# CARACTERIZACIÓN DE LOS EFECTOS BIOLÓGICOS DE LOS POLIFENOLES EN LA INFLAMACIÓN Y EL METABOLISMO : NUEVAS PERSPECTIVAS NUTRICIONALES

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TESIS DOCTORAL

dirigida por el

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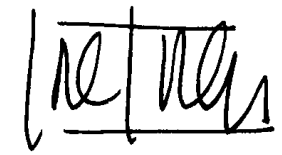
FEM CONSTAR que aquest treball, titulat “Caracterización de los efectos biológicos de los polifenoles en la inflamación y el metabolismo: nuevas perspectivas nutricionales.”, que presenta Raúl Beltrán Debón, ha estat realitzat sota la nostra direcció al Centre de Recerca Biomèdica de l’Institut d’Investigació Sanitària Pere Virgili i que acompleix els requeriments per poder optar al títol de Doctor.

Reus, 10 de juny de 2011

El director de la tesi doctoral

Dr. Carlos Alonso-Villaverde Lozano

El codirector de la tesi



Dr. Jorge Joven Maried

*A mi abuelo y*

*a mi tío Vicente*

# AGRADECIMIENTOS

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"*El agradecimiento es la memoria del corazón*" Lao Tse, pensador, filósofo y humanista chino.

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INTROD

# CAPÍTULO 1. LOS POLIFENOLES EN LA DIETA

Muchos estudios observacionales correlacionan la dieta mediterránea con un menor riesgo de padecer enfermedades cardiovasculares. Inicialmente se observó que la relación desfavorable de ácidos grasos poliinsaturados frente a los monoinsaturados contribuía significativamente con otros factores de riesgo conocidos como la presión sanguínea, la edad, el colesterol sérico o el tabaquismo sobre la incidencia de muerte de origen cardiovascular[1]. Aunque se demostró una asociación con la dieta no se pudieron establecer los mecanismos moleculares subyacentes relacionados con la patología ateromatosa[2]. Posteriormente, estudios observacionales señalaron una posible relación entre vitaminas antioxidantes y la reducción de la mortalidad cardiovascular. Sin embargo, este hallazgo no ha podido ser corroborado en estudios clínicos controlados[3,4].

Existen datos que apuntan a que el efecto beneficioso de la dieta mediterránea es debido probablemente a la combinación específica de numerosos micronutrientes. Por ejemplo, el aceite de oliva muestra efectos nutrigenómicos *in vivo*, regulando negativamente numerosos genes proaterogénicos[5], y uno de sus componentes, el ácido oleico, que inicialmente se postuló como ingrediente fundamental en la dieta mediterránea, muestra efectos similares aunque no todos los observados con el total el aceite de oliva, indicando la presencia de otros biocomponentes activos en el aceite de oliva[6].



## 1.1 Los polifenoles

Los polifenoles están presentes en las plantas como una mezcla y no como componentes aislados, un hecho que con frecuencia es ignorado en la realización de estudios experimentales. Varios miles de estos fitoquímicos ya han sido identificados. Los compuestos fenólicos pueden clasificarse de diversas formas,

por ejemplo, respecto a su unidad base o a la naturaleza de los grupos funcionales ligados a esta unidad base. La más utilizada es la clasificación que los divide en ácidos fenólicos, flavonoides y, los menos abundantes, estilbenos y lignanos, aunque existen muchos más tipos de estructuras.

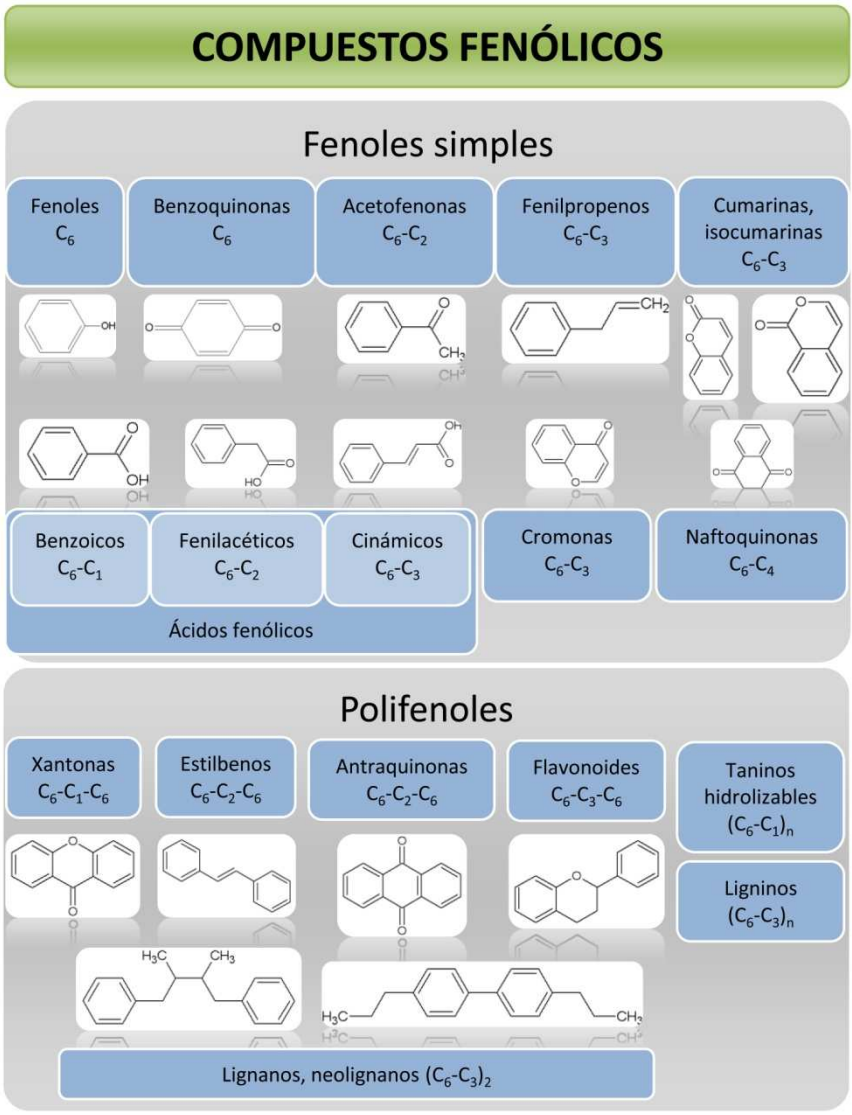


**Figura 1.** Clasificación de los compuestos fenólicos con los fenoles simples y polifenoles principales, entre los que se encuentran los flavonoides.

Desde un punto de vista estructural, los compuestos fenólicos pueden clasificarse en fenoles simples y polifenoles. Estos últimos se caracterizan por tener uno o más de un grupo fenol, y se dividen a su vez en xantonas, estilbenos, antraquinonas, lignanos (y polímeros de estos) y, los más abundantes, los flavonoides (Figura 1).

## 1.2 Flavonoides

Son los compuestos polifenólicos más abundantes en las plantas y pueden ser clasificados como flavonoles, flavonas, isoflavonas, flavanonas, antocianinas y flavanoles (catequinas y proantocianidinas). Estos compuestos están caracterizados por una estructura benzo-γ-pirona ($C_6$-$C_3$-$C_6$; Figuras 2 y 3). Esta estructura permite la sustitución en diversas posiciones de diferentes grupos funcionales como hidroxilos, metoxilos u *O*-glucósidos, lo que explica las más de 8000 estructuras caracterizadas hasta la fecha[7].



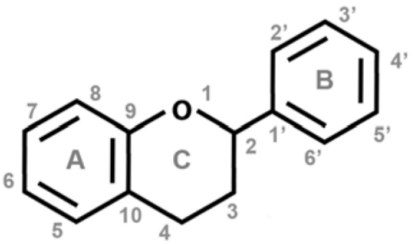


**Figura 2.** Estructura básica de los flavonoides.

Los flavonoides son productos del metabolismo secundario de las plantas sintetizados a partir de fenilalanina y desempeñan numerosas funciones. Casi todos son pigmentos que cubren todo el espectro de la luz visible y la ultra-violeta. Una de las funciones principales es la atracción de insectos, animales y aves polinizadoras, y la regulación de genes fotosensibles, aunque no participan en la fotosíntesis[8]. Los flavonoides inhiben o matan muchas cepas bacterianas, inhiben enzimas virales claves como la transcriptasa inversa y la proteasa, y

destruyen algunos protozoos patogénicos. Por otro lado, su toxicidad frente a células animales es baja.

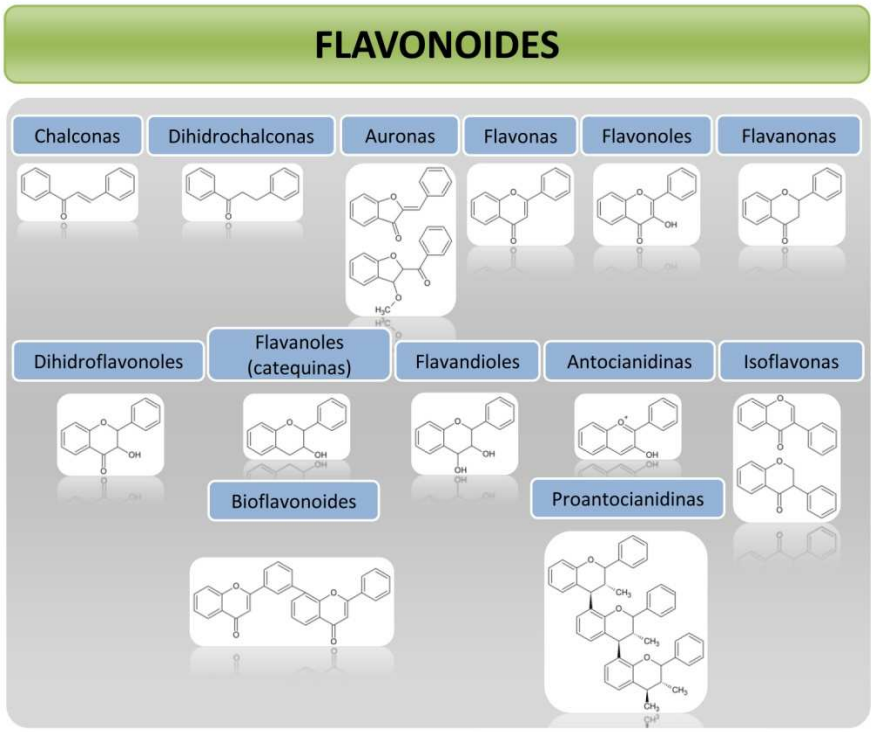


**Figura 3.** Estructuras base que caracterizan los diferentes tipos de flavonoides.

Actualmente se acepta que los polifenoles se sintetizan principalmente en respuesta a un gran estrés como periodos prolongados de sequía, la radiación ultravioleta o la infección por patógenos. Por lo tanto, en las frutas y verduras que se cultivan y consumen en las sociedades occidentales, donde las condiciones climáticas no son extremas y se controlan los agentes patógenos con el fin de aumentar la producción, la cantidad esperada de polifenoles es baja o muy baja (algunas excepciones son las cebollas, el ajo y las verduras crucíferas). Simplemente, no parece factible poder comer suficientes frutas y verduras para ingerir una cantidad efectiva de polifenoles, aun suponiendo que estos sean muy activos y fácilmente biodisponibles.

De este hecho se infiere que, por un lado, si los polifenoles se asocian con una serie de beneficios para la salud en los humanos, y por otro, el consumo debe ser mayor, la dosis necesaria no puede conseguirse mediante simples cambios en la dieta.

Para aumentar la ingesta de polifenoles, una estrategia complementaria sería la de cambiar ciertas prácticas agrícolas y la fabricación de suplementos dietéticos concentrados. Sin embargo, no sólo los factores cuantitativos, sino también cualitativos deben ser considerados. Ciertos polifenoles se encuentran ampliamente distribuidos, mientras que otros son específicos de determinados alimentos, y a menudo, asociados a una mezcla mal caracterizada. Además, el conocimiento de su composición se limita a unas pocas variedades para las que existe un grado aceptable de disponibilidad, precio y aceptación. Muchos tipos de productos derivados de las plantas exóticas o tropicales no han sido analizados y pueden representar una importante fuente de polifenoles.

Por otra parte, las normas actuales de fabricación de complementos dietéticos exigen una composición del producto bien definida, lo que es difícil de controlar en productos derivados de plantas, ya que el contenido de polifenoles depende de la madurez en el momento de la cosecha, el procesamiento y el almacenamiento[9-13], y puede llegar a ser muy variable de un año a otro o incluso entre lotes de fabricación. Los factores ambientales y los métodos de preparación culinaria también son importantes. Algunos de estos son los siguientes: la exposición a la luz, el cultivo orgánico (recordemos que cuanto mayor es el estrés mayor es el contenido en polifenoles), la lluvia, el tipo de suelo, la producción de frutos por árbol, hervir, pelar, freír o la utilización de un horno microondas[14]. La elaboración industrial de alimentos también afecta el contenido de polifenoles[15]. En algunos casos, este efecto es comercialmente inevitable, como en la producción de zumo de fruta, donde se eliminan ciertos polifenoles responsables de la decoloración y formación de precipitados. Por lo tanto, en la evaluación de los efectos biológicos derivados del consumo de plantas ricas en polifenoles la

caracterización química del producto final y los efectos de su posterior manipulación se deben tomar en cuenta.

# CAPÍTULO 2. LA HIPÓTESIS DE LA XENOHORMESIS

Desde hace miles de años las plantas tienen un uso terapéutico. Una tercera parte de los 20 fármacos más vendidos en el mercado son derivados de plantas, y cada semana se descubre una nueva molécula que puede ser beneficiosa para la salud[16]. La economía global y la salud humana dependen en parte de la capacidad para descubrir medicinas nuevas y efectivas. Sorprendentemente, no son muchos los esfuerzos destinados al hecho de que las plantas sinteticen moléculas beneficiosas para la salud de otros organismos. Una de las razones es la facilidad de patentar nuevos fármacos sintéticos (conocidos como "nuevas entidades químicas"), y otra es la *suciedad* de los compuestos derivados de las plantas. Un compuesto se considera *sucio* cuando interacciona con numerosas proteínas endógenas. A priori, un compuesto de este tipo, con varias dianas moleculares, puede tener un efecto imperceptible, o incluso adverso, frente a una molécula pura que específicamente interacciona con una proteína en concreto[16].



**Figura 4.** La corteza del sauce blanco contiene una gran cantidad de ácido salicílico.

Contrariamente a esta idea, existen numerosos ejemplos de moléculas vegetales que interaccionan con diversos enzimas y receptores y que son extraordinariamente seguras. Ya en el siglo V a. C., el griego Hipócrates escribió acerca del ácido salicílico "*un polvo amargo extraído de la corteza del sauce que alivia dolores y reduce la fiebre*". En 1763, el reverendo Edwar Stone experimentó con la corteza del sauce blanco (*Salix alba*, Figura 4) en el tratamiento de la fiebre y concluyó que era "*un remedio muy eficaz*". Desde entonces se ha aislado una gran variedad de salicilatos de diferentes plantas que han ayudado en el tratamiento de la gota, la fiebre reumática, el dolor y la artritis, y hoy en día se fabrican 45000

toneladas métricas anuales de un derivado acetilado del ácido salicílico para tratar una gran variedad de enfermedades: la aspirina.

Éste sólo es un ejemplo de las decenas de compuestos que se conocen hoy en día derivados de las plantas, que son beneficios para la salud y que además interaccionan con más de una diana molecular.

## 2.1 La hormesis

Los organismos vivos se topan continuamente con situaciones adversas o estímulos nocivos, y la adaptación a estos agresores externos, ya sean químicos, físicos, biológicos o sociales, es un principio clave de supervivencia.

Por otro lado, la exposición leve a un estímulo que a altas concentraciones puede ser perjudicial, puede proporcionar posteriormente resistencia o tolerancia a una agresión, incluso protagonizada por el mismo estímulo. Esta respuesta adaptativa al estrés se ha identificado como un proceso conservado evolutivamente.



En toxicología, el término hormesis, define una respuesta biológica de dos fases, no lineal, donde la exposición a una dosis baja (o a un estímulo débil) por parte de un tóxico ambiental (o una situación nociva) produce un efecto potencialmente beneficioso, mientras que una dosis alta produce efectos adversos[17] (Figura 5). En el campo de las disciplinas biomédicas, el término hormesis se define como una respuesta adaptativa de las células y organismos frente a un estrés moderado (o intermitente)[18].

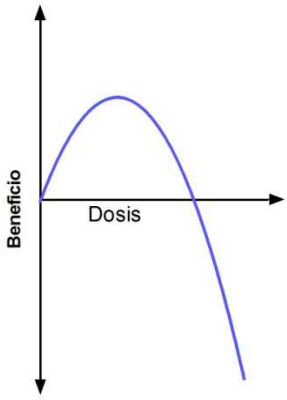


**Figura 5.** Una dosis muy baja de un agente perjudicial puede producir efectos beneficiosos en un organismo.

Así pues, la hormesis se podría definir como *un proceso en el que la exposición a una dosis baja a un compuesto químico o factor ambiental, que es perjudicial a altas concentraciones, induce un efecto beneficioso adaptativo sobre la célula u organismo*.

La hormesis representa un concepto fundamental en la teoría evolutiva, explicando cómo la vida en la tierra se ha adaptado a un ambiente a veces tan agresivo. Para sobrevivir a los venenos ambientales, los organismos han desarrollado diferentes vías de señalización celular que median las respuestas horméticas. Estos incluyen factores de transcripción y las cinasas que los regulan, modulando la expresión de genes codificantes de una batería de proteínas citoprotectoras y de resistencia al estrés (como chaperonas, enzimas antioxidantes y detoxificantes, etc.)[19].

El estrés oxidativo es uno de los factores principales en el envejecimiento y en las enfermedades crónicas. Uno de los factores principales que afectan al nivel de estrés oxidativo es la cantidad de energía ingerida y por ello, despierta un gran interés cómo pueden influir los factores dietéticos en el curso de una enfermedad modificando el estrés oxidativo. La cantidad de calorías consumidas se correlacionan inequívocamente con un riesgo más elevado de padecer enfermedades crónicas[20]. Por ejemplo, los ácidos grasos saturados y el colesterol pueden inducir enfermedades cardiovasculares[21,22] y dietas altas en azúcares simples incrementan el riesgo de diabetes[23].



En animales de experimentación, muchos estudios de restricción calórica (bien por reducción en la ingesta o por periodos de ayuno intermitentes) demuestran que las células de estos animales incrementan su resistencia a diferentes tipos de estrés. Por ejemplo, la mortalidad por causas naturales o inducida por temperatura o toxinas concretas, se reduce significativamente en aquellos animales sometidos a una restricción calórica frente a los animales que consumieron una dieta normal *ad libitum*[24-27]. La ingesta reducida de energía,

también protege a los animales de diferentes tipos de cáncer en varios tejidos como tumores pancreáticos[28], tumores mamarios[29] y cáncer de próstata[30], cada uno inducido mediante diferentes compuestos químicos. En humanos, el ayuno en días alternos mejora los síntomas y reduce marcadores de inflamación y de estrés oxidativo en pacientes con asma[31].

Otro ejemplo de proceso hormético además de la restricción calórica, es la exposición a bajas concentraciones a algunos fitoquímicos. Los organismos han evolucionado hasta adquirir la capacidad de detectar marcadores de estrés producidos por otras especies de su mismo hábitat. En esta línea, los organismos pueden prepararse con antelación frente a condiciones ambientales adversas. Esta hormesis entre especies, se conoce como xenohormesis, que describe un fenómeno donde un organismo detecta las señales químicas de otras especies acerca del estado del entorno o de la disponibilidad de alimentos y responde a ellas de forma que generen en él efectos beneficiosos[16,32].



## 2.2 La xenohormesis

Las plantas y animales comparten un alto grado de homología entre sus vías metabólicas de respuesta al estrés[33]. Cuando las plantas están sometidas a condiciones ambientales adversas, como la sequía, infecciones microbianas o plagas de insectos, producen compuestos químicos que les ayudan a resistir o incluso las protegen de dichas condiciones ambientales adversas. Por otro lado, como los animales dependen de las plantas como fuente alimenticia, han desarrollado mecanismos que les permiten detectar estas sustancias bioactivas producidas por las plantas estresadas, con la finalidad de estimar las cambiantes condiciones externas[33]. En este contexto, estos fitoquímicos producidos por las plantas como autodefensa frente a condiciones adversas son inherentemente fitoalexinas (toxinas vegetales). Estos **fitoquímicos xenohormético**s, que alertan a

los animales de la adversidad, pueden estimular sus vías metabólicas de respuesta al estrés y, temporalmente, fortalecer su capacidad de defensa celular. El término xenohormesis se acuñó para definir esta interacción entre especies.

Son miles los compuestos identificados provenientes de las plantas y que, en muchos casos, proveen de efectos beneficiosos a los animales[34,35]. Puesto que el estrés oxidativo es un factor importante en las enfermedades crónicas[36], y que las frutas y verduras contienen compuestos químicos que, a altas concentraciones, poseen la capacidad de eliminar radicales de oxígeno, en una primera hipótesis se atribuyeron los efectos beneficiosos para la salud de estos fitoquímicos a su capacidad antioxidante[37]. De hecho, concentraciones micromolares de numerosos polifenoles protegen frente al estrés oxidativo en experimentos realizados en varios modelos celulares, la aterosclerosis y enfermedades neurodegenerativas[38-40]. Sin embargo, los resultados obtenidos en ensayos clínicos y estudios primarios con dosis altas de dichos antioxidantes son muchas veces desilusionantes[41].

La hipótesis xenohormesis ha sido recientemente lanzada como posible mecanismo para explicar, al menos parcialmente, los efectos de las plantas en los animales[16].



