): silica gel *Merck* (40–63 μm). Prep. TLC: silica gel *60* $PF_{254+366}$ plates (20 × 20 cm, 1-mm thickness).

3.2. Plant Material

The plant material was identified by Dr. Rosa Febles. *T. proustii* was collected at Roque Agando (La Gomera, Canary Islands) in May 2009. A voucher specimen has been deposited at the Herbarium of the Viera y Clavijo Botanical Garden in Las Palmas de Gran Canaria (LPA 24194). *T. lagopoda* was collected at Tenteniguada (Gran Canaria, Canary Islands) in May 2008. A voucher specimen has been deposited at the Herbarium of the Viera y Clavijo Botanical Garden in Las Palmas de Gran Canaria (LPA 23596).

3.3. Extraction and Isolation

The aerial parts of *T. proustii* (3.0 Kg) were extracted with ethanol (4 L) in a Soxhlet until exhaustion. Solvent removal afforded a viscous residue (541.5 g) which was fractionated by means of CC (SiO_2; hexane/AcOEt step gradients). The fractions eluted with hexane-AcOEt (7:3) were submitted to a new MPLC chromatography with hexane-AcOEt (9:1), to give stigmasterol (30.6 mg); 3β-acetoxy-21α,22α-epoxy-20α-hydroxy-20(30)-dihydrotaraxasterol (6.3 mg), obtained by preparative TLC (benzene-ethyl acetate 8:2); ergosterol peroxide (obtained as its acetylated derivative upon acetylation of one of the obtained fractions (2.3 mg); 3β,30-dihydroxylup-20(29)-ene (31.1 mg); phytene-1,2-diol (7.6 mg); **3a** (2.3 mg), isolated by successive preparative TLCs (benzene-AcOEt, 9.9:0.1, 2 elutions; dichloromethane-hexane 1:1, 3 elutions) and **1** (3.7 mg), obtained by preparative TLC (dichloromethane, 3 elutions). Subsequent chromatography by MPLC with hexane-AcOEt (8.5:1.5) gave ursolic acid (193.8 mg); 21α-hydroxytaraxasterol (8,9 mg), obtained by preparative TLC (benzene-AcOEt, 8.5:1.5, 4 elutions); 11-oxo-β-amyryn (15.4 mg); 22-oxo-taraxasten-3β-ol (2.7 mg) isolated by preparative TLC (dichloromethane-AcOEt 9:1, 4 elutions); β-amyryn (5.2 mg); and **2** (2.1 mg), obtained upon crystallization in hexane-AcOEt and subsequent purification by preparative TLC (dichloromethane-AcOEt 8:2).

The fractions eluted with hexane-AcOEt (3:2) were partially rechromatographed by MPLC in hexane-AcOEt (7:3) giving, after preparative TLC (benzene-AcOEt, 8:2, 2 elutions), apigenin (41.2 mg) and scopoletin (6.7 mg). The remainder of these fractions was submitted to subsequent rechromatography by MPLC in hexane-AcOEt (8.5:1.5) to give 3β-acetoxy-1β,11α-dihydroxy-urs-12-ene (9.7 mg), isolated by preparative TLC (benzene-AcOEt, 8:2, 4 elutions), and 21α,22α-epoxy-20α-hydroxy-20(30)-dihydrotaraxasterol (3.6 mg), purified by preparative TLC (hexane-AcOEt, 8:2, 2 elutions). Finally, the fractions eluted with hexane-AcOEt (1:1) afforded aesculetin (566.8 mg), upon crystallization in hexane-AcOEt.