## 2.3 La inducción de genes sensibles al estrés celular mediante compuestos xenohorméticos.

La respuesta al estrés por parte de las plantas ha evolucionado durante al menos un millón de años. Ya que la gran mayoría de las plantas no pueden desplazarse físicamente, deben soportar el estrés ambiental que acontezca en cada momento. Este tipo de "vida sedentaria" puede explicar la complejidad de su respuesta al estrés[33]. Las plantas producen toxinas para protegerse contra hongos, insectos y predadores. En consecuencia, las plantas cultivadas destinadas al consumo contienen menos toxinas naturales que sus homólogas silvestres[42].

Cuando las plantas están bajo condiciones agresivas, se aprecia un aumento en la acumulación de pesticidas naturales (biopesticidas) que en ocasiones pueden llegar a producir intoxicaciones agudas en humanos. Algunos estudios estiman que el 99,99% de los pesticidas presentes en la dieta son compuestos químicos que producen las plantas para protegerse[43].

La xenohormesis puede explicar cómo los compuestos químicos que producen las plantas en condiciones ambientales adversas, y que utilizan como defensa ante éstas, pueden producir efectos beneficiosos en los animales que las consumen. Los animales aprovechan la información contenida en estos compuestos producidos por las plantas ante el estrés. De hecho, la mayoría de los efectos beneficiosos para la salud que se conocen de las plantas comestibles se atribuyen a las moléculas fabricadas en respuesta al estrés. Aunque los compuestos xenohormético poseen propiedades nocivas para los insectos y microorganismos, a las dosis subtóxicas ingeridas por los humanos como parte de su dieta, los mismos compuestos inducen respuestas de estrés celular moderadas[44,45]. Estas a su vez, activan vías de adaptación de respuesta al estrés, lo que deriva en un aumento de la expresión de genes codificantes de proteínas citoprotectoras como enzimas antioxidantes, chaperonas, factores de crecimiento, enzimas de detoxificación de fase-2, proteínas mitocondriales, etc. Por ejemplo, el estrés oxidativo causado por algunos flavonoides (con actividad prooxidante) contribuye a sus actividades beneficiosas para la salud induciendo enzimas antioxidantes, resultando en un efecto beneficioso, que partió de una supuesta reacción química tóxica.



Otro ejemplo es el resveratrol, que modula directamente alrededor de 30 enzimas y receptores sin ningún indicio de toxicidad, y los efectos observados radican en la inhibición de algunos o en la activación de otros[46]. Los polifenoles del té verde y la curcumina, interaccionan con decenas de dianas moleculares proporcionando numerosos beneficios para la salud[47,48].

# CAPÍTULO 3. POLIFENOLES Y ENFERMEDAD

La señal química de estrés ambiental en plantas son los polifenoles, los cuales otorgan protección frente a estrés en los humanos o animales que los ingieren. Esto sugiere la existencia de mecanismos que detecten este contenido polifenólico inducido por estrés. Así pues, el estrés sucede en las plantas, y los beneficiarios son los animales sensibles a estas señales químicas tras ingerirlas.

Un hecho a tener en cuenta es, contrariamente a lo que se creía hasta hace poco, que muchos de los efectos de los polifenoles pueden no estar ligados a sus propiedades antioxidantes intrínsecas, sino a cambios adaptativos que involucren a estas moléculas en la regulación de mecanismos de respuesta al estrés metabólico. Es decir, algunos mecanismos son químicamente simples (por ejemplo, actividad antioxidante), pero otros compuestos parecen actuar como moléculas de señalización o mensajeros químicos[49,50].

Las plantas estresadas proveen de una enorme fuente de moléculas xenohorméticas que pueden modular enzimas involucrados en la regulación de la respuesta al estrés y en la supervivencia celular. El interés en este tema emergió debido a hallazgos que indicaban que la ingesta de una dieta hipocalórica (como ejemplo de estrés moderado), incrementaba la supervivencia en numerosos modelos experimentales. Estos efectos fueron similares a los observados en los polifenoles a través de la activación de las sirtuinas[51,52].



Recientemente, experimentos de restricción de glucosa han otorgado una conexión plausible entre reguladores metabólicos críticos, indicando que los polifenoles deberían ser estudiados como moléculas de señalización[53]. La ausencia de glucosa aumenta la actividad de AMPK, y ésta induce la transcripción del enzima sintetizador de NAD, Nampt, necesario para la activación de SIRT1. Esto es todavía más importante considerando que ATP y NAD son indicadores del

estado energético celular, auditado por AMP-cinasa (ratio AMP/ATP) y las sirtuinas (que requieren de NAD para deacetilar substratos).

Muchos polifenoles son moduladores de la transcripción[54]. Aunque los mecanismos son difíciles de comprobar debido a las actividades pleiotrópicas de los polifenoles, la supresión de la actividad NF-κβ sugiere que los polifenoles poseen un papel importante en la modulación de la resistencia a la insulina y la inflamación[55]. Este hecho es particularmente importante porque estos efectos se solapan con los ya conocidos factores de riesgo de las enfermedades crónicas. El hecho de que los animales y plantas compartan un alto grado de homología en las secuencias de las vías de las cinasas reguladas por señales extracelulares (ERKs), explicaría que muchos polifenoles puedan modular vías de las cinasas, incluida AMPK, y que los polifenoles puedan modular simultáneamente la señalización redox e inhibir la función mitocondrial, son mecanismos potenciales a tener en cuenta[56,57]. Así pues, una reducción en la señalización inducida por estrés con un incremento en radicales libres mitocondriales y una subsecuente reducción en la producción de ATP, pueden ser efectos predecibles debidos a la ingestión de polifenoles, sugiriendo implicaciones importantes en la prevención de las enfermedades crónicas y el envejecimiento.



Los mecanismos moleculares sobre los que actúan los polifenoles todavía están por dilucidar. Son muchos los polifenoles y muchas sus dianas potenciales. Además, se debe tener en cuenta que los mecanismos que un polifenol pueda activar pueden no ser los mismos si es administrado individualmente o si lo hace formando parte de una mezcla compleja de polifenoles. Dependiendo de la patología que se estudie y de las vías metabólicas que estén alteradas en cada caso, la *receta óptima* de polifenoles puede variar sustancialmente.

## 3.1 Obesidad

La obesidad se define como una enfermedad crónica multifactorial caracterizada por un aumento de masa grasa, y por lo tanto del peso corporal, como consecuencia de un balance energético positivo mantenido en el tiempo[58]. Esta ganancia de peso supone normalmente un aumento de las reservas del organismo en forma de grasa en relación con el promedio normal para la edad, sexo, talla y complexión[59,60]. Actualmente se considera la obesidad como una de las alteraciones metabólicas de mayor repercusión no sólo desde el punto de vista sanitario, sino también desde ámbitos psicológicos, sociales y económicos[61]. Este fenómeno, de naturaleza epidémica, se debe en gran medida a los cambios ambientales y sociales que han tenido lugar en las últimas décadas y que han interaccionado con una determinada predisposición génica[62].

Actualmente, este trastorno se define como pandemia, con creciente prevalencia[63], que supone una grave amenaza para la salud pública, debido al riesgo a desarrollar enfermedades asociadas como la diabetes, hipertensión, enfermedades cardiovasculares, alteraciones inflamatorias, aumento del riesgo de padecer cáncer, insuficiencia respiratoria y osteoartritis, entre otras, y por el elevado coste sanitario que se deriva de las mismas[64].



Algunos de los flavonoides más estudiados en el tratamiento de la obesidad son los extraídos del té. Los compuestos mayoritarios del té son las catequinas, principalmente (-)-epicatequina (EC), (-)-epicatequina-3-gallato (ECG), (-)epigalo-catequina (EGC) y (-)-epigalocatequina-3-galato (EGCG)[65]. Los extractos de hojas del té mostraron una importante actividad hipolipemiante en ratas después de 63 semanas de tratamiento[66].

Los efectos de las catequinas purificadas sobre el peso de las ratas también fueron estudiados[67]. Cabe destacar que solamente la inyección intraperitoneal de EGCG, y no de las otras catequinas (EC, EGC y ECG), causó una pérdida de peso

corporal en ratas de ambos sexos en un periodo comprendido entre 2 y 7 días de tratamiento. Los mismos resultados se obtuvieron suplementando la dieta con EGCG en ratones alimentados con una dieta rica en grasa[68]. En este último estudio, los animales control ganaron el doble de peso que los animales tratados con EGCG, aunque la ingesta de comida no mostró diferencias entre grupos. La pérdida de peso se debió exclusivamente a una reducción en la cantidad de grasa corporal[68]. En experimentos realizados en líneas celulares de preadipocitos 3T3-L1, EGCG inhibió la proliferación de forma dosis-dependiente[69].

Los mecanismos moleculares encontrados muestran que los polifenoles del té estimulan la absorción de glucosa acompañada de un reducción en la translocación del transportador de glucosa 4 (GLUT4) en el tejido adiposo, mientras que en el músculo esquelético el aumento de absorción de glucosa se acompaña de un aumento en la translocación de GLUT4 a la membrana celular. Además, estos polifenoles suprimen la expresión de PPARγ y activan SREBP-1 en el tejido adiposo. En resumen, los polifenoles del té muestran efectos biológicos en la prevención de la obesidad modulando la absorción de glucosa en el tejido adiposo y en el músculo esquelético y suprimiendo la expresión de factores de transcripción relacionados con la adipogénesis[70].



Otros estudios se centran en la modulación de la actividad de la lipasa pancreática (LP)[71]. Este enzima, sintetizado en los humanos por el gen PNLIP, posee una función clave en la digestión de los triglicéridos[72]. La LP se secreta al duodeno y es la responsable de la hidrólisis del 50-70% de la grasas ingeridas[73]. Este enzima se utiliza a menudo para evaluar el potencial de eficacia de productos naturales como agentes antiobesidad[74]. El orlistat es uno de los fármacos aprobados en Europa que se utiliza en el tratamiento de la obesidad. Esta molécula inhibe la actividad de la LP reduciendo la absorción de triglicéridos[75].

Muchos polifenoles como los flavanoles, taninos y calconas inhiben la LP[73,76]. Un flavonoide con actividad inhibitoria de la LP es la hesperidina, obtenida de la

cáscara de *Citrus unshiu*[77]. Las proantocianidinas reducen la concentración plasmática de triglicéridos bloqueando la absorción de lípidos de la dieta[78] e inhiben enzimas digestivos como la tripsina, la amilasa y la lipasa[74].

## 3.2 La aterosclerosis

Los polifenoles han recibido una gran atención en la prevención de enfermedades cardiovasculares, derivada en parte de la llamada paradoja francesa, que se ha justificado en principio debido a la protección antioxidante que ofrecen los polifenoles presentes en el vino y las verduras. Tal vez el compuesto más estudiado en este contexto es el resveratrol. Se ha demostrado que el resveratrol ejerce efectos protectores en el modelo de ratones deficientes en la apolipoproteína E[79], actuando en la concentración sanguínea de lípidos y sobre el desarrollo de la placa aterosclerótica. El mecanismo parece ser, al menos en parte, antiinflamatorio, ya que disminuye la expresión de ICAM-1 y VCAM-1 en los vasos sanguíneos. Sin embargo, el resveratrol no afectó a los niveles sanguíneos de lípidos en conejos hipercolesterolémicos[80] o en ratas normales[81], aunque mejora la concentración lipídica en sangre en ratas Zucker, un modelo animal de obesidad que, en avanzada edad, muestran algunas alteraciones similares a las observadas en la diabetes tipo II[82]. De hecho, otro estudio mostró una extensión de las lesiones ateroscleróticas sin otro efecto significativo sobre los lípidos o la oxidación de las LDL[80]. Así pues, es poco probable que el resveratrol tenga efectos protectores cardiovasculares a través de cambios en el perfil lipídico. El resveratrol puede inhibir la producción de especies reactivas de oxígeno generadas a raíz de la captación de LDL oxidadas por los macrófagos[83]. Esta molécula también reduce la infiltración celular, la fibrosis y la expresión de citoquinas inflamatorias en un modelo de miocarditis autoinmune[84]. Otros polifenoles como la miricetina y la fisetina reducen la oxidación de las LDL y disminuyen la expresión de CD36[85]. Ambas acciones favorecen la reducción de la formación de células espumosas y por lo tanto son considerados protectores

contra la aterosclerosis. Otra forma que tienen los flavonoides de evitar la oxidación de las LDL es mediante el bloqueo de mieloperoxidasa de los neutrófilos. Este efecto es menos importante en los metabolitos de los flavonoides[86], que por lo general son menos bioactivos que sus precursores en forma de aglicona intacta[87]. Cabe señalar también que los macrófagos ubicados en las placas de ateroma pueden absorber conjugados de quercetina y convertirlos de nuevo a quercetina activa[88]. El mismo mecanismo (prevención de la oxidación de las LDL y reducción de la expresión de CD36) se ha descrito para ECG que, curiosamente, parece ejercer esta acción específicamente en las lesiones ateroscleróticas[89]. Lo mismo ocurre con el kaempferol y la rhamnocitrina[90], la quercetina[91], e incluso la quercetina-3-glucurónido[88].

Otros mecanismos de protección incluyen la activación del ABCA1, lo que posiblemente aumente la concentración sanguínea de las HDL[92]; la menor producción de NADPHox[93,94]; actividades antiaterotromboticas[95,96]; descenso de MCP-1[97] e inhibición de la proteína C reactiva[98].



Los polifenoles tienen efectos puramente vasculares que no se detallan en esta memoria[99]. Además, la mayor parte de la evidencia apunta a que los efectos antiateroscleróticos se deben a un mecanismo antinflamatorio, más que antioxidante.

## 3.3 Enfermedades inflamatorias crónicas.

La inflamación está presente en casi todas las enfermedades, pero en algunas como el asma, la artritis reumatoide o las alergias, el componente inflamatorio es especialmente importante.

Los polifenoles pueden prevenir la aparición de tejidos neoplásicos mediante la modulación de la respuesta inmune. Por ejemplo, se ha demostrado que la genisteína oral protege contra la carcinogénesis a través de la inmunoestimulación, reduciendo la expresión de Treg, y mejorando la

citotoxicidad de las células T y la función de las células *Natural Killer*[100]. La genisteína también evita la nefrotoxicidad mediante vías antinflamatorias[101] y previene la inflamación y ulceración de la mucosa gástrica[102], efecto compartido por otros flavonoides como la catequina[103]. Varios flavonoides protegen contra el shock inducido por LPS en modelos animales, incluida la rutina[104], la baicaleina[105], la apigenina[106], la hesperidina[107], el resveratrol[108], la quercetina 3-O-beta-(2(")-galoil)-rhamnopyranosido[109], y EGCG[110,111]. Estos efectos probablemente se deriven de la inhibición de las funciones de los monocitos. Un estudio realizado en ratones apunta que la quercetina protege contra el shock inducido mediante *Salmonella typhimurium*-aroA[112]. En cualquier caso, la quercetina no muestra ningún efecto protector frente a la fiebre inducida por LPS[113]. Además, la quercitrina, otro flavonoide, protege contra el shock anafiláctico[114]. El resveratrol[115,116] y la baicalina[117,118] muestran efectos protectores frente a la pancreatitis experimental, mientras que el ácido elágico es útil en el tratamiento la pancreatitis crónica[119]. La demetilnobiletina reduce la hipersensibilidad de tipo retardada o tardía, con una menor proliferación de células T, menor síntesis de citoquinas, y mayores niveles de IL-10[120]. El resveratrol[121] y la baicalina[122] tienen efectos protectores contra el daño hepático inducido por tetracloruro de carbono ($CCl_4$) y además el primero mejora la lesión hepática en un modelo de hemorragia traumática[123].



## 3.4 La enfermedad del hígado graso no alcohólica (NAFLD)

El hígado es el principal órgano metabólico y lleva a cabo una gran variedad de funciones bioquímicas necesarias para la homeostasis metabólica. Este órgano es sensible a muchos procesos patológicos, siendo la NAFLD la causa más común de disfunción hepática[124].

La NAFLD describe una condición clínico-patológica mayoritariamente asintomática, caracterizada por una significativa acumulación de lípidos en forma de vesículas (esteatosis, contenido graso > 5-10 % en peso) en los hepatocitos del parénquima del hígado y en ausencia de un consumo de alcohol significativo[125,126].

Esta enfermedad engloba la esteatosis con un pronóstico benigno, y la esteatosis acompañada de inflamación y de daño hepatocelular (esteatohepatitis no alcohólica o NASH), la cual puede desembocar en fibrosis avanzada, cirrosis y en último extremo en carcinoma hepatocelular[125]. Por tanto, debido a que esta acumulación lipídica en el hígado es un prerrequisito para el desarrollo de NASH y de sus complicaciones, el estudio de la esteatosis puede ser la base del desarrollo de nuevas estrategias de prevención y tratamiento de las enfermedades hepáticas crónicas.

Estudios recientes demuestran que algunos polifenoles[127-131] o extractos de polifenoles[132] pueden impedir, al menos parcialmente, la esteatosis hepática inducida por dietas ricas en grasa. De hecho, los polifenoles pueden actuar sobre el metabolismo lipídico incidiendo en la actividad del enzima desaturasa. Por ejemplo, algunos polifenoles reducen la expresión del gen de la Δ-9 desaturasa (estearoil-CoA desaturasa 1; SCD1)[68,133] y de la Δ-6 desaturasa[134]. Sin embargo, queda estudiar si los polifenoles pueden modular la composición de los ácidos grasos presentes en el hígado, no sólo el contenido total de lípidos. Los polifenoles también pueden actuar sobre las vías de señalización del metabolismo lipídico, incluyendo la síntesis y degradación de estos. Diferentes estudios[56,135] demostraron que niveles elevados de glucosa inhiben la actividad de la AMPK y la fosforilación de la acetil-CoA carboxilasa, aumentando por lo tanto el contenido de lípidos en los hepatocitos. Sin embargo, la administración de polifenoles revirtió la inhibición de la actividad de la AMPK y la fosforilación de la acetil-CoA carboxilasa[56]. Por otra parte, se ha demostrado que los polifenoles estimulan la AMPK y evitan la acumulación de lípidos en los hepatocitos, probablemente mediante la activación de sirtuina-1 deacetilasa[135]. Como las

mitocondrias son un orgánulo clave en el metabolismo lipídico y la principal fuente de especies reactivas de oxígeno y nitrógeno en los hepatocitos, se postulan como candidatas para desempeñar un papel central en la progresión de la esteatosis a esteatohepatitis no alcohólica y cirrosis[136]. Algunos polifenoles también han demostrado capacidad para modificar la actividad mitocondrial y aumentar la mitocondriogenesis[137]. Este efecto puede aumentar el catabolismo de lípidos mitocondrial y por lo tanto, atenuar la acumulación hepática de ácidos grasos.

La activación de AMPK puede considerarse una diana terapéutica adecuada para tratar esta enfermedad, y los polifenoles son compuestos naturales que actúan directamente sobre esta proteína[138].

## 3.5 Especies vegetales a estudio

En nuestra región geográfica, donde disfrutamos de la dieta mediterránea, es muy común el consumo de alimentos como las frutas, las verduras, el aceite o el vino. Desgraciadamente, el contenido de polifenoles en estos alimentos es muy bajo y su absorción es poco eficiente. Por otro lado, la absorción de una cantidad aceptable de polifenoles a partir de alimentos como el aceite o el vino, conllevaría la ingesta en cantidades nada saludables de otros componentes como grasas o alcohol. Así pues, es de gran interés encontrar e identificar plantas con un alto contenido en polifenoles que puedan ser incorporados a la dieta. En la presente tesis doctoral, se han centrado los esfuerzos tanto en *Hibiscus sabdariffa*, procedente del Sahel africano como en *Aspalathus linearis* (rooibos) procedente de un valle de la República de Sudáfrica (Figura 6).



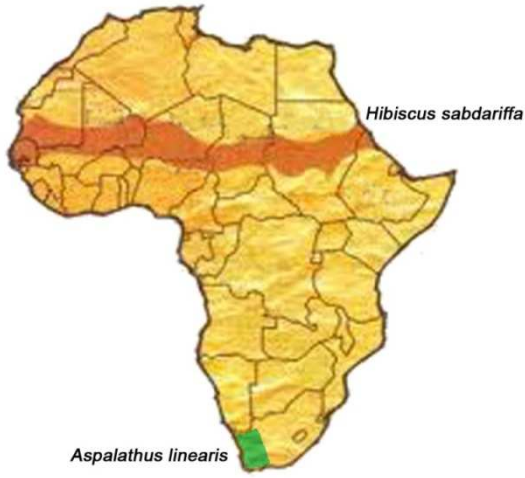


**Figura 6.** Distribución geográfica de las dos especies vegetales estudiadas, *Hibiscus sabdariffa* en el Sahel africano, y *Aspalathus linearis* en la costa oeste de Sudáfrica.

### 3.5.1 *Hibiscus sabdariffa*

**Figura 7.** Los cálices de *Hibiscus sabdariffa* poseen una gran cantidad de polifenoles.

*Hibiscus sabdariffa* L., (de la familia de las malváceas, Figura 7), generalmente llamado bissap, karkadé o flor de Jamaica, es una planta tropical consumida habitualmente como refresco o infusión. Tradicionalmente, se ha utilizado con eficacia contra la hipertensión, la inflamación y los trastornos hepáticos[139-141]. Estudios previos muestran que *H. sabdariffa* posee actividad antitumoral, antioxidante e hipolipemiante[142-147]. Recientemente, fue publicado que el extracto de *H. sabdariffa* inhibe la oxidación de las LDL y reduce la concentración de triglicéridos en suero[148], y el colesterol y el colesterol-LDL en animales de experimentación[149]. El examen histológico reveló que podría reducir la formación de células espumosas e inhibir la proliferación y migración de las células vasculares de músculo liso, lo que sugiere el efecto antiaterosclerótico de *H. sabdariffa*.



Además, los estudios en humanos muestran efectos antihipertensivos y antiinflamatorios[150,151]. Las antocianinas son los pigmentos que otorgan el color rojo a la planta y son los responsables de la amplia gama de colores en muchos alimentos. La fuente principal de flavonoides, incluyendo dichas antocininas, son los pétalos [152]. Las propiedades antioxidantes de *H. sabdariffa* y otras especies de hibisco han sido ampliamente estudiados[153,154].

### 3.5.2 *Aspalathus linearis*

El té rooibos, es una infusión derivada de *Aspalathus linearis* (Figura 8), nativa de la costa oeste de la República de Sudáfrica. Es un arbusto utilizado popularmente en la preparación de un té suave que no contiene cafeína, muy pocos taninos y grandes cantidades de polifenoles. Existen dos tipos de rooibos, el natural y el fermentado. El color natural del rooibos es verde, pero durante su fermentación, el color cambia de verde a rojo debido a la oxidación de los polifenoles[155]. Las características más relevantes del rooibos radican en su actividad antioxidante[156,157] y antimutagénica[158].

**Figura 8.** Flores de *Aspalathus linearis* en la costa oeste de Sudáfrica.



Estudios *in vitro* e *in vivo* demuestran que el rooibos posee una gran bioactividad. Previene la oxidación lipídica y el estrés oxidativo[159,160], la inflamación[161], y demuestra una gran capacidad hepatoprotectora[162].

Los compuestos mayoritarios identificados en esta planta son la orientina, la iso-orientina, la rutina y la aspalatina[163-165]. Algunos de estos compuestos son absorbidos efectivamente, lo que ratifica el valor de esta planta puesto que los polifenoles no se absorben con facilidad[166-169]. Algunos estudios *in vivo* muestran que la aspalatina posee efectos hipoglicemiantes en un modelo de ratón diabético[170]. La rutina en cambio, posee efectos antihipertensivos[171] y la orientina muestra actividad cardioprotectora[172].

# CAPÍTULO 4. TÉCNICAS ANALÍTICAS

Un parte importante en el estudio de los componentes biactivos de las plantas, es el desarrollo de técnicas analíticas que permitan determinar los compuestos presentes en los extractos vegetales. Estas técnicas deben ser robustas, reproducibles, rápidas y con el menor coste económico posible. Para llevar a cabo la identificación de los compuestos individualmente, deben utilizarse técnicas separativas como la electroforesis capilar (CE), la cromatografía de gases o la cromatografía líquida de alta resolución (HPLC).

La caracterización de los compuestos polifenólicos de las plantas utilizadas en esta memoria se ha llevado a cabo utilizando HPLC (Figura 9). A continuación se describen brevemente los sistemas de separación y detección utilizados.

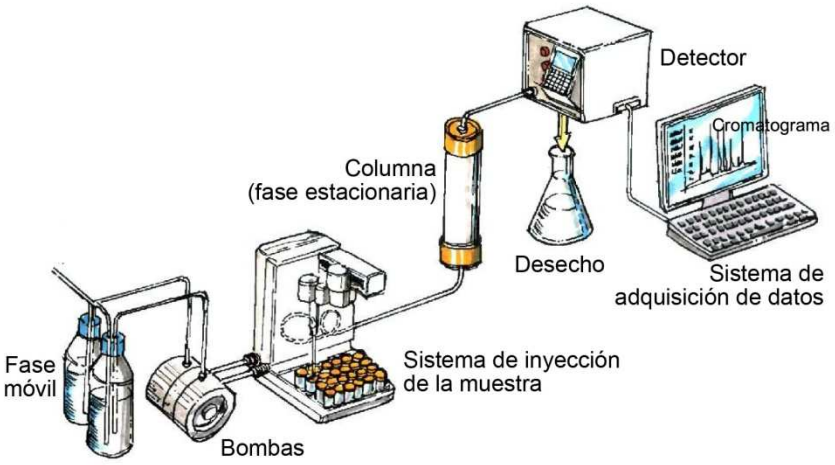


**Figura 9.** Esquema general de las partes principales de un sistema de análisis de muestras.

## 4.1 Cromatografía líquida de alta resolución (HPLC)

La HPLC (del inglés *high-performance liquid chromatography*) es una técnica de separación utilizada para analizar la composición de mezclas complejas. En esta técnica, la mezcla a resolver se inyecta en un sistema formado por un fluido, fase móvil, que circula en contacto con una fase fija o inmóvil (fase estacionaria). En el caso de la cromatografía líquida, la fase estacionaria es un sólido o un líquido fijado en un sólido y la fase móvil es un líquido. Las dos fases se eligen de forma que los componentes de la mezcla que se quiere analizar se distribuyan de distinto modo entre la fase móvil y la estacionaria. Aquellos componentes que son fuertemente retenidos por la fase estacionaria se mueven lentamente con el flujo de la fase móvil; por el contrario, los componentes que se unen débilmente a la fase estacionaria se mueven con rapidez. Como consecuencia de la distinta movilidad, los componentes de la mezcla se separan en bandas o zonas discretas que pueden analizarse cualitativa o cuantitativamente (Figura 10).



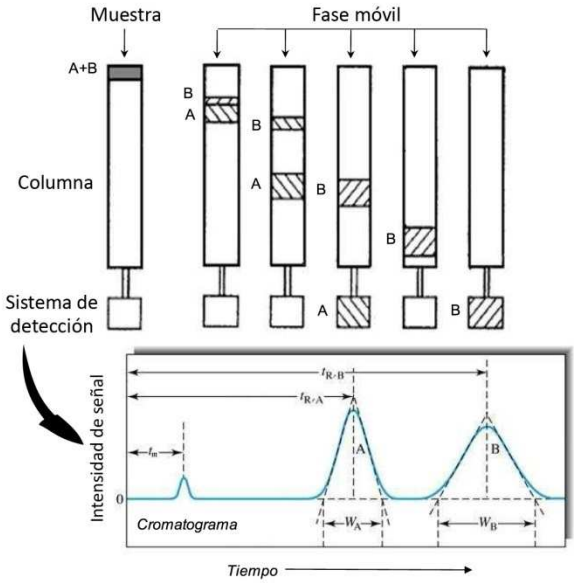


**Figura 10.** Los componentes de una mezcla se separan al pasar a través de una columna dependiendo de la fuerza de interacción con la misma.

La cromatografía líquida clásica o a baja presión se lleva a cabo en una columna generalmente de vidrio rellena con la fase estacionaria. La muestra se introduce por la parte superior y la fase móvil se hace fluir a través de la columna por gravedad. Las separaciones requieren mucho tiempo y además presentan baja eficacia. Con el objeto de aumentar la eficacia en las separaciones, el tamaño de las partículas de fase estacionaria se fue disminuyendo, lo que generó la necesidad de utilizar un sistema de bombeo a alta presión para conseguir un flujo razonable de la fase móvil. De esta manera nació la técnica de cromatografía líquida de alta resolución (HPLC) que, en fase inversa, es la técnica cromatográfica más empleada en la actualidad.

La cromatografía de fase normal o "Normal phase HPLC" (NP-HPLC) fue el primer tipo de sistema HPLC utilizado en el campo de la química, y se caracteriza por separar los compuestos en base a su polaridad. Esta técnica utiliza una fase estacionaria polar y una fase móvil apolar, y se emplea cuando el compuesto de interés es bastante polar. El compuesto polar se asocia y es retenido por la fase estacionaria. La fuerza de absorción aumenta a medida que aumenta la polaridad del compuesto, y la interacción entre el compuesto polar y la fase estacionaria polar (en comparación a la fase móvil) aumenta el tiempo de retención.



Esta técnica cayó en desuso con la aparición de la cromatografía de fase reversa a finales de los años 70. La HPLC de fase reversa (RP-HPLC) consiste en una fase estacionaria apolar y una fase móvil de polaridad moderada. El tiempo de retención es mayor para las moléculas de naturaleza apolar, mientras que las moléculas de carácter polar eluyen más rápidamente.

La cromatografía de fase reversa es tan utilizada que a menudo se le denomina HPLC sin ninguna especificación adicional. Esta técnica se basa en el principio de las interacciones hidrofóbicas que resultan de las fuerzas de repulsión entre un disolvente relativamente polar, un compuesto relativamente apolar, y una fase estacionaria apolar.

## 4.2 Espectrometría de masas (MS)

En la actualidad, la MS es probablemente la herramienta analítica con mayores aplicaciones. Es capaz de analizar prácticamente cualquier molécula[173] debido a su selectividad y es uno de los pocos sistemas de detección que proporciona información estructural.

La MS se basa en la separación al vacío de iones en fase gaseosa de acuerdo con su relación masa/carga (*m/z*). Su combinación con una técnica de separación presenta la ventaja de proporcionar una segunda dimensión de separación, ya que tras separar los compuestos según su tiempo de retención, el espectrómetro de masas produce una separación en función de la relación *m/z*, siendo muy útil en el análisis de muestras complejas[174].

La versatilidad de la MS se debe, en parte, al amplio abanico de posibilidades de cada una de las secciones del espectrómetro, en concreto el resultado que se obtenga va a depender notablemente de cuáles sean la interfase (método de ionización) y el analizador utilizados (Figura 11).



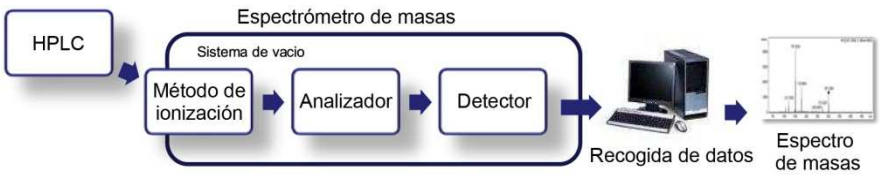


**Figura 11.** Un espectrómetro de masas consta de una interfase (método de ionización) que vaporiza la muestra, un analizador que se encarga de separar las moléculas en base a su relación *m/z* y un detector.

Para llevar a cabo el acoplamiento de las técnicas separativas empleadas que trabajan en fase líquida (HPLC y CE), con un espectrómetro de masas, en el que las sustancias para ser analizadas deben estar en fase gaseosa, es necesaria la utilización de una interfase adecuada que transfiera los iones desde una fase a

otra. A pesar de la variedad de interfases que se han desarrollado para estos acoplamientos[175,176], la más utilizada es la ionización por electrospray (ESI). Esta interfase permite un análisis eficaz de compuestos polares, lábiles y de compuestos con un alto peso molecular (normalmente hasta 100.000 Da). Por otro lado, es fácil de implementar, es sensible y puede utilizarse en un amplio espectro de aplicaciones.

En el analizador de masas se produce la discriminación entre los iones de diferente *m/z* al ser sometidos a campos eléctricos o magnéticos constantes, por pulsos o que varían periódicamente en el tiempo[177]. Los más usados son: cuadrupolo, trampa de iones (IT), triple cuadrupolo (QqQ), tiempo de vuelo (TOF), transformada de Fourier-resonancia de ión ciclotrónica, orbitrap y algunos de los acoplamientos entre varios de los mencionados[178]. Todos ellos se diferencian en tamaño y precio, resolución, rango y exactitud de masa que ofrecen, y en la capacidad de determinar distribuciones isotópicas y de realizar experimentos de masa en tándem (MS/MS o MSn)[173].



A continuación se detallan en líneas generales la interfase y los analizadores utilizados en los trabajos presentados en esta tesis.

### 4.2.1 Ionización por electrospray (ESI)

Esta técnica de ionización suave se utiliza para acoplar técnicas de separación que trabajan en fase líquida y MS para el análisis de biomoléculas polares, no volátiles y térmicamente lábiles, en parte por su capacidad de formar iones mono- o multicargados.

En el proceso de formación del electrospray, el cual se lleva a cabo a presión atmosférica, intervienen diversos mecanismos al mismo tiempo[179].

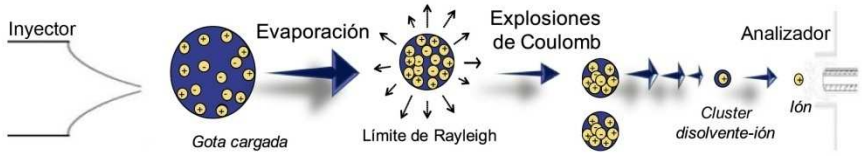


**Figura 12.** Proceso de ionización en una interfase de ionización por electrospray (ESI).

El primer paso en la creación de iones aislados es la formación de un cono de líquido en la punta de la aguja de la interfase, desde donde se expelen microgotas cargadas (Figura 12); es entonces cuando la muestra procedente del capilar o columna de separación y generalmente con la ayuda de un gas nebulizador, se carga y dispersa simultáneamente. El disolvente se va evaporando de las microgotas, aumentando su densidad de carga eléctrica, de modo que los iones de la igual polaridad son atraídos hacia la superficie de la misma debido al campo electrostático que se aplica entre la salida de la interfase y la entrada al espectrómetro de masas (±2-5 kV). Cuando las fuerzas de repulsión electrostáticas de los iones son mayores que la tensión superficial que mantiene unidas las gotas de forma esférica (límite de Rayleigh), las microgotas se van dividiendo en otras aún más pequeñas (explosiones de Coulomb), que seguirán sufriendo procesos de evaporación sucesivos hasta que finalmente se forman iones cargados desnudos que pasan a fase gaseosa, siendo atraídos hacia la entrada del espectrómetro de masas como consecuencia del voltaje aplicado[180,181].



La ionización puede llevarse a cabo en el modo positivo o negativo. En el modo positivo, se producirá la formación de iones protonados $[MH]^+$ o múltiplemente protonados. En el modo negativo, se observa normalmente la desprotonación de las moléculas, pudiéndose formar también iones múltiplemente desprotonados[179].

## 4.2.2 Analizadores de masas

Una vez que en la interfase se ha llevado a cabo la transferencia de los iones desde la fase líquida a la fase gaseosa, los iones son dirigidos hacia el analizador de masas. Los analizadores de masas permiten la separación, fragmentación, detección y cuantificación de los analitos en estudio con un grado de sensibilidad y selectividad muy elevado, proporcionando información sobre su masa molecular.

### A *Trampa de iones (IT)*

El analizador IT consiste fundamentalmente en un electrodo anular y dos electrodos laterales de geometría hiperbólica, que poseen una perforación que permite la entrada y la salida de los iones. Cuando los iones están dentro, se aplican diferentes voltajes generando un campo eléctrico tridimensional en la cavidad de la trampa. Este campo atrapa y concentra los iones dada su trayectoria de oscilación estable. La naturaleza de la trayectoria depende del potencial y de la relación *m/z* de los iones. Para llevar a cabo la determinación de las especies que entran o se forman en la trampa, los potenciales de los electrodos se alteran sometiendo a los iones confinados a una rampa lineal de radiofrecuencia (RF), de manera que son expulsados progresivamente en la dirección axial en función de su relación *m/z* (Figura 13), como resultado de desestabilizaciones de las trayectorias que mantienen dentro de la trampa. Una vez que estos iones llegan al detector, la señal se procesa y da lugar al espectro de masas.



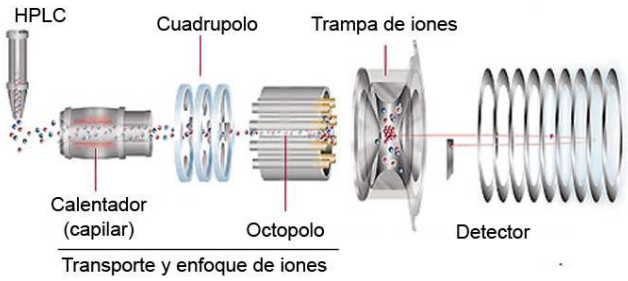


**Figura 13.** Los iones son expulsados de la trampa hacia el detector mediante radiofrecuencia.

## B *Tiempo de vuelo (TOF)*

En el TOF (del inglés, *time of flight*), la separación se basa en un principio bastante simple: la distinta velocidad que adquieren los iones en el interior del analizador en función de su relación *m/z*. Si todos los iones comienzan su vuelo por el analizador al mismo tiempo y con la misma energía cinética, los de menor *m/z* llegarán al detector antes (a mayor velocidad) que los de mayor *m/z*[182] (Figura 14).

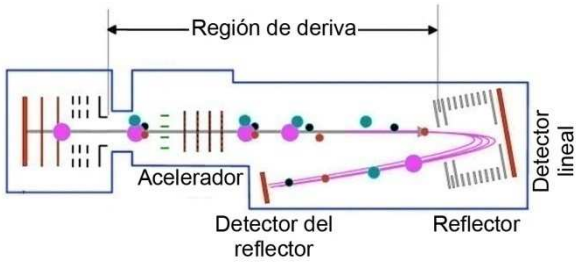


**Figura 14.** En el analizador TOF, los iones se aceleran y se separan dependiendo de su relación *m/z*. De esta manera pueden ser detectados individualmente.

Para ello es necesario emplear una fuente de ionización por pulsos ya que el uso de una fuente de ionización continua, como el ESI, puede ocasionar grandes pérdidas de intensidad. Para eliminar este problema, los iones que provienen del electrospray se introducen y aceleran en el tubo de vuelo en dirección perpendicular al eje del espectrómetro. Este diseño ortogonal en el TOF proporciona una alta eficacia a la hora de transmitir en modo de pulsos los iones que vienen en forma de haz continuo; además, produce dispersiones pequeñas en la velocidad de los iones, obteniéndose una mayor resolución[183].

Una de sus principales ventajas es que proporciona una resolución elevada (Figura 15), lo que permite obtener valores de masa molecular muy exactos; además permite obtener espectros de masas con una transmisión iónica eficaz y proporciona ciclos muy rápidos[182].

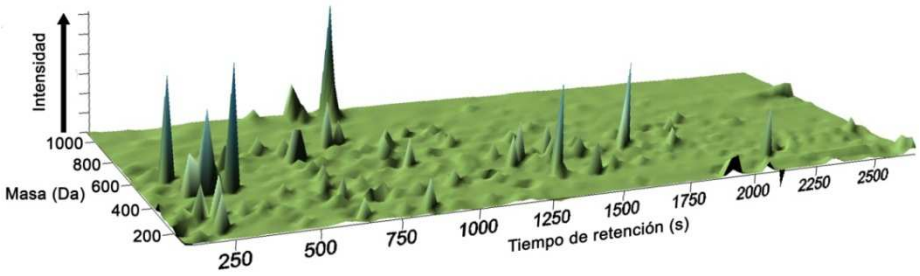


**Figura 15.** Cromatograma en tres dimensiones resultante de la separación de los componentes de una mezcla.

El desarrollo de estas técnicas permite caracterizar de manera relativamente fácil y en poco tiempo los compuestos presentes en un extracto vegetal determinado. Es necesario el desarrollo de éstas técnicas en el análisis de muestras biológicas para determinar qué compuestos son absorbidos y si sufren modificaciones antes de pasar a la sangre. Conocer qué compuestos son retenidos en los tejidos y sus mecanismos moleculares de actuación, puede ser clave en el estudio y desarrollo de terapias eficaces en el tratamiento de enfermedades crónicas como la obesidad, la enfermedad del hígado graso no alcohólica y los procesos inflamatorios asociados.

# HIPÓTESIS Y OBJETIVOS

# Hipótesis

El consumo de extractos ricos en polifenoles de origen vegetal pueden tener efectos beneficiosos en la salud humana.

# Objetivos

1. Desarrollar y estandarizar técnicas analíticas reproducibles para la caracterización y cuantificación de los compuestos presentes en los extractos de plantas.

2. Evaluar la actividad antiinflamatoria.



3. Asesorar la capacidad de extractos vegetales de actuar en la prevención de esteatosis hepática en modelos animales.

4. Establecer posibles mecanismos moleculares de actuación.

# RESULTADOS

RESULT

# ESTUDIO 1

**Direct characterization of aqueous extract of *Hibiscus sabdariffa* using HPLC with diode array detection coupled to ESI and ion trap MS.**

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Short Communication

# Direct characterization of aqueous extract of *Hibiscus sabdariffa* using HPLC with diode array detection coupled to ESI and ion trap MS

The phenolic fraction and other polar compounds of the *Hibiscus sabdariffa* were separated and identified by HPLC with diode array detection coupled to electrospray TOF and IT tandem MS (DAD-HPLC-ESI-TOF-MS and IT-MS). The *H. sabdariffa* aqueous extract was filtered and directly injected into the LC system. The analysis of the compounds was carried out by RP HPLC coupled to DAD and TOF-MS in order to obtain molecular formula and exact mass. Posterior analyses with IT-MS were performed and the fragmentation pattern and confirmation of the structures were achieved. The *H. sabdariffa* samples were successfully analyzed in positive and negative ionization modes with two optimized linear gradients. In positive mode, the two most representative anthocyanins and other compounds were identified whereas the phenolic fraction, hydroxycitric acid and its lactone were identified using the negative ionization mode.