The aerial parts of *T. lagopoda* (2.7 Kg) were extracted with ethanol (4 L) in a Soxhlet until exhaustion. Removal of the solvent afforded a viscous residue (279.8 g), which was fractionated by CC (SiO_2; hexane/AcOEt step gradients). Fractions eluted with hexane-ethyl acetate (4:1) were subsequently chromatographed by MPLC with hexane-ethyl acetate (7.5:2.5) to give five fractions (numbered from fraction 1 to 5). From fraction 1 stigmasterol (14.3 mg) was purified by crystallization in hexane-AcOEt, while fraction 3 afforded 2,4′-dihydroxy-4-methoxybenzophenone**,** obtained by crystallization with hexane-AcOEt (15.1 mg) and from its mother liquors α-amyrin (2.9 mg) was obtained by preparative TLC with (benzene-AcOEt, 9:1, 5 elutions), as its acetylated derivative, after

acetylation. From fraction 4 ursolic acid (6.1 mg) was purified after a methylation process. Fraction 5 afforded ergosterol peroxide (11 mg) by preparative TLC (hexane-AcOEt, 9:1, 2 elutions), purified after acetylation as its acetyl derivative. Finally, fraction 2 afforded compound **2** (1.8 mg) by preparative TLC (benzene-AcOEt, 9:1). Fractions eluted with hexane-AcOEt (7:3) were subsequently chromatographed by MPLC with hexane-AcOEt (8:2), the most polar fractions affording the mixture of 7-oxo-β-sitosterol and 7-oxo-stigmasterol (18.1 mg) were purified by preparative TLC (hexane-AcOEt, 7:3, 5 elutions).

30-Chloro-3β-acetoxy-22α-hydroxyl-20(21)-taraxastene (**1**). Amorphous white solid; $[\alpha]_D^{25}$ = +6.0 ($CHCl_3$, c 0.001); for ^{1}H and ^{13}C-NMR data, see Table 1. IR, ν max (KBr): 3446, 2924, 2853, 1732, 1439, 1244, 1172 cm^{-1}. EIMS *m/z* 520 (5.7), 518 (15.5), 502 (13.5), 500 (35.2), 483 (7.3), 460 (4.3), 458 (10.5), 464 (13.6), 404 (5.1) 249 (21.0), 190 (36.5) 189 (100.0), 187(40.0) 161 (17.0), 133(29.0) 121 (36.0), 81(31.6). HREIMS *m/z* 520.3497 $[M]^+$ (calcd for $C_{32}H_{51}O_3{}^{37}Cl$ 520.3497), 518.3517 $[M]^+$ (calcd for $C_{32}H_{51}O_3{}^{35}Cl$ 518.3527), 502.3414) $[M-H_2O]^+$ (calcd for $C_{32}H_{49}O_2{}^{37}Cl$ 502.3392), 500.3436 $[M-H_2O]^+$ (calcd for $C_{32}H_{49}O_2{}^{35}Cl$ 500.3421.

Acetylation of **1**. Compound **1** (2.0 mg) was dissolved in pyridine (1 mL) and acetic anhydride (2 mL), and the reaction was further stirred at room temperature for 12 h and after usual work-up. The product was dried under vacuum to furnish **1a** (1.8 mg). For ^{1}H and ^{13}C-NMR spectroscopic data, see Table 1.

30-Chloro-3β,22α-diacetoxy-20(21)-taraxastene (**1a**). Amorphous white solid; $[\alpha]_D^{25}$ = +86.7 ($CHCl_3$, c 4.5 × 10^{-3}); for ^{1}H and ^{13}C-NMR data, see Table 1. IR, ν max (KBr): 2920, 2852, 1731, 1652, 1540, 1456, 1372, 1247, 1016, 982 cm^{-1}. EIMS *m/z* 562 (0.7), 560 (1.7), 525 (34.7), 500 (27.0), 466 (10.4), 404 (6.4), 189 (100), 95 (52.8), 69 (56). HREIMS m/z 562.3624 $[M]^+$ (calcd for $C_{34}H_{53}O_4{}^{37}Cl$ 562.3603), 560.3644 $[M]^+$ (calcd for $C_{34}H_{53}O_4{}^{35}Cl$ 560.3632).

3β,22α-Diacetoxy-30-ethoxy-20(21)-taraxastene (**2**). Colourless amorphous solid; $[\alpha]_D^{25}$ = +4.8 ($CHCl_3$, c 0.015); for ^{1}H and ^{13}C-NMR data, see Table 1. IR, ν max (KBr): 2931, 2872, 1732, 1652, 1464, 1456, 1671, 1244, 1016 cm^{-1}. EIMS *m/z* 510 (100) $[M-OAc]^+$, 450 (40) $[M-2OAc]^+$. HRESIMS positive ion *m/z* 593.4176 $[M+Na]^+$ (Calcd. for $C_{36}H_{58}O_5Na$, 593.4182).