**Keywords:** Anthocyanins / DAD / ESI-TOF-MS / *Hibiscus sabdariffa* / HPLC / IT-MS / Polyphenols

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## 1 Introduction

*Hibiscus sabdariffa* L. (family Malvaceae), commonly known in English as roselle or red sorrel and in Arabic as karkadeh, is widely grown in Central and West Africa, South East Asia, and elsewhere. *H. sabdariffa* (Malvaceae) is an annual, erect, bushy, herbaceous sub-shrub that grows to 8 ft (2.4 m) in height and typically consists of a red calyx with five large sepals. It is a tropical plant native to India and Malaysia. It grows widely in the tropics and subtropics of both hemispheres and has become naturalized in many areas of America [1] where the plant (family Malvaceae) has been extensively described [2]. The thick, red and fleshy, cup-shaped calyces of the flower are consumed worldwide as a cold beverage and as a hot drink (sour tea). These extracts are also used in folk medicine which include remedies for high blood pressure, liver diseases, and fever [2–4]. It is also used against inflammation [5] and mutagenicity [6]. The red anthocyanin pigments present in their calyces are used as food coloring agents [7]. Chemical composition of *H. sabdariffa* has been widely reported for long time. Chemical verification using TLC and HPLC fingerprint analysis revealed the presence of flavonoids (quercetin, luteolin and its glycoside) as well as chlorogenic acid [8]. Some other flavonoids such as gossypetin, hibiscetin and their respective glycosides [9], phenols and some phenolic acids (protocatechuic and pelargonidic acid [10]), eugenol [11], and the sterols β-sitosterol and ergosterol [12] have also been previously reported. Glycons, such as quercetin was also recorded in roselle color [13]. Hydroxycitric acid (HCA) has been reported to be present as a major acid component in the fruit rinds of *Garcinia* species as well as in *H. sabdariffa* [14]. It is also well known that *H. sabdariffa* contains hibiscus acid which is the lactone of the above-mentioned HCA [14]. Anthocyanins such as delphinidin-3-sambubioside and cyanidin-3-sambubioside have been previously detected as main components in the aqueous extract of *H. sabdariffa* [15].

The purpose of the present work was to develop a very simple qualitative methodology to simultaneously separate and identify the most representative compounds in *H. sabdariffa* aqueous extract. The procedure consisted in diluting the sample leaves and filtering it followed by RP HPLC coupled with the photodiode array detection (DAD) and ESI TOF MS in negative and positive polarity modes. Finally, the fragmentation pattern obtained in MS/MS experiments by IT-MS confirmed the proposed struc-

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**Abbreviations: DAD,** diode array detection; **HCA,** hydroxycitric acid

tures. UV-VIS spectrophotometry was a valuable tool to identify the class of phenolic and anthocyan compounds, whereas the two analyzers TOF-MS and IT-MS were useful for their structural characterization.

## 2 Materials and methods

### 2.1 Chemicals and standards

All chemicals were of analytical HPLC reagent grade and used as received. Formic acid and ACN used for preparing mobile phases were purchased from Fluka, Sigma–Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Solvents were previously filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). The standards, chlorogenic acid, quercetin 3-rutinoside, quercetin 3-glucoside, kaempferol 3-*O*-rutinoside and kaempferol 3-(*p*-coumarylglucoside), quercetin, and delphinidin-3-sambubioside were purchased either from Fluka, Extrasynthese (Genay Cedex, France) or Polyphenols (Polyphenols Laboratories AS, Hanaveien Sandnes, Norway). The tuning mix solution to optimize the TOF parameters was purchased from Agilent Technologies (Palo Alto, CA, USA). The stock solutions containing these analytes were prepared in methanol also purchased from Lab-Scan. As a vortex mixer a G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used. Distilled water with a resistance of 18.2 M was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA). Filters to filter the sample prior the injection into the HPLC system were Filters Millex (Millipore, Bedford, MA, USA) (0.20 μm, 30 mm).

### 2.2 Sample preparation

The *H. sabdariffa* plant is originally from the village of Guerle in Senegal. It was kindly provided by Clinical Laboratories, Saint John Hospital, Reus, Tarragona, Spain. Sample preparation consisted in a very simple procedure in which the dry leaves of the plant are manually mill ground until almost converted into a very thin powder. An amount of *H. sabdariffa* was weighed and mixed with ultrapure water at room temperature, up to a concentration of 50 g/L. The aqueous extract of *H. sabdariffa* was freshly prepared for each analysis. 0.25 g was mixed with 5 mL of water. The aqueous extract was stirred in a vortex for 10 min, until diluted, filtered with units of single use filters and directly injected into the HPLC system.

### 2.3 Instrumentation

HPLC analyses were performed with a RRLC 1200 series (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump with Zorbax Eclipse Plus $C_{18}$ 4.6 × 150 mm, 1.8 μm column. Prefilters were used as precolumn, RRLC in-line filters, 4.6 mm, 0.2 μm supplied by Agilent Technologies. HPLC was equipped with DAD and coupled to a TOF mass spectrometer equipped with an orthogonal electrospray interface ESI, model G1607A from Agilent Technologies, operating in both negative and positive modes. MS-Instrument: TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). MS/MS analyses were carried out with a P/ACE System MDQ coupled with an Esquire 2000 ion trap (IT)-mass spectrometer (Bruker Daltonik).

### 2.4 Chromatographic conditions

The compounds of the aqueous extract of *H. sabdariffa* were separated by the C18 column at room temperature at a flow rate of 0.5 mL/min and the injection volume was 10 μL for both gradient elution programs. The use of the prefilters as guard column also provided some protection against decomposition and blocking of the working column, bearing in mind the *H. sabdariffa* samples were quite sticky at the working concentration of 50 g/L. The linear gradient used for the analysis, separation and identification of the polyphenols, HCA, and its lactone (gradient program 1) was as follows: mobile phases: A: water/ACN (ACN) 90:10 (1% HCOOH) and B: ACN. The linear gradient elution program was run as stated: 0 min, 5% (B); 20 min 20% (B); 25 min 40% (B); 30 min 5% (B); 35 min, isocratic of B 5%. Anthocyanins, due to their acid–base equilibrium, need a more acidic pH to be resolved. Subsequently, a different chromatographic method (gradient program 2) was employed. Solvents that constituted the mobile phases were: A: water (10% HCOOH) and B: ACN. The applied elution conditions were: 0 min, 0% (B); 13 min 20% (B); 20 min 30% (B); 25 min 80% (B); 30 min 0% (B); 35 min, isocratic of B 0%. The two different methods were chosen as they both provided short analysis times and good chromatographic separations.

### 2.5 ESI-TOF-MS and IT-MS conditions

TOF-MS transfer parameters were optimized by direct infusion experiments with Tuning mix (Agilent Technologies). The trigger time was set to 53 s (50 s for setting transfer time and 3 s pre-pulse storage time), corresponding to a mass range of 50–1000 *m/z*. The other optimum values of the ESI-MS parameters were capillary, 4500 V: gas heater temperature, 200°C; drying gas flow, 7 L/min; nebulizing gas pressure, 1.5 bar; and the spectra rate was 1 Hz. At this stage the use of a splitter was required to the coupling with the MS detector from the HPLC as the flow which arrived at the ESI-TOF detector had to be no more than 0.25 mL/min in order to obtain reproducible results and stable spray. This splitter (1:2) provided the required separation of the working flow needed to reach the detector. The TOF mass spectrometer was equipped with an

 

**Table 1.** Mass spectral and UV data negative mode (gradient program 1) in the *Hibiscus sabdariffa* aqueous extract DAD-ESI-TOF/IT (M-1)

| Compound | RT | Molecular formula | *m/z* experimental | *m/z* calculated | UV (nm) | MS/MS fragments | Proposed compound |
|---|---|---|---|---|---|---|---|
| 1 | 3.20 | $C_6H_7O_8$ | 207.0140 | 207.0146 | – | 189, 127 | Hydroxycitric acid |
| 2 | 3.42 | $C_6H_5O_7$ | 189.0035 | 189.0041 | – | 127 | Hibiscus acid |
| 3 | 5.20 | $C_{16}H_{17}O_9$ | 353.0891 | 353.0878 | 297<br>324 | 191, 179,<br>135 | Chlorogenic acid isomer I |
| 4 | 7.10 | $C_{16}H_{17}O_9$ | 353.0872 | 353.0878 | 297<br>324 | 191 | Chlorogenic acid[a)] |
| 5 | 7.60 | $C_{16}H_{17}O_9$ | 353.0871 | 353.0878 | 297<br>324 | 191, 179,<br>135 | Chlorogenic acid isomer II |
| 6 | 10.00 | $C_{26}H_{27}O_{17}$ | 611.1271 | 611.1254 | 352 | 316, 317 | Myricetin 3-arabinogalactoside |
| 7 | 12.60 | $C_{26}H_{27}O_{16}$ | 595.1309 | 595.1305 | 345 | 463, 445,<br>300, 301 | Quercetin 3-sambubioside |
| 8 | 13.40 | $C_{16}H_{15}O_8$ | 335.0768 | 335.0772 | 296<br>326 | 161, 135 | 5-*O*-Caffeoylshikimic acid |
| 9 | 14.50 | $C_{27}H_{29}O_{16}$ | 609.1462 | 609.1461 | 255<br>353 | 301 | Quercetin 3-rutinoside[a)] |
| 10 | 16.00 | $C_{21}H_{19}O_{12}$ | 463.0873 | 463.0882 | 253<br>356 | 301 | Quercetin 3-glucoside[a)] |
| 11 | 17.50 | $C_{27}H_{29}O_{15}$ | 593.1512 | 593.1512 | 265<br>350 | 285 | Kaempferol 3-*O*-rutinoside[a)] |
| 12 | 26.70 | $C_{18}H_{19}NO_4$ | 312.1234 | 312.1241 | 286<br>316 | 178, 135 | *N*-Feruloyltyramine |
| 13 | 27.60 | $C_{30}H_{25}O_{13}$ | 593.1312 | 593.1301 | 258<br>315 | 447, 285 | Kaempferol 3-(*p*-coumarylglucoside)[a)] |
| 14 | 28.40 | $C_{15}H_9O_7$ | 301.0339 | 301.0354 | 253<br>372 | 179, 151 | Quercetin[a)] |
| a | 4.40 | $C_{26}H_{27}O_{16}$ | – | (595) | 520<br>280 | – | Delphinidin 3-sambubioside[a)] |
| b | 5.70 | $C_{26}H_{27}O_{15}$ | – | (579) | 520<br>280 | – | Cyanidin 3-sambubinoside |

[a)] Standard available.
a, b: The compounds could not be fully assigned.

ESI interface operating in negative or positive ion polarity. To tune the detector to optimal conditions calibration was performed with sodium formate clusters (5 mM sodium hydroxide in water/isopropanol 1:1 v/v, with 0.2% v/v of formic and acetic acids) in quadratic + high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior to the polyphenol identification. The accurate mass data for the molecular ions were processed using the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formula by using the GenerateMolecularFormula™ editor. The GenerateFormula™ editor uses the sigmaFit™ algorithm, CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue™) for increased confidence in the suggested molecular form [16]. The use of isotopic abundance patterns as a single further constraint removes >95% of false candidates. This orthogonal filter can condense several thousand candidates down to only a small number of molecular formulas. The analyses carried out in the IT-MS were run in the negative and positive ion modes and the capillary voltage was set at 3500 V. The IT scanned at 50 – 1000 *m/z* range. The other parameters were dry temperature, 300° C, drying gas flow, 7 L/min, and nebulizing gas pressure, 1.5 bar. The instrument was controlled by a PC running the Esquire NT software from Bruker Daltonik. The choice of these variables for the MS instrumentation (TOF and IT) was a compromise between maintaining efficient and well-resolved chromatographic separation and improving ionization performance.

## 3 Results and discussion

### 3.1 Profile and compounds characterization

Figure 1 shows the ESI-TOF base peak chromatogram (BPC) obtained using the two linear gradient elution pro-

 

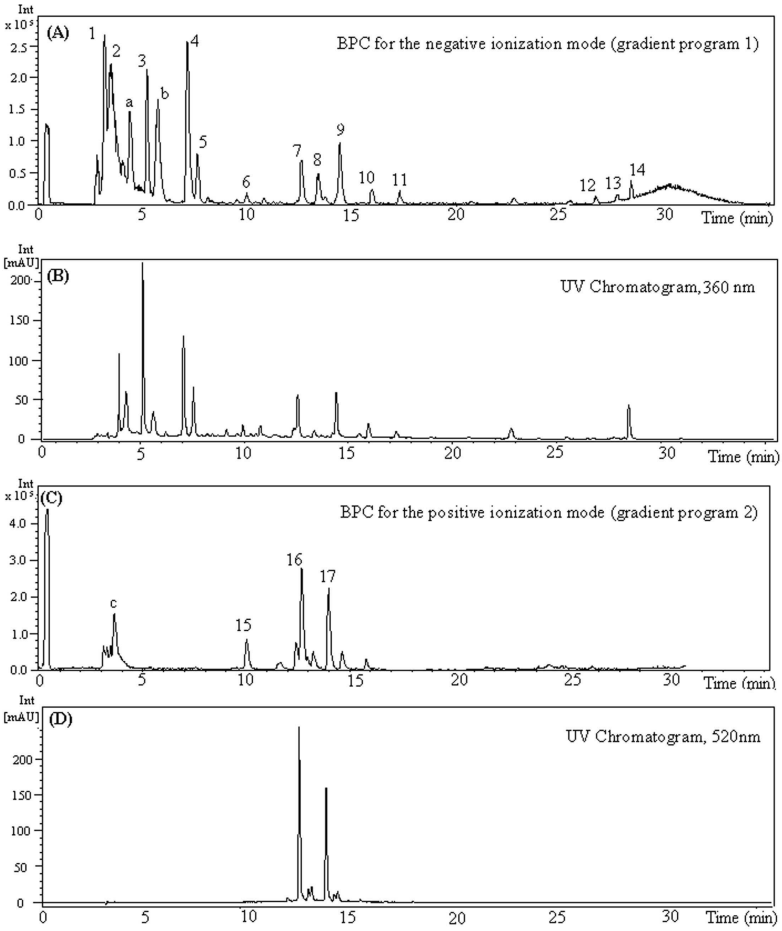


**Figure 1.** Chromaographic profiles using the optimized gradient programs as described in Section 2.4. Compounds are signed by numbers as stated in Tables 1 and 2. (A) Base peak chromatogram (BPC) for the gradient program 1, negative mode (B) UV chromatogram at 360 nm for the gradient program 1. (C) Base peak chromatogram (BPC) for the gradient program 2, positive mode. (D) UV chromatogram at 520 nm for the gradient program 2.

grams (1 and 2) in negative and positive ionization modes, respectively. The compounds are signed by numbers and letters as stated in Tables 1 and 2. It also shows the UV chromatograms obtained for the more representative absorption bands for the compounds of interest for the aqueous *H. sabdariffa* extract. The peak in the segment between 0 and 0.25 min corresponds to the abovementioned calibrant. The identification of phenolic compounds, HCA and its lactone, and the anthocyanins was carried out comparing their retention times and mass spectra provided by TOF-MS and IT-MS with those of authentic standards when available. Remaining compounds were identified by the interpretation of their mass spectra obtained by the TOF-MS and the MS/MS spectra acquired with the IT-MS and also bearing in mind all the data provided by the literature and the UV informa-

 

**Table 2.** Mass spectral and UV data positive mode (gradient program 2) in the *Hibiscus sabdariffa* aqueous extract DAD-ESI-TOF/IT

| Compound | RT | Molecular formula | *m/z* experimental | *m/z* calculated | UV (nm) | MS/MS fragments | Proposed compound |
|---|---|---|---|---|---|---|---|
| 15 | 10.07 | $C_9H_7O_3$ | 163.0386 | 163.0390 | 254<br>325 | 135, 106 | 7-Hydroxycoumarin |
| 16 | 12.71 | $C_{26}H_{29}O_{16}$ | 597.1446 | 597.1450 | 520<br>280 | 303 | Delphinidin 3-sambubioside[a)] |
| 17 | 14.00 | $C_{26}H_{29}O_{15}$ | 581.1493 | 581.1501 | 520<br>280 | 287 | Cyanidin 3-sambubinoside |
| c | 3.70 | $C_6H_7O_7$ | 191.0181 | 191.0186 | – | – | Hibiscus acid |

a) Standard available.
c: Compound already characterized in the negative mode of ionization.

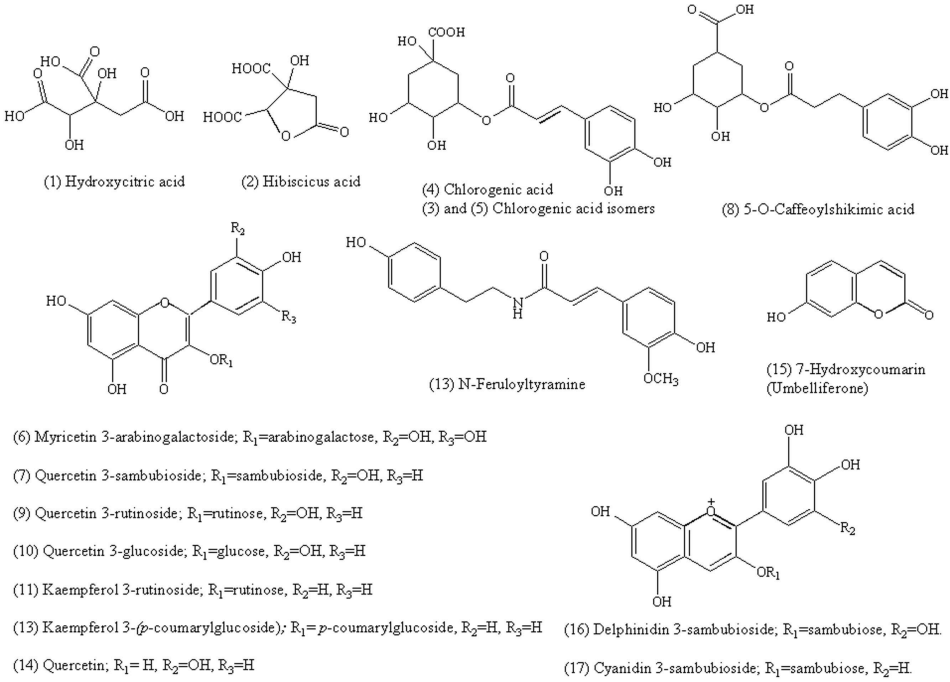


**Figure 2.** Proposed structures for the identified compounds in the *Hibiscus sabdariffa* aqueous extract.

tion provided by DAD. All the compounds studied are summarized in Table 1 (elution gradient at negative mode for the polyphenols, HCA and its lactone) and Table 2 (elution gradient at positive mode for the anthocyanins). Table 1 also showed the two major anthocyanins present in the aqueous extract of *H. sabdariffa*, separated with elution gradient 1 and negative mode with masses 595 and 579 (compounds a and b, Fig. 1 A) and absorbance of 280 and 520 nm which they are characteristic of the anthocyanins family. However, the error values for these two compounds were considerably high. Subsequently, the full characterization was performed with the elution gradient 2 and positive mode of ionization as stated in Table 2. The tables include molecular formula, selected ion, calculated and experimental *m/z*, MS/MS fragments, error, sigma value, retention times, and finally the proposed compounds. The absorbance UV bands are also shown for most of the compounds. It is

 

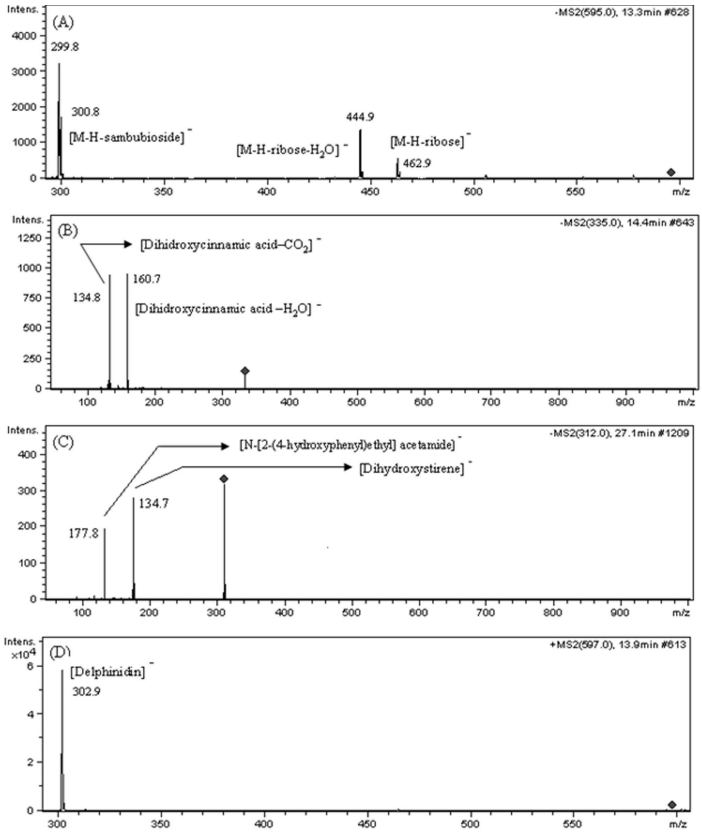


**Figure 3.** MS/MS spectra for some compounds. The spectra correspond to the fragmentation pattern for: (A) Quercetin 3-sambubioside, (B) 5-*O*-Caffeoylshikimic acid, (C) *N*-Feruloyltyramine, (D) Delphinidin 3-sambubioside.

needed to point out that the given names for the compounds in which no standards were available are just proposed ones after the information from MS/MS fragmentation and bearing in mind all the data reported in the literature. The latter also apply for the isomers of the mentioned compounds in the way it was not possible to elucidate between them. The tolerance in generating the molecular formula was 5 ppm for all the compounds considered. Figure 2 shows the proposed structures of the identified compounds (signed by numbers). Figure 3 shows some of MS/MS spectra acquired with the IT-MS.

### 3.2 Identification of compounds in negative mode

At 3.20 min, the ion found at *m/z* 207 corresponded to the HCA ($C_6H_7O_8$), compound 1. It was consistent with its fragments found at *m/z* 189 and 127. This corresponded to the loss of a molecule of water and the successive losses of water and $CO_2$, respectively, from the main ion. At 3.42 min, the ion found at *m/z* 189 (compound 2) corresponded to the lactone of the previous compound. It presented a fragment at *m/z* 127. These two compounds did not show any UV absorbance bands. The presence of these two compounds in the *H. sabdariffa* has been reported previously in the literature [14]. The presence of chlorogenic acid and possible isomers (compounds 3 – 5) was found *via* *m/z* 353 at 5.20, 7.10, and 7.60 min, respectively. Their fragmentation pattern was consistent with the proposed structures as they presented fragments at *m/z* 191 for the chlorogenic acid and 135, 179, and 191, respectively, for the two possible isomers. The fragment at *m/z* 191 represented the deprotonated quinic acid ($C_7H_{12}O_6$). The fragment at *m/z* 179 corresponded to the deprotonated form of dihydroxycin-

 

namic acid ($C_9H_7O_4$). The one at *m/z* 135 is the result of the decarboxylation from the previous acid. All these facts confirmed the structure. On the other hand, the presence of chlorogenic acid at 7.10 min was corroborated by comparison with the corresponding standard in terms of retention time and fragmentation pattern obtained by IT-MS. The ion found at *m/z* 611 presented a fragment at *m/z* 317, so the myricetin derivative was proposed as structure (compound 6). The difference represented the loss of the sugar (arabino-galactose) moiety. Moreover, the presence of myricetin has been reported to be present in *H. sabdariffa* [17]. This compound also presented a fragment at *m/z* 316 which represented the major fragmentation peak. However, the one at *m/z* 317 is the consistent one for the cleavage into the aglycone-myricetin. This anomalous fragmentation has been reported previously for quercetin derivatives in which the product ion spectra gave as most abundant product ion *m/z* 300 by the homolytic cleavage to the remaining quercetin fragment $[M-H]^-$ after the loss of the sugar moieties, whereas *via* heterolytic cleavage the product ion of *m/z* 301 occurred [18]. At 12.60 min, the ion found at *m/z* 595 (compound 7) presented four main product ions at *m/z* 463, 445, 301, and 300. The first one corresponded to the loss of the sugar, the ribose, moiety from the main ion. The second one at *m/z* 445 represented the loss of a water molecule from the first product ion. The fragment found at *m/z* 300 again represented the anomalous fragmentation reported for quercetin derivatives [18]. At last the one at *m/z* 301 represented the deprotonated aglycone-quercetin. The proposed structure was quercetin 3-sambubioside. The latter has been reported previously in other variety of *Hibiscus*, the *Hibiscus mutabilis* (Gulzuba) [19]. Compound 8, in its mass spectra, had a $[M-1]^-$ ion at *m/z* 335 in accordance with a $C_{16}H_{16}O_8$ formula. In the MS/MS spectrum, $[M-1]^-$ of the compound gave ions at *m/z* 161 and 135 corresponding, respectively, to diagnostic fragments $[179\text{-}H_2O]^-$, the deprotonated dihydroxycinnamic acid less a molecule of water and 135 ($C_8H_7O_2$) derived from the caffeoyl moiety. The latter is the result of decarboxylation of the deprotonated dihydroxycinnamic acid $[179\text{-}CO_2]^-$. Compounds 9 and 10 with ions found at *m/z* 609 and 463, respectively, presented both the same fragment at *m/z* 301. The difference between the precursors, 609 and 463 with 301 represented the loss of the sugar moieties, rutinose, and glucose, respectively. The quercetin derivatives were proposed as structures as they were confirmed with the corresponding standards in terms of retention times and fragmentation patterns. Moreover, these quercetin derivatives have been reported to be present in the extracts of *Hibiscus* [20]. Compounds 11 and 13 presented ions at *m/z* 593. The fragmentation patterns were consistent with the loss of the sugar moiety, rutinoside (*m/z* 285 as product ion), and the loss of rhamnose (*m/z* 447 and 285). They both gave the product ion at *m/z* 285 corresponding to the aglycone-kaempferol. They were both also confirmed with the commercial standards. The ion found at *m/z* 312, corresponding to compound 12 presented two products ions at *m/z* 178 and 135. The former corresponded to the deprotonated amide, *N*-[2-(4-hydroxyphenyl)ethyl] acetamide ($C_{10}H_{13}O_2N$). The ion at *m/z* 135 corresponded to the deprotonated form of the dihydroxystyrene ($C_8H_8O_2$) after the loss of a methyl group (17 μ) from the methoxy group of the benzene ring. The last eluting peak at *m/z* 301 corresponding to quercetin, compound 14, presented a fragmentation pattern which consisted in the products ions at *m/z* 151 and 179, corresponding, respectively, to the $A^-$ ring fragment released after RDA fission and the retrocyclization after fission on bonds 1 and 2. This fragmentation pattern has been previously reported [21]. It was also confirmed by comparing with the corresponding standard.

### 3.3 Identification of compounds in positive mode

At 10.07 min, the ion found at *m/z* 163 corresponded to 7-hydroxycoumarin (umbelliferone), compound 15. It presented two fragments at *m/z* 135 and 107. This is consistent with the two successive losses of 28 μ (CO). This fragmentation pattern has been reported previously [22]. Moreover, the presence of umbelliferone has been reported previously to be present in plants [23]. At 12.71 min, the ion found at *m/z* 597 corresponded to delphinidin 3-sambubioside ($C_{26}H_{29}O_{16}$), compound 16. It was consistent with its fragment found at *m/z* 303 which corresponded to the loss of the sugar, the sambubiose moiety from the main ion. This was also confirmed with the comparison with the standard. Compound 17 with ion found at *m/z* 581 presented the fragment at *m/z* 287 corresponding to the aglycone-cyanidin. This is consistent with the loss of the sambubiose moiety, so the cyanidin derivative was proposed as structure. Moreover, the presence of cyanidin 3-sambubioside in *H. sabdariffa* extract has been previously reported [15, 24, 25].

## 4 Concluding remarks

The proposed methods exhibited excellent performance in the determination of the different families of phenolic compounds as well as the HCA, its lactone and also the two major anthocyanins present in the aqueous extract of *H. sabdariffa*. Seventeen compounds were successfully separated and identified using two optimized gradients programs and both, negative and positive modes of ionization. The use of TOF-MS provided excellent information about the exact mass and molecular formula of the compounds and the use of the IT-MS gave important information about the fragmentation of the compounds

 

in order to elucidate the structure and disregard false positives. All these facts are also complemented by the use of DAD which gave additional information about the type of compounds regarding the absorbance bands. It is also important to highlight that, to our knowledge, the compounds 5-*O*-caffeoylshikimic acid and *N*-feruloyltyramine were described for the first time in *H. sabdariffa* aqueous extract.

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*The authors have declared no conflict of interest.*

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# ESTUDIO 2

## Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT).

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ORIGINAL PAPER

# Identification of phenolic compounds in aqueous and ethanolic rooibos extracts (*Aspalathus linearis*) by HPLC-ESI-MS (TOF/IT)

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**Abstract** Rooibos (*Aspalathus linearis*) is a rich source of polyphenols and used to make a mild-tasting tea containing no caffeine, is low in tannins compared to green or black teas, and has antioxidant and antimutagenic/antitumoral properties. In vivo results show that rooibos has beneficial effects upon the lipid profile by decreasing serum triglycerides and cholesterol. In this sense, we have developed a simple and rapid method to separate and characterize simultaneously the polyphenolic compounds in aqueous and ethanolic rooibos extracts using high-performance liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (HPLC-ESI-TOF-MS) and ion trap multiple mass spectrometry (HPLC-ESI-IT-$MS^2$). The phenolic compounds were separated on a $C_{18}$ column (4.6×150 mm, 1.8 μm) with 1% formic acid in water/acetonitrile 90:10 *v/v* and acetonitrile as mobile phases. The accuracy mass data generated by TOF-MS together with the fragmentation pattern obtained by IT-$MS^2$ experiments confirmed the presence of 25 and 30 phenolic compounds in the aqueous and ethanolic extracts, respectively.

**Keywords** Rooibos tea · Polyphenols · High-performance liquid chromatography (HPLC) · Mass spectrometry (MS)

## Introduction

Polyphenols are widely distributed throughout the plant kingdom and represent an abundant antioxidant component of the human diet. These compounds are generally classified into flavonoids, phenolic acids, lignans, and stilbenes. During the past decade, interest in the possible health benefits of polyphenols has increased due to their antioxidant capacity, and many polyphenols, particularly the flavonoids, have been found to possess relatively potent antioxidant, antiatherosclerotic, anti-inflammatory, antitumoral, antithrombogenic, anti-osteoporotic, and antiviral activities [1–3].

*Aspalathus linearis* (Fabaceae) is a flowering shrub-like leguminous bush native to the Cedarberg Mountains in South Africa's Western Cape and used to make a mild-tasting tea, known as rooibos, containing no caffeine and very little tannin but significant amounts of polyphenolic antioxidants. There are two types of rooibos tea: unfermented and fermented. The unfermented product remains green in color and is referred to as green rooibos. During fermentation, the color changes from green to red, with oxidation of the constituent polyphenols, so the final product is often referred to as red tea or red bush tea [4]. The most important characteristics of rooibos are its antioxidant [5, 6] and antimutagenic/antitumoral properties [7].

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Koeppen et al. were the first chemists to attempt to determine phenolic compounds in rooibos tea, and they were able to identify the presence of orientin, iso-orientin (formerly known as homo-orientin), isoquercitrin, rutin [8], orientin, iso-orientin [9], and aspalathin [10]. Hydroxylated benzoic and cinnamic acids, luteolin, chrysoeriol, quercetin, isoquercitrin, and the C–C linked *β*-D-glucopyranosides have also been isolated by various extraction methods [11]. Gradient separation of the *C*-glucoside dihydrochalcones, aspalathin and nothofagin, was later achieved on a reversed-phase $C_{18}$ column [7]. Multilayer counter-current chromatography and preparative high-performance liquid chromatography (HPLC) have recently been used to isolate the polyphenols present in rooibos [12]. In vivo studies have shown that aspalathin has hypoglycemic effects in a diabetic mouse model [13]. Other studies conducted in rats have revealed that the whole extract of rooibos tea had hepatoprotective effects on $CCl_4$-induced liver damage, partially prevented oxidative stress in streptozotocin-induced diabetes, and increased serum SOD activity [14–16].

Beltrán-Debón et al. have assessed the effects of rooibos tea extract on cholesterol and triglyceride serum concentrations in a hypercholesterolemic LDLr knockout mouse model. Rooibos tea extract did not change the serum concentration of these metabolites when the mice were fed on the chow diet (3% fat and 0.03% cholesterol *w/w*), there being no significant differences between the control and the rooibos groups. Rooibos tea extract did however show high bioactivity in those groups fed on the high-fat high-cholesterol diet (20% fat and 0.25% cholesterol *w/w*), in which it lowered serum cholesterol and triglyceride concentrations compared to the control. These in vivo results suggest that rooibos tea may exert beneficial effects upon the lipid profile by decreasing serum triglycerides and cholesterol, thus protecting the mice against harm caused by LDL cholesterol [17].

HPLC hyphenated to mass spectrometry (MS) detection is one of the most important analytical techniques used for the analysis of phenolic compounds. Recently, an improvement in chromatographic performance has been achieved by using columns packed with small particles. The newest column packed with particle size less than 2 μm, operated at a pressure up to 600 bar, thus allowing high resolution analysis in shorter times [18]. The on-line coupling of HPLC with MS using electrospray ionization (ESI) as an interface yields a powerful method because of its high efficient resolution and characterization of a wide range of polar compounds. ESI is one of the most versatile ionization techniques and is the preferred one for detection of polar compounds separated by liquid chromatography. The advantages of MS detection include the ability to determine the molecular weight and to obtain structural information. Time-of-flight (TOF)-MS can provide excellent mass accuracy over a wide dynamic range and allow measurements of the isotopic pattern, providing important additional information for the determination of the elemental composition. Furthermore, TOF-MS permits the rapid and efficient confirmation of the elemental composition of ions when carrying out fragmentation studies and also provides high selectivity in the determination of compounds in complex matrices using the extracted ion chromatogram (EIC) mode when there are overlapping peaks. Otherwise, IT-MS can be used to obtain fragmentation ions of structural relevance for identifying target compounds in a highly complex matrix.

The aims of our study have been to contribute to the phenolic profile of rooibos and to characterize polyphenolic compounds in rooibos extracts (aqueous and ethanolic) using HPLC coupled with two different detection systems: diode array (DAD) and MS with TOF and IT analyzers. UV/visible has proved to be a valuable tool for identifying the family of these phenolic compounds, while TOF-MS and IT-MS allow accurate mass measurements and complementary structural information.

## Experimental

### Chemicals

All chemicals were analytical HPLC reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were from Fluka, Sigma-Aldrich (Steinheim, Germany), and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively. Solvents were filtered before use with a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). The tuning mix solution to optimize the TOF parameters was from Agilent Technologies (Palo Alto, CA, USA). A G560E Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA) was used as a vortex mixer. Distilled water with a resistance of 18.2 MΩ was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA).

### Sample preparation

Fermented rooibos plant material (red-brownish dry leaves) was provided by Arend Redelinghuys (Rooibos BPK Ltd., South Africa). The aqueous extract was prepared in aqueous solution at a concentration of 1,000 ppm, filtered on a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size), and injected directly into the HPLC system. The ethanolic extract was prepared by boiling 20 g of rooibos leaves in 100 mL of distilled $H_2O$ for 5 min. Subsequently, extract was brought down to room temperature and filtered. Forty milliliters of the extract were collected, and to precipitate the polysaccharides, 160 mL of ethanol (80% *v/v*) was added. The extract was conserved at 4 °C overnight; afterwards, the extract was centrifuged at 3,000 rpm for

5 min to remove the precipitate and evaporated at 40 °C to recover 40 mL. The extract was aliquotted and lyophilized. Same as preparing aqueous extract, the ethanolic extract sample was prepared in aqueous solution at concentration of 1,000 ppm, filtered on a polytetrafluoroethylene (PTFE) syringe filter (0.2 μm pore size), and injected directly into the HPLC system.

### Determination of total phenols

The total phenolic content of rooibos tea was determined in triplicate by colorimetric assay using Folin–Ciocalteu reagent [19]. The absorbance of the solution was measured after incubation for 2 h against caffeic acid concentration at a wavelength of 725 nm in a Spectronic Genesys™ 5 spectrophotometer. The results are expressed as the equivalent of caffeic acid. The absorbance curve versus concentrations for the caffeic acid is described by the equation $y=0.027x-0.0373$ ($R^2=0.995$).

### HPLC conditions

LC analyses were made with an Agilent 1200 series rapid-resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler, and a diode array detector (DAD). Separation was carried out with a Zorbax Eclipse Plus $C_{18}$ analytical column (150×4.6 mm, 1.8 μm particle size). Gradient elution was conducted with two mobile phases consisting of 1% formic acid in water/acetonitrile 90:10 *v/v* (phase A) and acetonitrile (phase B) using the following gradient: 0–20 min, linear gradient from 5% B to 20% B; 20–25 min, linear gradient from 20% B to 40% B; 25–30 min, linear gradient from 40% B to 5% B; and 30–35 min, isocratic of 5% B. The flow rate used was set at 0.50 mL/min throughout the gradient. The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer. Thus, in this study, the flow which arrived into the ESI-TOF-MS and ESI-IT-$MS^2$ detector was 0.2 mL/min. The column temperature was maintained at 25 °C, and the injection volume was 10 μL.

### TOF-MS conditions

TOF-MS was conducted using a microTOF™ (Bruker Daltonics, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with an ESI interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range of 50–1,000 *m/z*. The other optimum values of the ESI-MS parameters were capillary voltage, +4.5 kV; dry gas temperature, 200 °C; dry gas flow, 7.0 L/min; nebulizer pressure, 1.5 bar; and spectra rate, 1 Hz.

The accurate mass data of the molecular ions were processed through the software Data Analysis 3.4 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formula by using the Smart Formula Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (*v/v*). Using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the microTOF, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

### IT-MS conditions

The identical HPLC system was coupled to a Bruker Daltonics Esquire 2000™ ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray interface (Agilent Technologies, Palo Alto, CA, USA) in negative ion mode. The ion trap scanned at 50–1,000 *m/z* range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 200 ms, the target count at 20,000, and compound stability was set at 50%. The optimum values of the ESI-MS parameters were capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 1.5 bar. The instrument was controlled by Esquire NT software from Bruker Daltonics.

## Results and discussion

### Total polyphenolic contents

The Folin–Ciocalteu test is an established method to give a rough estimate of the total polyphenolic content. The calibration curve showed good linearity between caffeic acid concentration and absorbance. The average total polyphenol contents in the aqueous and ethanolic rooibos extracts, as evaluated by the Folin–Ciocalteu assay, were 252.07±8.01 and 233.80±7.71 mg/g, respectively. The

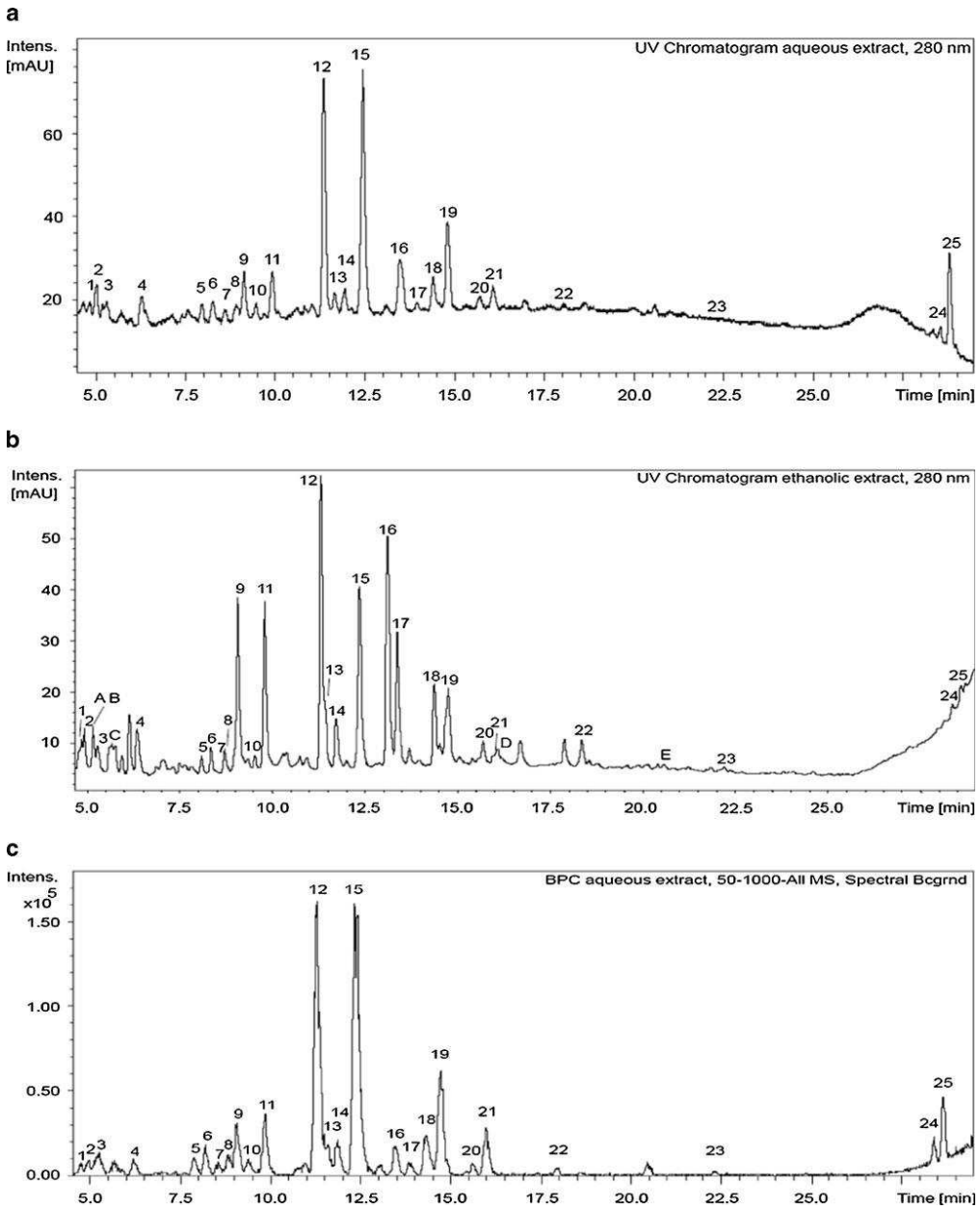


**Fig. 1** UV chromatograms of aqueous (**a**) and ethanolic (**b**) rooibos extracts at 280 nm. Base peak chromatograms of aqueous (**c**) and ethanolic (**d**) rooibos extracts

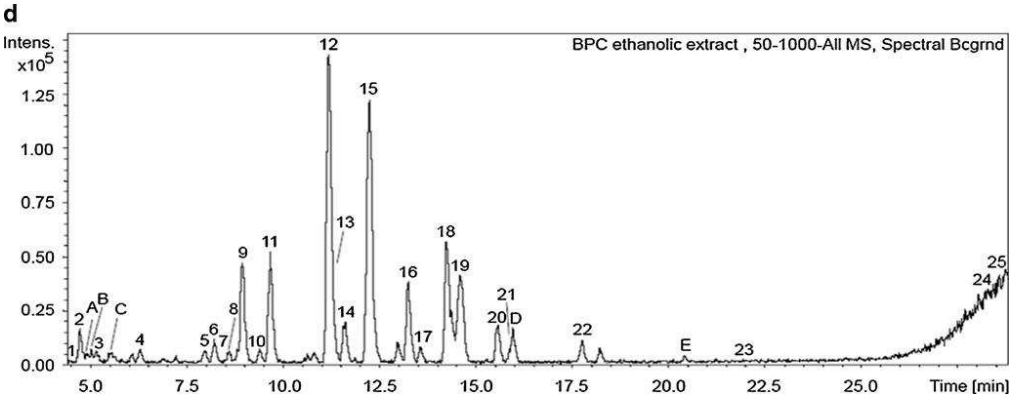


**Fig. 1** (continued)

concentrations observed were multiplied by the dilution factor of the original sample. The Folin–Ciocalteu reagent is nonspecific for any phenolics, and the color yielded depends on hydroxyl groups and their place in molecules. Despite the fact that phenolic reagents are unspecific, in certain circumstances (e.g., absence of interfering substances), we may get only relative results for phenolics. Therefore, by the Folin–Ciocalteu test, we can observe that the total polyphenolic content in fermented rooibos (*A. linearis*) is higher than in the fermented *Cyclopia* species.