3β,28-Diacetoxy-11α-hydroperoxy-12-ursene **3a**. Colourless amorphous solid; $[\alpha]_D^{25}$ = +9.5 ($CHCl_3$, c 0.014); for ^{1}H and ^{13}C-NMR data, see Table 2. IR, ν max (KBr): 3391, 2951, 2925, 2875, 1728, 1392, 1244, 1032, 983, 903 cm^{-1}. HREIMS *m/z* 540.3829 $[M-H_2O]^+$ (Calcd. for $C_{34}H_{52}O_5$, 540.3815); m/z 524.3873 $[M-H_2O_2]^+$ (Calcd. for $C_{34}H_{52}O_4$, 524.3866). HRESIMS positive ion *m/z* 581.3801 $[M+Na]^+$ (Calcd. for $C_{34}H_{54}O_6Na$, 581.3818).

3.4. Antioxidant Experiments

3.4.1. Chemicals

Methanol HPLC grade (Panreac, Barcelona, Spain) and Milli-Q water (18MQ, Millipore, Billerica, MA, USA) were always used in this study. Formic acid and sodium acetate (Merck, Darmstadt, Germany) were analytical quality reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tri (2-

pyridyl)-1,3,5-triazine (TPTZ) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany); rutin and gentisic and caffeic acids were supplied by Merck. Ferric chloride ($FeCl_3 \cdot 6H_2O$), ferrous sulphate ($FeSO_4 \cdot 7H_2O$) and glacial acetic acid were obtained from Panreac.

3.4.2. Preparation of Extracts for Antioxidant Assays

The ethanolic extract residue was solved in methanol by stirring at room temperature. After centrifugation at 7,000 rpm for 10 min, the supernatant was collected and the antioxidant activity was measured. Aesculetin, α-tocopherol and BHA (5 mg) were dissolved in methanol (10 mL). The solution was diluted to 0.1 mg mL^{-1} to be used for antioxidant assays.

3.4.3. Free Radical Scavenging Activity on DPPH

The reducing ability of antioxidants towards DPPH radical was evaluated by measuring the loss of 1,1-diphenyl-2-picrylhydrazyl (DPPH) colour at 515 nm after reaction with test extracts [29]. The sample solution (30 μL) was rapidly mixed with 1 mL of a 0.1 mM DPPH solution. After 25 min incubation time in the dark at ambient temperature (23 °C), the decline in absorbance against a methanol blank was recorded. The inhibition percentage values were calculated by equation: Radical Scavenging Activity (RSA) = 100 (1–Abs in the presence of sample/Abs in the absence of sample).

3.4.4. Ferric Reducing Antioxidant Power Assay (FRAP)

Reducing power was determined according to [30]. This method is based on the reduction of Fe^{3+} to Fe^{2+}, which is recorded by measuring the formation of a blue colored Fe^{2+}-tripyridyltriazine compound from the colourless oxidized Fe^{3+} form by the action of electron donating antioxidants. The FRAP reagent consists of 300 mM acetate buffer (3.1 g sodium acetate + 16 mL glacial acetic acid, made up to 1 litre with distilled water; pH = 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ in the ratio of 10:1:1.

Extract (10 μL) was added to 1.0 mL freshly prepared and prewarmed (37 °C) FRAP reagent. The mixture was incubated at 37 °C for 10 min and the absorbance was measured against a reagent blank (1.0 mL FRAP reagent + 10 μL distilled water) at 593 nm. A standard curve of Fe^{2+} was constructed over the concentration range of 0.2 μmol L^{-1} to 1 μmol L^{-1}. The results were determined by the regression equation of the curve ($y = 0.00221x + 0.00999$, $r = 0.9969$) and expressed as μmol ferric ions reduced to ferrous form per g of dry plant material.

3.5. Cytotoxic Experiments

3.5.1. Cell Culture

Human leukemia K-562 cells (DSMZ No: ACC 10, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 containing 2 mM L-glutamine supplemented with 10% (v/v) heat-inactivated fetal bovine serum. The K-562/ADR cell line was kindly provided by Professor Lisa Oliver (INSERM, Nantes, France) and cultured as above.

3.5.2. Assay for Growth Inhibition and Cell Viability

The cytotoxicity of compounds was assessed using a 3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Briefly, 1×10^4 exponentially growing cells were seeded in 96-well microculture plates with various compounds concentrations (3–100 μM) in a volume of 200 μL. DMSO concentration was the same in all the treatments and did not exceed 0.1% (v/v). After 72 h, surviving cells were detected based on their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide into formazan crystals. Optical density was read with an ELISA reader at a wavelength of 570 nm and was used as a measure of cell viability. The MTT dye reduction assay measures mitochondrial respiratory function and can detect the onset of cell death earlier than dye-exclusion methods. Cell survival was calculated as the fraction of cells alive relative to control for each point: cell survival (%) = mean absorbance in treated cells/mean absorbance in control wells × 100. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically using the curve fitting algorithm of the computer software Prism 4.0 (GraphPad). Values are means ± SEM from three independent experiments, each performed in triplicate.

4. Conclusions

We have studied two endemic Canary plants of the *Tolpis* genus and two new compounds tolpidiol A (**2**) and B (**3**) and a novel chloro derivative chlorotolpidiol (**1**) were isolated from *T. proustii* and *T. lagopoda*. The isolation of halogenated triterpenoids from terrestrial sources is very rare, and few cases have been reported. To the best of our knowledge, chlorotolpidiol is the first example of a pentacyclic triterpene of the taraxastane-ursane series chlorinated at the position C-30. Herein we present a tentative explanation of the biogenesis of this class of compounds. The presence of chlorine atoms provides chemically addressable handles for further work in organic-medicinal chemistry. The antioxidant activities of the ethanolic extract were evaluated and it showed relative efficiency. We found weak cytotoxic activity against K562 and K-562/ADR cell lines of some of the isolated and derivative compounds.

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Sample Availability: Samples of the compounds **1a**, **2** and **3** are available from the authors.

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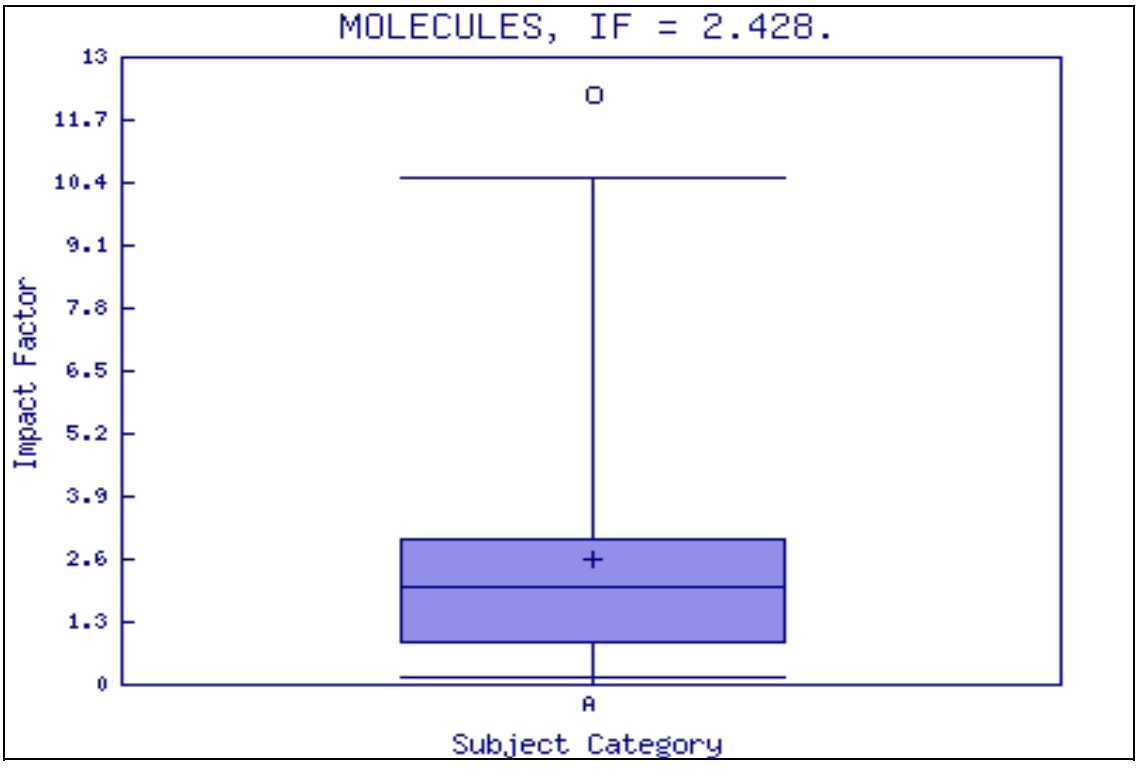