## Profile and compound characterization

Polyphenolic compounds were successfully separated and identified using HPLC-ESI-MS (TOF/IT). The UV chromatogram at 280 nm and the TOF-MS base peak chromatogram (BPC) of aqueous and ethanolic rooibos extracts are shown in Fig. 1a–d, respectively. The phenolic compounds were identified by interpreting their mass spectra obtained via their MS (TOF), MS/MS (IT), and UV spectra, taking into account all the data provided by the literature. Table 1 provides a summary of all the compounds studied, including retention times, experimental and calculated *m/z*, molecular formula, error, sigma value, MS/MS fragments, UV bands, as well as their proposed identities. It must be emphasized that the names attributed to the polyphenolic compounds are only proposed ones after the information provided by TOF-MS, the MS/MS fragmentation by IT-MS, and bearing in mind all the data reported in the literature. This also applies to the isomers of these compounds since it was impossible to distinguish between them. Figure 2 shows the proposed structures of the compounds identified (by number).

The fragmentation observed for each ion was useful in order to confirm the proposed structures. Thus, losses of 120 and 90 u were observed, corresponding to cross-ring cleavages in the sugar moiety. Figure 3 illustrates the theoretical fragmentation pattern of a glycosyl flavonoid. The description of the cleavage and the nomenclature follow the system adopted by Waridel et al. [20].

Peak 1 (RT, 4.60 min) gave a molecular mass of *m/z*493. It was tentatively assigned to patuletin 7-glucoside according to the molecular formula provided for its accurate mass and corroborated by the HPLC profile of this compound, in this case with RT at 4.40 min which has been encountered previously in other species such as *Centaurea ruthenica* [21]. Its MS/MS spectrum showed fragments at *m/z*475, 403, 385, 373, and 355, corresponding to the loss of water (475), the $[M\text{-}H\text{-}90]^-$ (403) and $[M\text{-}H\text{-}90\text{-}H_2O]^-$ (385) ions, as well as the successive loss of the $[M\text{-}H\text{-}120]^-$ ion (373) and water (355) from the main fragment.

Peak 2 (RT, 4.77 min) had $[M\text{-}H]^-$ at *m/z*339, which gave daughter ions at *m/z*249 $[M\text{-}H\text{-}90]^-$ and 219 $[M\text{-}H\text{-}120]^-$. These fragmented ions were also derived from cross-ring cleavage in its sugar moiety. It was assigned tentatively to esculin, which has been previously reported in other sweet grass species such as Cortex Fraxini and corroborated with the HPLC profile of this compound with RT at 5.89 min [22].

The ion found at *m/z*611 was assigned to safflomin A (peak 3; RT, 5.19 min), which presented four product ions at *m/z*521, 491, 401, and 371. The first one, at *m/z*521, corresponded to the loss of the $[M\text{-}H\text{-}90]^-$ ion, and the others corresponded to the loss of the $[M\text{-}H\text{-}120]^-$ (491), $[M\text{-}H\text{-}120\text{-}90]^-$ (401), and $[M\text{-}H\text{-}120\text{-}120]^-$ (371) ions. This compound has been reported elsewhere in *Carthamus tinctorius* L. [23].

MS/MS delivered a molecular mass of *m/z*609 for peak 4 (RT, 6.32 min), which we assigned to quercetin-3-*O*-robinobioside with the fragmentation pattern at *m/z*519 and 489 [24, 25]. The former corresponded to the loss of $[M\text{-}H\text{-}90]^-$ and the latter to the loss of $[M\text{-}H\text{-}120]^-$.

**Table 1** Phenolic compounds characterized in rooibos extract by HPLC-ESI-MS (TOF/IT)

| Peak | RT (min) | *m/z* | | Error (ppm) | mSigma value | Formula $[M-H]^-$ | $\lambda_{max}$ (nm) | MS/MS fragments | Proposed compound |
|---|---|---|---|---|---|---|---|---|---|
| | | Experimental | Calculated | | | | | | |
| 1 | 4.60 | 493.0990 | 493.0988 | 0.4 | 42.1 | $C_{22}H_{21}O_{13}$ | 278 | 475, 403, 373, 385, 355 | Patuletin 7-glucoside |
| 2 | 4.77 | 339.0717 | 339.0722 | −1.4 | 7.1 | $C_{15}H_{15}O_9$ | 257, 284, 314 (sh) | 249, 219 | Esculin |
| 3 | 5.19 | 611.1620 | 611.1618 | 0.3 | 22.7 | $C_{27}H_{31}O_{16}$ | 284 | 521, 491, 401, 371 | Safflomin A |
| 4 | 6.32 | 609.1506 | 609.1461 | 7.3 | 12.6 | $C_{27}H_{29}O_{16}$ | 261, 341 | 519, 489 | Quercetin-3-*O*-robinobioside |
| 5 | 7.98 | 579.1336 | 579.1355 | −3.3 | 37.9 | $C_{26}H_{27}O_{15}$ | 271, 341 | 561, 519, 489, 459, 441, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 6 | 8.23 | 593.1482 | 593.1512 | −5.1 | 24.1 | $C_{27}H_{29}O_{15}$ | 271, 333 | 575, 503, 473, 413, 383, 353 | Vicenin-2 |
| 7 | 8.61 | 579.1334 | 579.1355 | −2.0 | 33.9 | $C_{26}H_{27}O_{15}$ | 271, 341 | 561, 519, 489, 459, 429, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside 2″-*O*-*β*-arabinopyranosylorientin |
| 8 | 8.83 | 579.1355 | 579.1355 | 0.1 | 41.6 | $C_{26}H_{27}O_{15}$ | 271, 341 | 559, 519, 489, 459, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 9 | 8.97 | 449.1087 | 449.1089 | −0.5 | 20.0 | $C_{21}H_{21}O_{11}$ | 287 | 359, 329 | (*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside |
| 10 | 9.40 | 579.1335 | 579.1355 | −3.5 | 30.1 | $C_{26}H_{27}O_{15}$ | 271, 341 | 561, 519, 489, 459, 429, 399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 11 | 9.68 | 449.1082 | 449.1089 | −1.7 | 15.4 | $C_{21}H_{21}O_{11}$ | 287 | 359, 329 | (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside |
| 12 | 11.21 | 447.0935 | 447.0933 | 0.5 | 11.7 | $C_{21}H_{19}O_{11}$ | 269, 348 | 429, 411, 357, 327 | Iso-orientin |
| 13 | 11.34 | 449.1020 | 449.1089 | −1.2 | 18.2 | $C_{21}H_{21}O_{11}$ | 285 | 359, 329 | (*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside |
| 14 | 11.62 | 449.1084 | 449.1089 | −0.2 | 43.8 | $C_{21}H_{21}O_{11}$ | 286 | 359, 329 | (*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside |
| 15 | 12.26 | 447.0930 | 447.0933 | −0.7 | 6.6 | $C_{21}H_{19}O_{11}$ | 254, 267 (sh), 349 | 357, 327 | Orientin |
| 16 | 13.26 | 451.1253 | 451.1246 | 1.5 | 1.6 | $C_{21}H_{23}O_{11}$ | 280 | 361, 331 | Aspalathin |
| 17 | 13.53 | 449.1082 | 449.1089 | −1.6 | 34.3 | $C_{21}H_{21}O_{11}$ | 282 | 359, 329, 285 | Aspalalinin |
| 18 | 14.32 | 609.1452 | 609.1461 | −1.5 | 15.2 | $C_{27}H_{29}O_{16}$ | 267, 348 | 301 | Rutin |
| 19 | 14.58 | 431.0991 | 431.0984 | 1.8 | 7.2 | $C_{21}H_{19}O_{10}$ | 270, 336 | 413, 341, 311 | Isovitexin |
| 20 | 15.57 | 463.0880 | 463.0882 | −0.5 | 24.5 | $C_{21}H_{19}O_{12}$ | 276 | 301, 343, 373 | Quercetin-3-*O*-glucoside/galactoside |
| 21 | 15.89 | 447.0932 | 447.0933 | −0.2 | 22.3 | $C_{21}H_{19}O_{11}$ | 270, 342 | 285 | Luteolin-7-*O*-glucoside |
| 22 | 17.76 | 435.1309 | 435.1297 | 2.8 | 39.8 | $C_{21}H_{23}O_{10}$ | 283 | 345, 315 | Nothofagin |
| 23 | 22.01 | 361.1644 | 361.1657 | −3.4 | 88.3 | $C_{20}H_{25}O_6$ | 280 | 346, 331 | Secoisolariciresinol |
| 24 | 28.20 | 285.0348 | 285.0405 | −2.0 | 45.5 | $C_{15}H_9O_6$ | 241, 269, 335 | 151, 107 | Luteolin |
| 25 | 28.41 | 301.0292 | 301.0354 | −6.2 | 24.8 | $C_{15}H_9O_7$ | 254, 271 (sh), 371 | 179, 151 | Quercetin |
| A | 4.94 | 339.0705 | 339.0722 | −3.0 | 34.9 | $C_{15}H_{15}O_9$ | 235, 284 | – | 5,7,Dihydroxy-6-*C*-glucosyl-chromone |
| B | 5.05 | 611.1600 | 611.1618 | −4.9 | 53.3 | $C_{27}H_{31}O_{16}$ | 235, 284 | 449, 287 | Eriodictyol 5,3′di-*O*-glucoside |
| C | 5.50 | 595.1285 | 595.1305 | −3.3 | 60.5 | $C_{26}H_{27}O_{16}$ | 235, 274 | 301 | Quercetin-3-*O*-arabinoglucoside |
| D | 15.97 | 463.0891 | 463.0882 | 1.9 | 17.0 | $C_{21}H_{19}O_{12}$ | 245, 264 (sh), 284 (sh), 349 (sh) | 301 | Isoquercitrin |
| E | 20.40 | 461.1070 | 461.1089 | −4.2 | 53.9 | $C_{22}H_{21}O_{11}$ | 247, 286 | 371, 341 | Scoparin |

*sh* shoulder

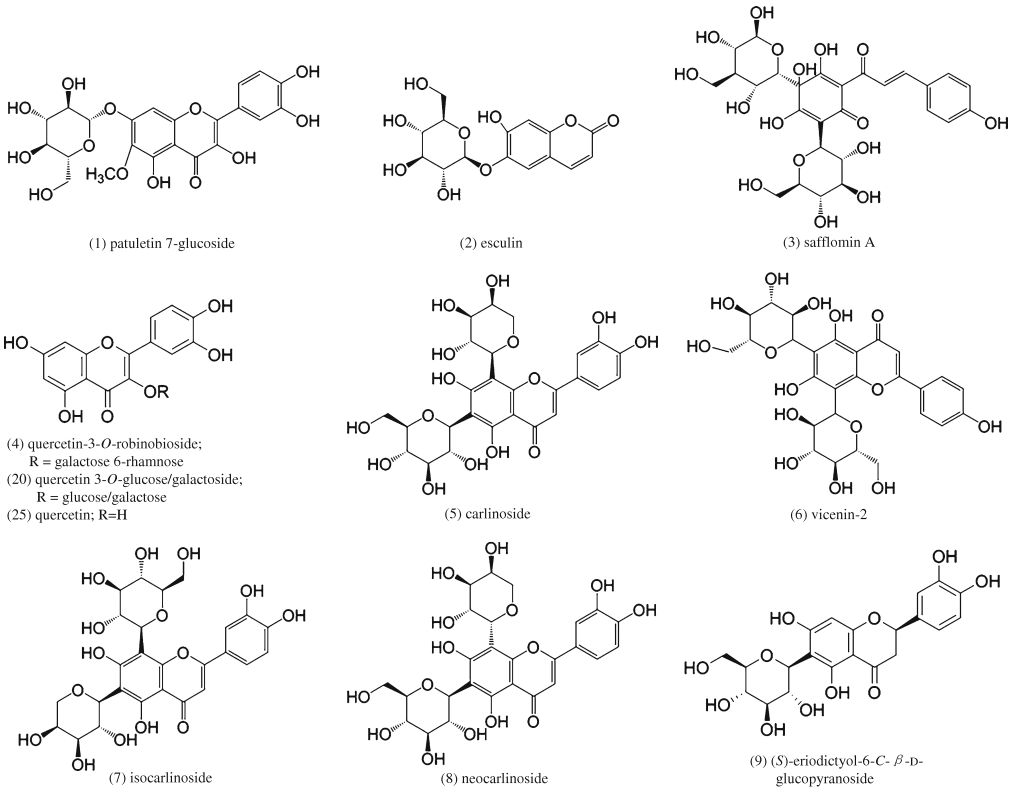


**Fig. 2** Chemical structures of the proposed compounds

Peaks 5, 7, 8, and 10, with *m/z*579, were detected at RT, 7.98, 8.61, 8.83, and 9.40 min and showed the same molecular formula according to Smart Formula Editor. All these compounds responded in a similar way to UV at 271 and 341 nm as their rutinoside counterparts, which were tentatively proposed as being carlinoside [26], isocarlinoside [26, 27], neocarlinoside [28], and/or 2″-*O*-*β*-arabinopyranosyl-orientin [29]. In the absence of standards, it was impossible to distinguish between isomers and to ascertain the position of conjugating groups on the flavonoid skeleton. Thus, they could only be partially identified on the basis of their MS fragmentation pattern. The fragment at *m/z*561 represented the loss of water. The fragments at *m/z*489 and 459 were consistent with the loss of the $[M-H-90]^-$ and $[M-H-120]^-$ ions, respectively, from the precursor. The fragments at *m/z*441 and 399 corresponded to the loss of the $[M-H-18-120]^-$ and $[M-H-90-90]^-$ ions, respectively.

The ion found at *m/z*593 (peak 6; RT, 8.23) was tentatively assigned to vicenin-2, which was reinforced by its fragmentation pattern. This compound has previously been found in *Lychnophora ericoides* Mart. [30]. It presented fragments at *m/z*575, 503, 473, 413, and 383, corresponding to a loss of water from the precursor followed by a loss of the $[M-H-90]^-$, $[M-H-120]^-$, $[M-H-90-90]^-$, and $[M-H-90-120]^-$ ions.

The Smart Formula Editor provided the same molecular formula for peaks 9, 11, 13, and 14 (RT, 8.97, 9.68, 11.34, and 11.62 min, respectively) with *m/z*449. These compounds showed the same UV absorbance band at 287 nm, thus revealing that they have a similar structure and the same substituents, which we tentatively identified as (*R*/*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside and (*R*/*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside. Based on literatures [24, 31], by using reversed-phase chromatography, compound eriodictyol-6-*C*-*β*-D-glucopyranoside was eluted earlier than eriodictyol-8-*C*-*β*-D-glucopyranoside. Krafczyk and Glomb have studied four flavanone-C-glycosides, which gave different retention times in HPLC analysis [12]. Although the absolute stereochemistry was not determined, the peak eluting first was tentatively assigned to S-isomer, the basis of Philbin and Schwartz [32].

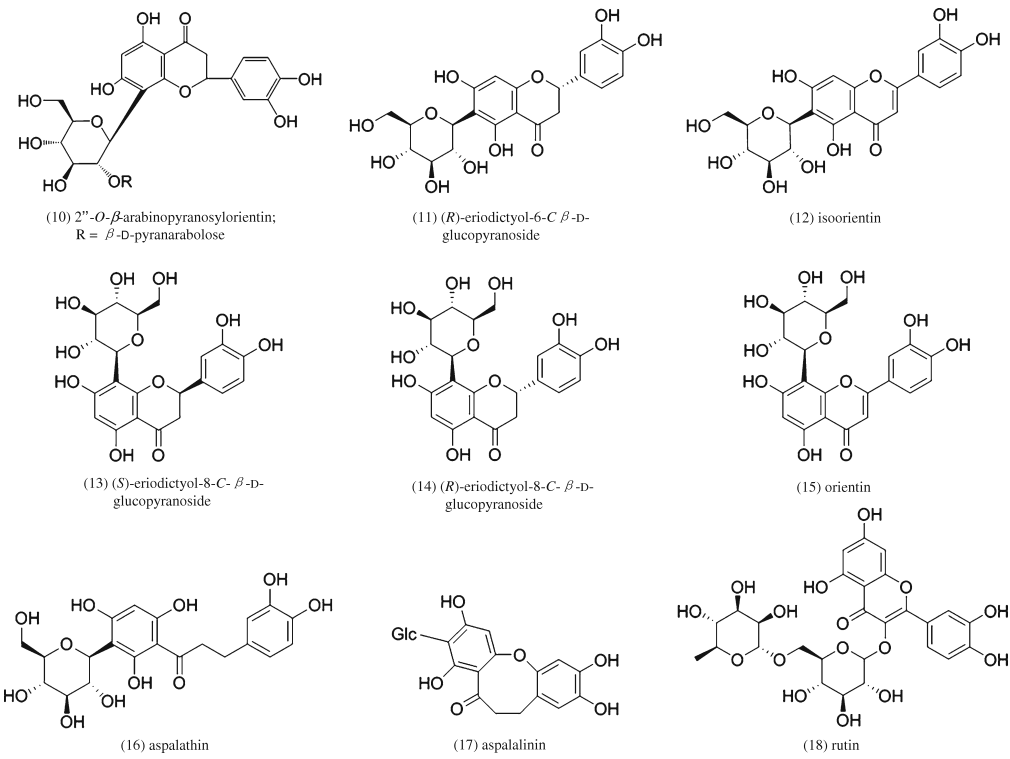


**Fig. 2** (continued)

In addition, during the process, (*R*)/(*S*)-eriodictyol-6-*C*-*β*-D-glucopyranosides were the major products coinciding with minor concentrations of (*R*)/(*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside [12]. Hence, we tentatively identified peaks 9, 11, 13, and 14 as (*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside, followed by (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside, (*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside, and (*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside, respectively. Each of them gave the same fragments at *m/z*359 and 329, which represented a loss of the $[M-H-90]^-$ and $[M-H-120]^-$ ions, respectively, from the precursor.

Peaks 12 and 15 (RT, 11.21 and 12.26 min) presented iso-orientin and orientin, with an ion found at *m/z*447, and showed the same fragmentation pattern at *m/z*357 and 327, corresponding to $[M-H-90]^-$ and $[M-H-120]^-$, respectively, from the main fragment. In addition, peak 12 showed an ion at *m/z*429 and 411, which is consistent with the successive loss of a molecule of water and two molecules of water (Fig. 4a). Waridel et al. have studied in more detail for the differentiation of isomer pairs orientin/iso-orientin. In the negative mode, a clear loss of water (*m/z*429) and a low abundance of *m/z*411 were specific to 6-*C*-glycosides and can be used for discrimination [20]. These losses were not recorded for the 8-*C*-isomer orientin (Fig. 4b). It was possible as well to distinguish them by comparing UV absorbance where only peak 15 showed UV absorbance spectrum at 267 nm (shoulder) which corresponded to orientin. These compounds have also been described elsewhere in the literature [8–12, 24, 31]. Snijman et al. have studied the antimutagenic activity of orientin and iso-orientin, containing the catechol moiety, which were more effective inhibitors of 2-AAF-induced mutagenesis than the C4-monohydroxylated flavonoids [33].

Peak 16 (RT, 13.26 min) was assigned to aspalathin [10–12, 24, 31] with an ion found at *m/z*451. It presented fragments at *m/z*361 and 331, corresponding to the $[M-H-90]^-$ and $[M-H-120]^-$ ions, respectively, from the main fragment (Fig. 4c).

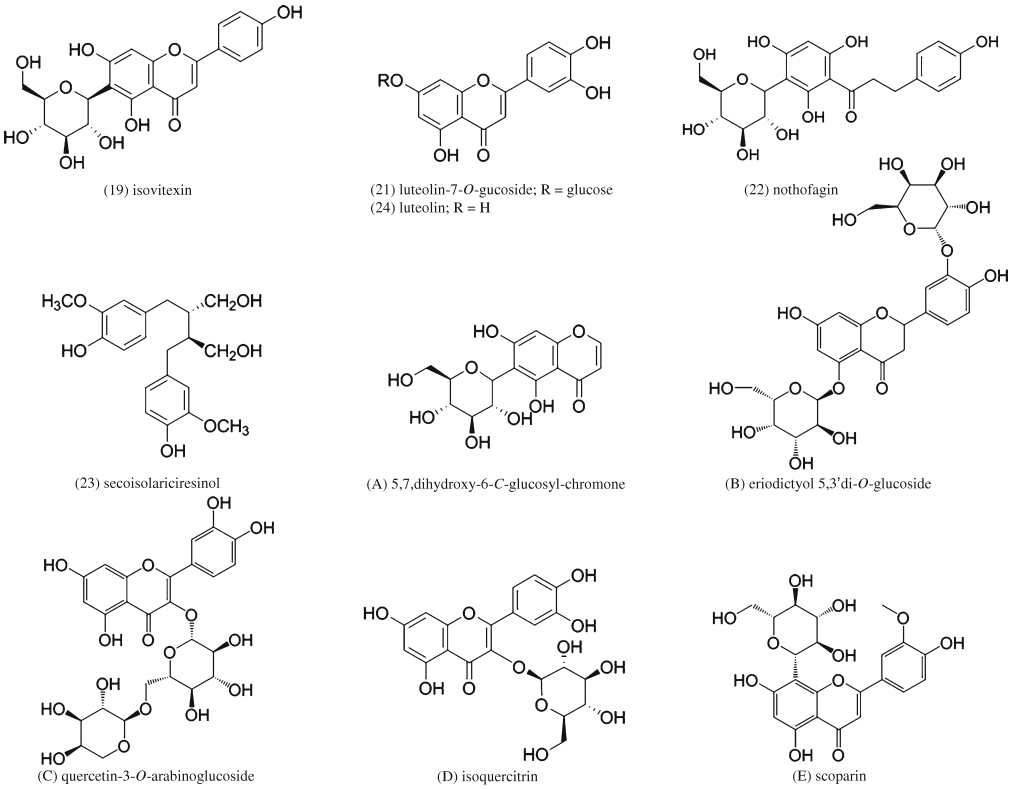


**Fig. 2** (continued)

Peak 17 (RT, 13.53 min) had a $[M-H]^-$ ion at *m/z*449 and was assigned to aspalalinin [24]. It presented daughter ions at *m/z*359, 329, and 285. The first two fragments corresponded to the $[M-H-90]^-$ and $[M-H-120]^-$ ions, respectively, from the main fragment, and *m/z*285 corresponded to the loss of $CO_2$.

Peak 18 (RT, 14.23 min), with precursor and product ions at *m/z*609 and 301, respectively, corresponds to the characteristic fragment mass of *O*-diglycoside, which is the neutral loss of rutinose (Fig. 4d). It was identified as rutin [8, 12, 31].

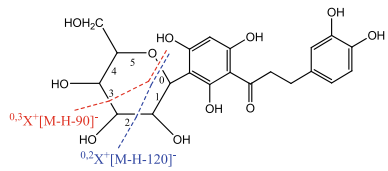


**Fig. 3** Fragmentation of a glycosil flavonoid illustrated for aspalathin

Peak 19 (RT, 14.58 min), with an ion at *m/z*431, was assigned to isovitexin [8, 10, 11, 24, 31]. It presented fragments at *m/z*413, 341, and 311, corresponding to a successive loss of water and the $[M-H-90]^-$ and $[M-H-120]^-$ ions. It was corroborated by its UV absorbance spectrum at 272 and 337 nm, which distinguish it from vitexin.

Peak 20 (RT, 15.57 min), with an ion found at *m/z*463, showed fragments at *m/z*373, 343, and 301, corresponding to the $[M-H-90]^-$ and $[M-H-120]^-$ ions, and the loss of sugar moiety. We assigned it to either quercetin-3-*O*-glucoside or quercetin-3-*O*-galactoside [8, 11, 12, 24, 31].

The ion found at peak 21 (RT, 15.89 min) corresponded to luteolin-7-*O*-glucoside [34] with the precursor and product ions at *m/z*447 and 285, respectively, which is the characteristic fragment mass of *O*-monoglycoside, representing luteolin.

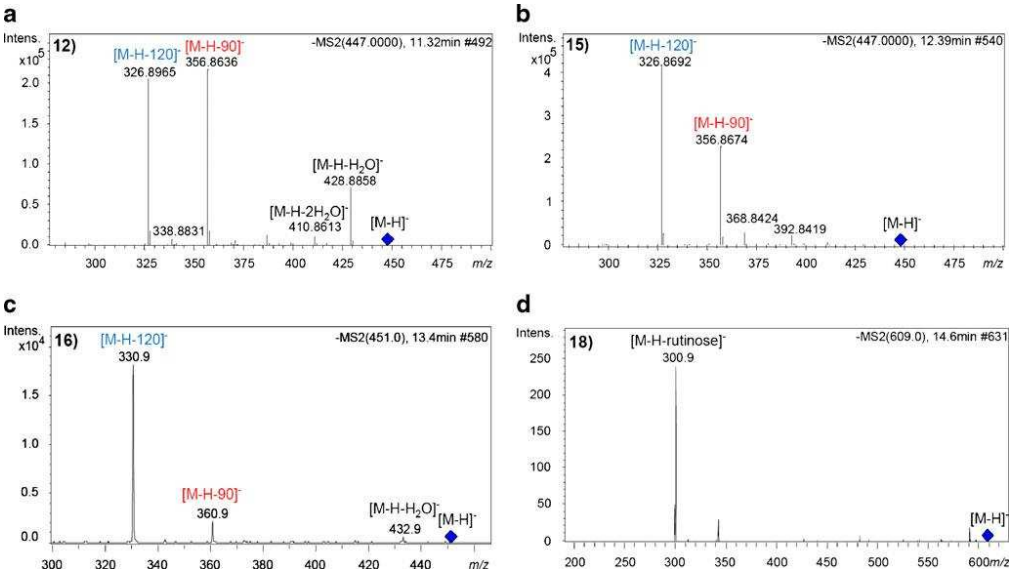


**Fig. 4** MS/MS spectra of the most representative phenolic compounds: iso-orientin (**a**), orientin (**b**), aspalathin (**c**), and rutin (**d**)

The presence of nothofagin [12, 24, 31] was identified at peak 22 (RT, 17.76 min), and the ion was found at *m/z*435. It presented two main product ions at *m/z*345 (loss of 90 u) and *m/z*315 (loss of 120 u) from the main fragment.

Peak 23 (RT, 22.01 min), with an ion found at *m/z*361, corresponded to secoisolariciresinol [24]. It presented two main product ions at *m/z*346 $[M\text{-}H\text{-}CH_3]^-$ and *m/z*331 $[M\text{-}H\text{-}OCH_3]^-$ from the main fragment.

Peaks 24 and 25 were assigned to aglycone. The fragmentation pattern of the $[M\text{-}H]^-$ ion at *m/z*285 (peak 24; RT, 28.20 min) produced MS/MS fragments at *m/z*151 and 107, corresponding to an $A^-$ ring fragment released after RDA fission followed by the loss of $CO_2$. It was assigned to luteolin [11, 12, 24, 31]. The ion found at *m/z* 301 (peak 25; RT, 28.41 min) was assigned to quercetin [11, 12, 24, 31], which presented fragments at *m/z*151 and 179, corresponding to an $A^-$ ring fragment released after RDA fission and retrocyclization after fission on bonds 1 and 2 (Fig. 5) [35–37].

Another five compounds were tentatively identified in ethanolic extract, which were not detected in aqueous rooibos extract. Peak A (RT, 4.94 min) and peak D (RT, 15.97 min) were assigned to 5,7,dihydroxy-6-*C*-glucosyl-chromone [38] and isoquercitrin [8, 11, 12, 24, 31], respectively. These compounds have been also described previously in rooibos. A signal at *m/z*611 (peak B) was observed at 5.05 min. This compound showed a molecular formula $C_{27}H_{32}O_{16}$. It presented fragments at *m/z*449 and 287, corresponding to the loss of a sugar moiety and two sugar moieties consecutively from eriodictyol (aglycone). It was tentatively identified as eriodictyol 5,3′di-*O*-glucoside [39]. At a retention time of 5.50 min, a signal with *m/z*595.1285 (peak C) was detected as quercetin-3-*O*-arabinoglucoside corroborated with its fragment ion at *m/z*301 corresponded to quercetin [40].

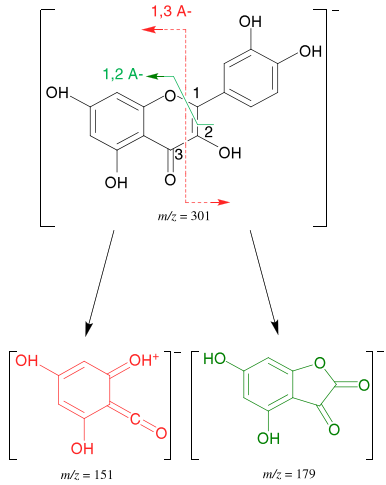


**Fig. 5** Proposed fragmentation pathway of quercetin

Finally, peak E (RT, 20.40 min) with molecular formula $C_{22}H_{22}O_{11}$ was tentatively identified as scoparin [41]. It presented two main product ions at *m/z*371 $[M-H-90]^-$ and *m/z*341 $[M-H-120]^-$ from the main fragment.

## Conclusions

In this work, a powerful analytical method has been used to separate and characterize phenolic compounds in aqueous and ethanolic rooibos extracts by combining HPLC-ESI-TOF-MS and HPLC-ESI-IT-$MS^2$. Identification was accomplished with the determination of accurate mass of the deprotonated molecules in the studied phenolic compounds by TOF-MS and confirmation of their fragmentation ions by IT-$MS^2$ further. This analytical technique has proved to be a useful tool for the identification of 25 major compounds in both extracts and five more compounds in ethanolic extract for HPLC runs for less than 30 min. Orientin and iso-orientin with their antimutagenic activity were found as two predominant compounds in both extracts. It is also important to highlight that, to our knowledge, the compounds patuletin 7-glucoside, esculin, safflomin A, carlinoside, isocarlinoside, neocarlinoside, 2″-*O*-*β*-arabinopyranosylorientin and vicenin-2, eriodictyol 5,3′di-*O*-glucoside, quercetin-3-*O*-arabinoglucoside, scoparin are reported here for the first time in rooibos. The Folin–Ciocalteu assay was used to determine the total polyphenolic contents as 25% and 23% *w*/*w* of aqueous and ethanolic extracts, respectively.

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# ESTUDIO 3

**Continous administration of polyphenols from aqueous rooibos (*Aspalathus linearis*) extract ameliorates dietary-induced metabolic disturbances in hyperlipidemic mice.**

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# Continuous administration of polyphenols from aqueous rooibos (*Aspalathus linearis*) extract ameliorates dietary-induced metabolic disturbances in hyperlipidemic mice

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ABSTRACT

The incidence of obesity and related metabolic diseases is increasing globally. Current medical treatments often fail to halt the progress of such disturbances, and plant-derived polyphenols are increasingly being investigated as a possible way to provide safe and effective complementary therapy. Rooibos (*Aspalathus linearis*) is a rich source of polyphenols without caloric and/or stimulant components. We have tentatively characterized 25 phenolic compounds in rooibos extract and studied the effects of continuous aqueous rooibos extract consumption in mice. The effects of this extract, which contained 25% w/w of total polyphenol content, were negligible in animals with no metabolic disturbance but were significant in hyperlipemic mice, especially in those in which energy intake was increased via a Western-type diet that increased the risk of developing metabolic complications. In these mice, we found hypolipemiant activity when given rooibos extract, with significant reductions in serum cholesterol, triglyceride and free fatty acid concentrations. Additionally, we found changes in adipocyte size and number as well as complete prevention of dietary-induced hepatic steatosis. These effects were not related to changes in insulin resistance. Among other possible mechanisms, we present data indicating that the activation of AMP-activated protein kinase (AMPK) and the resulting regulation of cellular energy homeostasis may play a significant role in these effects of rooibos extract. Our findings suggest that adding polyphenols to the daily diet is likely to help in the overall management of metabolic diseases.



## Introduction

Oxidation and inflammation are major pathogenic factors involved in metabolic disorders in which excessive energy consumption plays a major role. These disorders include obesity, liver steatosis, type 2 diabetes, dyslipidemia and probably cancer, which exhibit increases in morbidity and mortality rates not only in Western societies but also throughout the world (http://www.cdc.gov). Caloric intake is a major contributor to these diseases, but the type of diet consumed also plays a role. It has been known for decades that a diet rich in fruits, vegetables and olive oil may help to reduce cardiovascular and metabolic complications, but the components of these foods that confer health benefits are currently unknown (Hu 2003). Recent interest has been focused on the biological activities of plant-derived phenolic compounds, but safety and efficacy issues remain unresolved in humans. For instance, it has already been documented that polyphenols from green tea or wine may confer a cardiovascular protective effect (Feillet-Coudray et al. 2009; Li et al. 2004), and other medicinal plants are currently being considered for anti-inflammatory purposes (Beltran-Debon et al. 2010).

Rooibos (*Aspalathus linearis*) (Fig. 1) grows naturally in certain areas of South Africa (Joubert et al. 2008) and is widely used to

*Abbreviations:* AMPK, AMP-activated protein kinase; BAT, brown adipose tissue; CD, chow diet; C/EBP, CCAAT/enhancer-binding protein; DAD, diode array detector (analyzer); eWAT, epididymal white adipose tissue; FASN, fatty acid synthase; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; HF, high-fat, high-cholesterol diet; HPLC, high performance liquid chromatography; iWAT, inguinal white adipose tissue; LDL, low density lipoprotein; $LDLr^{-/-}$, low density lipoprotein receptor knock-out mice; MCP-1, monocyte chemoattractant protein-1; MS, mass spectrometry; TOF, time of flight (analyzer); UV, ultraviolet; VLDL, very low density lipoprotein.

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**Fig. 1.** Rooibos plant (*Aspalathus linearis*).

prepare infusions that represent a rich source of polyphenols without caloric value (Krafczyk and Glomb 2008). Moreover, *in vitro* and *in vivo* studies have demonstrated that rooibos has significant bioactivity, with described effects of preventing lipid oxidation and oxidative stress (Fukasawa et al. 2009; Ulicna et al. 2006), inflammation (Baba et al. 2009), hyperglycemia (Kawano et al. 2009) and chemically induced liver damage (Ulicna et al. 2003). In this study, we have characterized phenolic compounds in rooibos extract and found that the continuous administration of this extract has no effect in metabolically normal mice. However, we found a significant metabolic modulatory effect of rooibos extract administration in low-density lipoprotein receptor (LDLr) deficient mice, a model for metabolic disturbances that can be further exacerbated by feeding the animals a Western-type diet (Rodríguez-Sanabria et al. 2010).

## Materials and methods

### *Identification of phenolic compounds*

Rooibos plant material was kindly provided by Arend Redelinghuys (Rooibos BPK, Ltd., South Africa). Samples were prepared in an aqueous solution at a concentration of 1000 ppm; they were then filtered and injected into the HPLC system, an Agilent 1200-series RRLC system (Agilent Technologies, Palo Alto, CA, USA) that was equipped with a binary pump and DAD. Injected samples (10 μl) were passed through a Zorbax Eclipse Plus $C_{18}$ (4.6 mm × 150 mm, 1.8 μm) column at room temperature at a constant flow of 0.5 ml/min. A solution of 1% formic acid in water/acetonitrile 90:10 v/v and acetonitrile were used as mobile phases A and B, respectively, and programmed as follows: gradient elution from 5% to 20% B for 20 min, from 20% to 40% B for 5 min, from 40% to 5% B for 5 min, and an isocratic solution of 5% B for 5 min.

TOF-MS was conducted using a microTOF™ (Bruker Daltonik GmbH, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with an electrospray ionization (ESI) interface. Parameters for analysis were set using the negative ion mode with a capillary voltage of 4500 V, a dry gas temperature of 200 °C, a dry gas flow of 7.0 l/min, a nebulizer pressure of 1.5 bar and a spectra rate of 1 Hz. The spectra were acquired over a mass range of 50–1000 *m*/*z*. All operations were controlled by DataAnalysis 3.4 software (Bruker Daltonik), which provided a list of possible elemental formulas using the GenerateMolecularFormula™ editor.

UV–visible spectrophotometry delimited the class of phenolic compounds, and the accurate mass measurements on the TOF-MS enabled us to identify the compounds in the extract. Finally, the fragmentation pattern obtained in the MS/MS experiments performed with an Esquire 2000 ion trap (IT)-mass spectrometer (Bruker Daltonik) confirmed the proposed structures. The ion trap scanned at a 50–1000 *m*/*z* range with a gradient optimized for a negative ionization polarity. The optimum values of the ESI-MS parameters were as follows: capillary voltage, +3.5 kV; dry gas temperature, 300 °C; dry gas flow, 7 l/min; and nebulizer pressure, 1.5 bar.

The total phenolic content of the rooibos extract was determined by colorimetric assay using Folin–Ciocalteu reagent (Singleton and Rossi 1965). The absorbance of the solution was measured at a wavelength of 725 nm in a Spectronic Genesys™ 5 spectrophotometer.

### *Animal experimental models*

Studies on C57BL/6J male mice showed a lack of toxicity with the continuous administration of aqueous rooibos extract, and we found no effect (for clarity, data are not shown because changes were negligible). To test our hypothesis, we decided to use male $LDLr^{-/-}$ mice as a model of intense metabolic alterations that in some aspects resemble the human metabolic syndrome (Rodríguez-Sanabria et al. 2010). They were the progeny of animals obtained from the Jackson Laboratory in a C57BL/6J background. The number of animals used in the experiment ($n = 32$) was determined based on previous data (Joven et al. 2007). Animals were housed under a 12-h light/dark cycle at a constant temperature of 25 °C with free access to water and a commercial mouse diet (14% Protein Rodent Maintenance Diet, Harlan, Barcelona, Spain). At 10 weeks of age, animals with equivalent body weights were randomly assigned to two dietary study groups. The chow diet group (CD, $n = 16$) continued to receive the same maintenance diet (3% fat and 0.03% cholesterol, w/w), and the second group ($n = 16$) was fed a high-fat, high-cholesterol diet (HF, 20% fat and 0.25% cholesterol, w/w). Each dietary group was divided into two treatment groups ($n = 8$). One of these treatment groups received tap water as a unique liquid source (control), while the other group received a rooibos extract (10 g/l). To prevent possible degradation, fresh extracts were prepared every two days. The material was weighted, boiled in tap water, cooled down to room temperature and filtered. The animals had free access to liquid sources and food; body weight and food intake were measured weekly. Treatments were maintained over 14 weeks, after which the animals were sacrificed under xylazine–ketamine anesthesia. All procedures were performed in accordance with institutional guidelines.

### *Cell culture*

3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dexamethasone, 3-isobutyl-1-methylxanthine, insulin and crystal violet were obtained from

Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, calf serum, fetal bovine serum, and antibiotic mixtures (penicillin–streptomycin) were purchased from PAA Laboratories (GmbH, Linz, Austria). Sodium pyruvate and trypsin-EDTA were obtained from the Invitrogen Co. (Carlsbad, CA, USA). The AdipoRed™ Assay Reagent was purchased from Lonza (Walkersville, MD, USA).

The 3T3-L1 pre-adipocyte line, maintained at low passage number, was grown at 37 °C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 1% pyruvate, 100 μg/ml streptomycin and 100 units/ml penicillin. The cells were subcultured before reaching a confluence of 80%. Pre-adipocytes were differentiated using standard protocols (Maslowska et al. 2006). The cells were harvested 10 days after the initiation of differentiation, at which time 90% of cells were mature adipocytes with accumulated fat droplets (as determined by microscopic evaluation). To test the effects of rooibos on adipogenesis, rooibos extract was added to the medium at a final concentration of 600 μg/ml. Extracts were prepared with distilled water, filtered through a 0.22-μm filter and lyophilized. Cytotoxicity was measured by the crystal violet method as previously described (Ishiyama et al. 1996). Lipid content was measured using the Adipored assay following the manufacturer's protocol.

*Sample collection and laboratory measurements*

Blood and tissue samples were obtained and processed as previously described (Rull et al. 2007). Serum cholesterol, triglycerides, free fatty acids and bilirubin concentrations and AST activity were determined using an automatic analyzer Synchron LXi 725 system (Beckman Coulter, IZASA, Barcelona, Spain). A glucose tolerance test was performed as described elsewhere (Rull et al. 2007).

*Histology*

Liver steatosis was qualitatively evaluated on a scale of 0–3, where 0 represented an absence of steatosis and 3 indicated a major grade of steatosis (>66%). Immunohistochemistry and measurements of the area of adipocytes were performed as described elsewhere (Rull et al. 2007).

*FPLC lipoprotein fractionation*

Plasma lipoproteins were separated using a Bio-Rad BioLogic DuoFlow 10 system (Bio-Rad, Spain). Plasma pooled samples from each group (100 μl) were injected into a Superose 6/300 GL column (GE Healthcare Europe GmbH, Barcelona, Spain), and 500-μl fractions were collected. Cholesterol and triglycerides in each fraction were determined as described above.

*Western blot analysis*

Liver and eWAT tissues were weighted and homogenized in 5 ml/g of lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 1 mM Pefabloc and 1% phosphatase inhibitor cocktail no. 2, Sigma–Aldrich Inc., Steinheim, Germany) using the Precellys 24 system (Bertin Technologies, France). Samples were delipidated using a methanol/diethyl ether (3:7) extraction. Proteins were quantified using a 2D Quant kit (GE Healthcare, Piscataway, NJ, USA) and electrophoresed in NuPAGE 4–12% Bis–Tris gradient polyacrylamide gels (Invitrogen, Barcelona, Spain). MES was used for AMPK, pAMPK, C/EBPβ and actin electrophoresis, while MOPS buffer (Invitrogen) was used for FASN. Proteins were transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Detection antibodies were rabbit anti-AMPK (2532, Cell Signaling Tech., Danvers, MA, USA), rabbit anti-pAMPK (2531, Cell Signaling Tech.), rabbit anti-C/EBPβ (Δ198, St. Cruz Biotech., Heidelberg, Germany), rabbit anti-FAS (3180, Cell Signaling Tech.) and rabbit anti-actin (H-300, St. Cruz Biotech.). The secondary antibody was goat anti-rabbit-HRP (Dako, Glostrup, Denmark). Chemiluminescent detection was performed using the ECL Advance Western Blotting Detection kit (Amersham, GE Healthcare, Barcelona, Spain), and membranes were analyzed in a VersaDoc system (Bio-Rad, Spain).

*Statistical analyses*

Values are represented as mean ± SEM. Differences between groups were compared using non-parametric tests and were considered statistically significant when $p < 0.05$. All statistical analyses were carried out using the Statistical Package for Social Sciences version 15.0 (SPSS, Chicago, IL, USA).

## Results

*Rooibos extract is composed of multiple distinct phenolic compounds*

A total of 25 phenolic compounds were successfully separated and identified with a gradient optimized for negative ionization polarity in less than 30 min. The base peak chromatogram obtained using an ESI-TOF mass spectrometer and a three-dimensional chromatogram showed a correlation between retention time, $m/z$, and intensity, as depicted in Fig. 2A and B, respectively. Identified and characterized compounds of each peak are summarized in Table 1. The average total polyphenol content, as evaluated by the Folin–Ciocalteu assay, was 252.07 ± 8.01 mg/g of rooibos tea extract, expressed as the equivalent of caffeic acid.