Key

A - CHEMISTRY, ORGANIC

Capítulo 5:

CONCLUSIONES

Todas las metodologías desarrolladas para la identificación y cuantificación simultánea de dieciocho polifenoles en macroalgas y en microalgas son rápidas, precisas y presentan buena sensibilidad y reproducibilidad.

Los resultados muestran que el alga parda *S. scoparium* representa una fuente natural de compuestos polifenólicos con elevada y reconocida actividad antioxidante tales como el ácido gálico, que constituye prácticamente el 0,1% en peso del alga, (+)-catequina y (-)-epicatequina.

Nuestros resultados aportan argumentos suficientes que apoyan la implicación de los polifenoles en los mecanismos de detoxificación de metales y reguladores de la biodisponibilidad del hierro en las microalgas *P. tricornutum* y *D. tertiolecta*.

Tanto las diatomeas *P. tricornutum* como la microalga *D. tertiolecta* hacen un esfuerzo metabólico extra y exudan mayor cantidad de polifenoles por célula en presencia de cobre que en el experimento control, con el objetivo de complejar el cobre presente en el agua de cultivo para disminuir su efecto tóxico.

La diatomea *P. tricornutum* produjo 2,4 veces más polifenoles en presencia de cobre que en el control, lo que puede reflejar la participación de estos compuestos en alguno de los mecanismos de defensa contra la toxicidad del cobre a nivel del interior de la célula. Sin embargo, en la microalga *D. tertiolecta*, las cantidades de los compuestos identificados en el interior de la

célula disminuyeron en presencia de cobre debido a la presencia de otros mecanismos de defensa que no implican polifenoles y a su capacidad de excluir el cobre.

El contenido fenólico de la diatomea también se incrementa en presencia de altas concentraciones de hierro respecto al control, debido, probablemente, a que estos compuestos son capaces de complejar el metal y evitar la reacción de Fenton. Cuando la microalga *D. tertiolecta* fue cultivada en presencia de hierro, la cantidad de polifenoles identificados en el interior de la célula disminuyó ligeramente respecto al control.

En ambas microalgas cultivadas en presencia de hierro, la cantidad de compuestos fenólicos exudados por célula disminuye drásticamente debido a la alta densidad celular que ayuda al reducir al mínimo el esfuerzo metabólico necesario para mantener las concentraciones de polifenoles en el agua de mar y favorecer la persistencia y asimilación del Fe(II).

Los extractos de las diatomeas expuestas a cobre presentaron mayores actividades antioxidantes que el resto, mayor al incrementar la concentración de cobre, probablemente, debido al requerimiento de la célula en esas condiciones de mayor estrés.

Las actividades antioxidantes de ambas microlgas y la presencia de polifenoles las señalan como potenciales fuentes de los mismos para su uso en la industria farmacéutica y alimentaria.

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"Lo que sabemos es una gota de agua;

lo que ignoramos es el océano"

- Isaac Newton -