*Effects on body weight and food intake were apparently contradictory and evident after a short period of treatment*

The extract is a complex mixture of polyphenolic compounds (Fig. 2 and Table 1) in which the quantitatively important components are orientin, isoorientin, vitexin, aspalathin, rutin and quercetin (and derivatives). The continuous ingestion of such a mixture under the study conditions did not exhibit toxic effects. Metabolic effects, however, were clearly dependent upon the type of diet consumed, even in a mouse model that was already metabolically compromised. This was immediately evident in the amount of body weight gain and food intake observed in the animals. Surprisingly, when mice were fed a high-fat, high-cholesterol diet, the rooibos extract increased the consumption of food, and a subsequent increase in body weight was observed (Fig. 3A). Conversely, when fed a chow diet, the rooibos extract had the opposite effect; the mice did not gain body weight after 4–7 weeks of treatment. Such an effect was not due to a generally decreased food intake (Fig. 3B). These diet-conditioned effects were further analyzed by weighting individual organs and tissues (Table 2). Notably, under both diets, the weight of the pancreas was significantly higher when supplemented with the rooibos polyphenolic mixture. There were also trends towards lower muscle weight and higher brown adipose tissue (BAT) weight. However, the two diets elicited opposite effects in other tissues. The rooibos extract decreased liver weight and increased the weight of epididymal and inguinal white adipose tissue (WAT) in mice fed CD. However, when fed a HF diet, liver weight increased and WAT weight decreased significantly, indicating that the effect of rooibos is sensitive to the characteristics and function of the analyzed metabolic tissue.

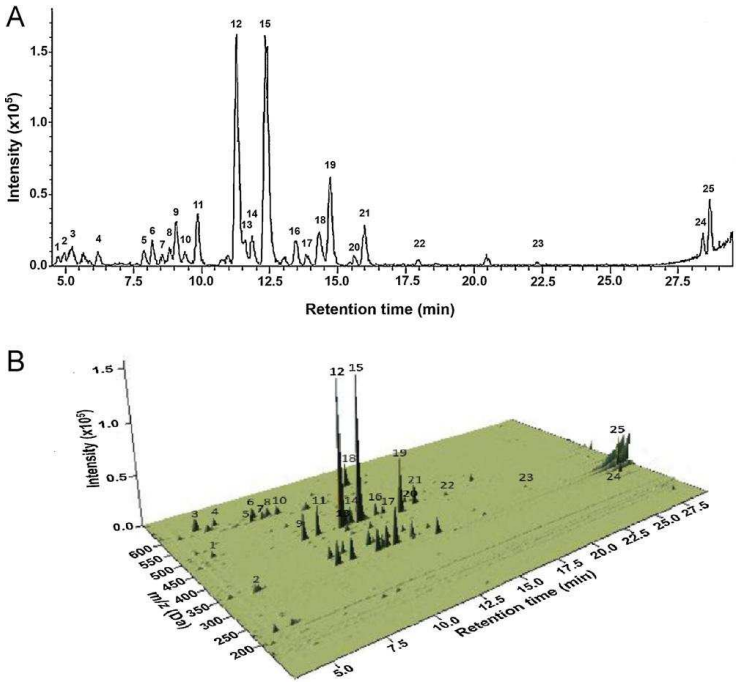


**Fig. 2.** The ESI-microTOF base peak chromatogram of rooibos extract obtained using negative ion mode (A). A three-dimensional chromatogram of the rooibos extract (B).

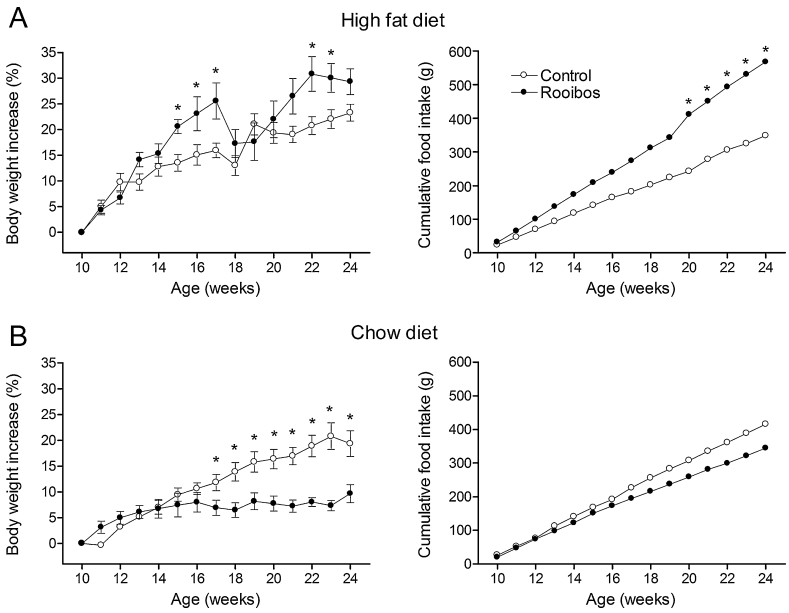


**Fig. 3.** Body weight gain and cumulative food intake in mice fed either HF (A) or CD (B). Open circles represent the control group and closed circles the group supplemented with aqueous rooibos extract. $^{*}p < 0.05$ for differences between groups.

**Table 1**
Mass spectral data obtained in the rooibos tea extract and identified by ESI-microTOF-IT.

| Compound | RT | Molecular formula | *m/z* experimental | *m/z* calculated | UV (nm) | MS/MS fragments | Proposed compound |
|---|---|---|---|---|---|---|---|
| 1 | 4.79 | $C_{22}H_{21}O_{13}$ | 493.0968 | 493.0988 | 278 | 475, 403, 373, 385, 355 | Patuletin 7-glucoside |
| 2 | 4.99 | $C_{15}H_{15}O_9$ | 339.0710 | 339.0722 | 257<br>284<br>314(sh) | 249, 219 | Esculin |
| 3 | 5.30 | $C_{27}H_{31}O_{16}$ | 611.1600 | 611.1618 | 284 | 521, 491, 401, 371 | Safflomin A |
| 4 | 6.22 | $C_{27}H_{29}O_{16}$ | 609.1446 | 609.1461 | 261<br>341 | 519, 489 | Quercetin-3-*O*-robinobioside |
| 5 | 7.91 | $C_{26}H_{27}O_{15}$ | 579.1359 | 579.1355 | 271<br>341 | 561, 519, 489, 459, 441,<br>399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-β-arabinopyranosylorientin |
| 6 | 8.20 | $C_{27}H_{29}O_{15}$ | 593.1496 | 593.1512 | 271<br>333 | 575, 503, 473, 413, 383,<br>353 | Vicenin-2 |
| 7 | 8.58 | $C_{26}H_{27}O_{15}$ | 579.1356 | 579.1355 | 271<br>341 | 561, 519, 489, 459, 429,<br>399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 8 | 8.85 | $C_{26}H_{27}O_{15}$ | 579.1353 | 579.1355 | 271<br>341 | 559, 519, 489, 459, 399,<br>369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 9 | 9.10 | $C_{21}H_{21}O_{11}$ | 449.1088 | 449.1089 | 287 | 359, 329 | (*S*)-eriodictyol-6-*C*-*β*-D-glucopyranoside |
| 10 | 9.40 | $C_{26}H_{27}O_{15}$ | 579.1365 | 579.1355 | 271<br>341 | 561, 519, 489, 459, 429,<br>399, 369 | Carlinoside or isocarlinoside or neocarlinoside or 2″-*O*-*β*-arabinopyranosylorientin |
| 11 | 9.89 | $C_{21}H_{21}O_{11}$ | 449.1072 | 449.1089 | 287 | 359, 329 | (*R*)-eriodictyol-6-*C*-*β*-D-glucopyranoside |
| 12 | 11.27 | $C_{21}H_{19}O_{11}$ | 447.0923 | 447.0933 | 269<br>348 | 429, 411, 357, 327 | Isoorientin |
| 13 | 11.63 | $C_{21}H_{21}O_{11}$ | 449.1055 | 449.1089 | 285 | 359, 329 | (*S*)-eriodictyol-8-*C*-*β*-D-glucopyranoside |
| 14 | 11.88 | $C_{21}H_{21}O_{11}$ | 449.1069 | 449.1089 | 286 | 359, 329 | (*R*)-eriodictyol-8-*C*-*β*-D-glucopyranoside |
| 15 | 12.34 | $C_{21}H_{19}O_{11}$ | 447.0939 | 447.0933 | 254<br>267(sh)<br>349 | 357, 327 | Orientin |
| 16 | 13.43 | $C_{21}H_{23}O_{11}$ | 451.1253 | 451.1246 | 280 | 361, 331 | Aspalathin |
| 17 | 13.83 | $C_{21}H_{21}O_{11}$ | 449.1100 | 449.1089 | 282 | 359, 329, 285 | Aspalalinin |
| 18 | 14.32 | $C_{27}H_{29}O_{16}$ | 609.1465 | 609.1461 | 267<br>348 | 301 | Rutin |
| 19 | 14.74 | $C_{21}H_{19}O_{10}$ | 431.1002 | 431.0984 | 270<br>336 | 413, 341, 311 | Vitexin/isovitexin |
| 20 | 15.59 | $C_{21}H_{19}O_{12}$ | 463.0897 | 463.0882 | 276 | 301, 341, 371 | Quercetin-3-*O*-glucoside/galactoside |
| 21 | 15.97 | $C_{21}H_{19}O_{11}$ | 447.0921 | 447.0933 | 270<br>342 | 285 | Luteolin-7-*O*-glucoside |
| 22 | 17.93 | $C_{21}H_{23}O_{10}$ | 435.1287 | 435.1297 | 283 | 345, 315 | Nothofagin |
| 23 | 22.29 | $C_{20}H_{25}O_6$ | 361.1633 | 361.1657 | 280 | 346, 331 | Secoisolariciresinol |
| 24 | 28.37 | $C_{15}H_9O_6$ | 285.0386 | 285.0405 | 241<br>269<br>335 | 151, 107 | Luteolin |
| 25 | 28.60 | $C_{15}H_9O_7$ | 301.0335 | 301.0354 | 254<br>271(sh)<br>371 | 179, 151 | Quercetin |

*Note*: sh = shoulder.

*Effects of aqueous rooibos extract in hepatic and adipose tissue*

We examined liver histology and found that dietary-induced liver steatosis was completely prevented by the rooibos extract (Fig. 4A) and that this was associated with a significant decrease in macrophage recruitment as assessed by F4/80 immunostaining (Fig. 4B). In mice fed CD, the degree of steatosis was significantly lower, and the effect of the rooibos extract was not appreciable. Further, we did not find significant alterations in serum biomarkers of liver injury, mainly bilirubin and AST, in any of the conditions assayed (Fig. 4C).

We also assessed the size and number of adipocytes in adipose tissue. As expected, the adipocyte area was increased in control mice fed HF relative to mice fed CD. Despite a significant trend towards lower WAT weight in response to rooibos treatment, the differences in adipocyte size and number were not significant. However, the opposite effect was observed in mice fed CD (Fig. 5A), where rooibos supplementation resulted in a significant increase in the adipocyte area of epididymal WAT. The large variability in adipocyte size in inguinal WAT prevented the difference between the groups in adipocyte area in this tissue from reaching significance (data not shown). To further assess this effect, we used an

**Table 2**
The course of individual weights of organs and selected tissues (mg/g body weight) were dependent on the type of diet (#) and on treatment with rooibos extract (*). ($^{*,\#}p < 0.05$; $^{**,\#\#}p < 0.01$, denote differences respect to the corresponding group).

| | Chow diet | | High fat diet | |
|---|---|---|---|---|
| | Control | Rooibos | Control | Rooibos |
| Liver | 42.2 ± 1.4 | 38.9 ± 1.7 | 41.9 ± 1.3 | 46.5 ± 0.7$^{*,\#\#}$ |
| Pancreas | 9.4 ± 0.8 | 13.6 ± 0.4$^{**}$ | 9.5 ± 0.9 | 13.9 ± 0.6$^{**}$ |
| Muscle | 13.5 ± 0.3 | 12.8 ± 0.3 | 13.4 ± 0.4 | 12.1 ± 0.2$^{*}$ |
| Epididimal WAT | 13.5 ± 1.1 | 15.6 ± 2.3 | 18.5 ± 1.9 | 14.5 ± 1.7 |
| Inguinal WAT | 11.8 ± 0.8 | 15.9 ± 0.2$^{**}$ | 15.1 ± 1.0$^{\#}$ | 12.7 ± 0.9$^{\#}$ |
| BAT | 2.5 ± 0.2 | 3.2 ± 0.6 | 2.1 ± 0.2 | 2.4 ± 0.1$^{\#}$ |

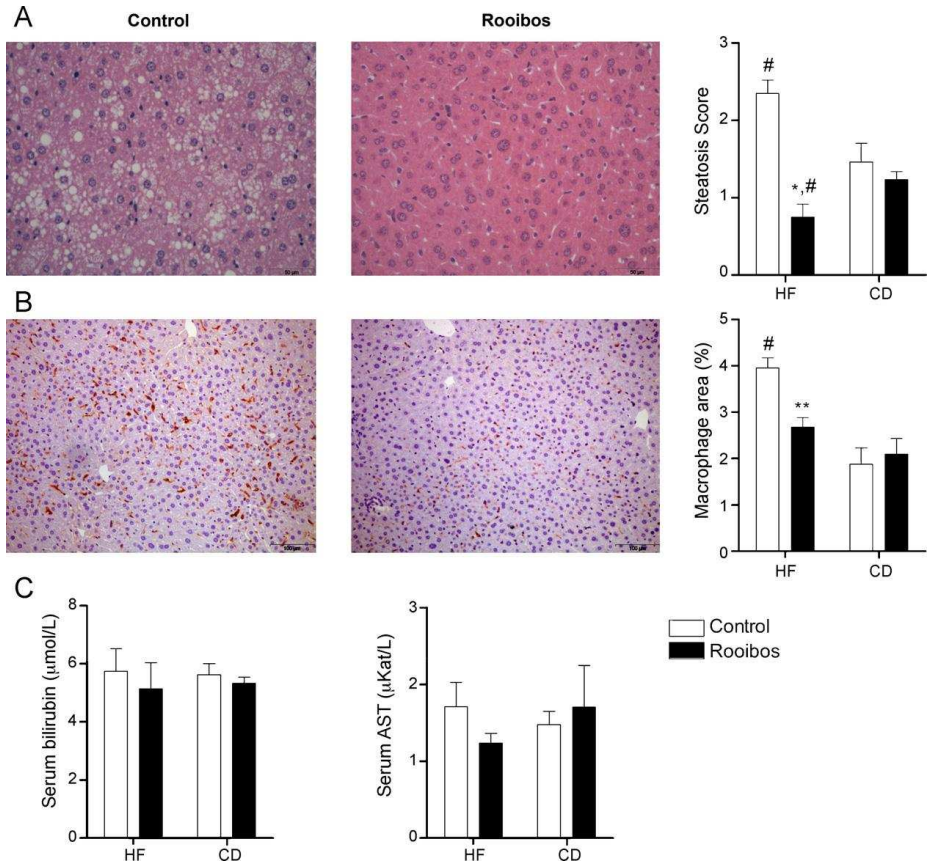


**Fig. 4.** Representative microphotographs illustrating the effect of rooibos extract consumption in livers of mice fed HF, decreasing steatosis (A) and macrophage infiltration (B) without affecting serum biomarkers of liver injury (C). $^{*}p < 0.05$; $^{**}p < 0.01$ for differences between control and rooibos treatment for the same dietary group; $^{\#}p < 0.05$ for differences between dietary groups.

*in vitro* model with cultured 3T3-L1 mouse adipocytes. We found no effect of rooibos on differentiation from pre-adipocytes, but in mature adipocytes, which readily accumulate large intracellular fat droplets, rooibos extract significantly inhibited the accumulation of triglycerides in a dose-dependent manner as assessed by AdipoRed staining (Fig. 5B) without affecting cell viability (Fig. 5C and D).

*Other metabolic effects and possible mechanisms*

Despite such obvious metabolic effects, these were not related to changes in insulin resistance as assayed by glucose tolerance tests (Fig. 6). Effects of rooibos on glucose metabolism are therefore unlikely, but we found significant changes in lipoprotein metabolism associated with rooibos extract consumption. Specifically, rooibos extract had marked hypolipidemic effects in this particular model. This finding was limited to mice fed a HF diet and consisted of significant reductions in serum cholesterol, triglycerides and free fatty acids (Fig. 7A). As observed in the FPLC distribution, the reduction in cholesterol was evident in all lipoprotein particles, while the reduction in triglycerides was limited to very low density lipoproteins (VLDL) (Fig. 7B).

Taken together, our results suggest that possible molecular targets of the polyphenols contained in rooibos extract are mainly located in the liver and adipose tissue, and we assessed the expression of relevant candidate proteins in both of these tissues. We found that rooibos extract significantly activated the AMP-activated protein kinase (AMPK) in the liver but not in the eWAT, and this effect was not dependent on the type of diet. Similarly, the expression of CCAAT/enhancer-binding protein (C/EBPβ) was higher in the eWAT of rooibos-treated animals. Again, this effect was not affected by diet and was not evident in the liver. Finally, there was a trend towards lower expression of fatty acid synthase (FASN) in the liver, but the difference was not significant. Conversely, FASN expression was increased in adipose tissue, but this was only evident in the eWAT of mice fed CD under rooibos treatment (Fig. 8).

## Discussion

Our data indicate that the continuous administration of polyphenolic compounds may play a complementary role in the treatment of metabolic diseases. Although a preventive effect can-

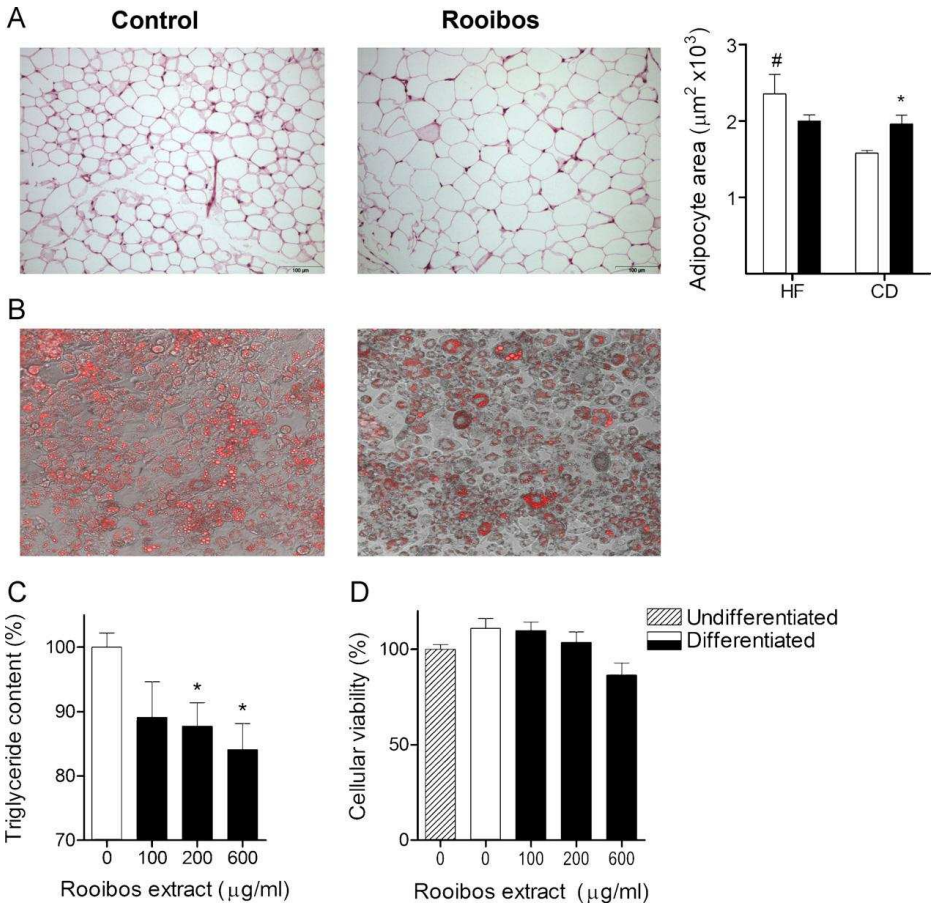


**Fig. 5.** The rooibos extract increased the adipocyte size of animals fed on CD (A, representative microphotographs for eWAT). 3T3-L1 adipogenesis was analyzed using the AdipoRed stain (B, adipocytes treated with 600 μg/ml of rooibos extract), showing dose-dependent anti-adipogenic activity of rooibos extract (C). No toxic effects were observed at the assayed concentrations (D). Adipocyte area differences are marked with # between dietary groups and with * between treatment groups for the same diet (both $p < 0.05$). For cellular experiments, differences between groups are marked with $^{*}(p < 0.05)$.

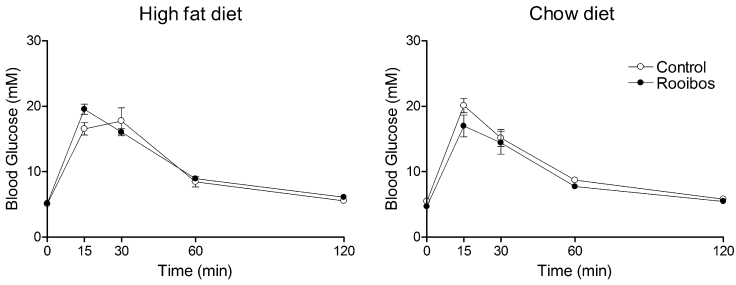


**Fig. 6.** The glucose tolerance tests showed no differences derived from diet type or rooibos extract supplementation.

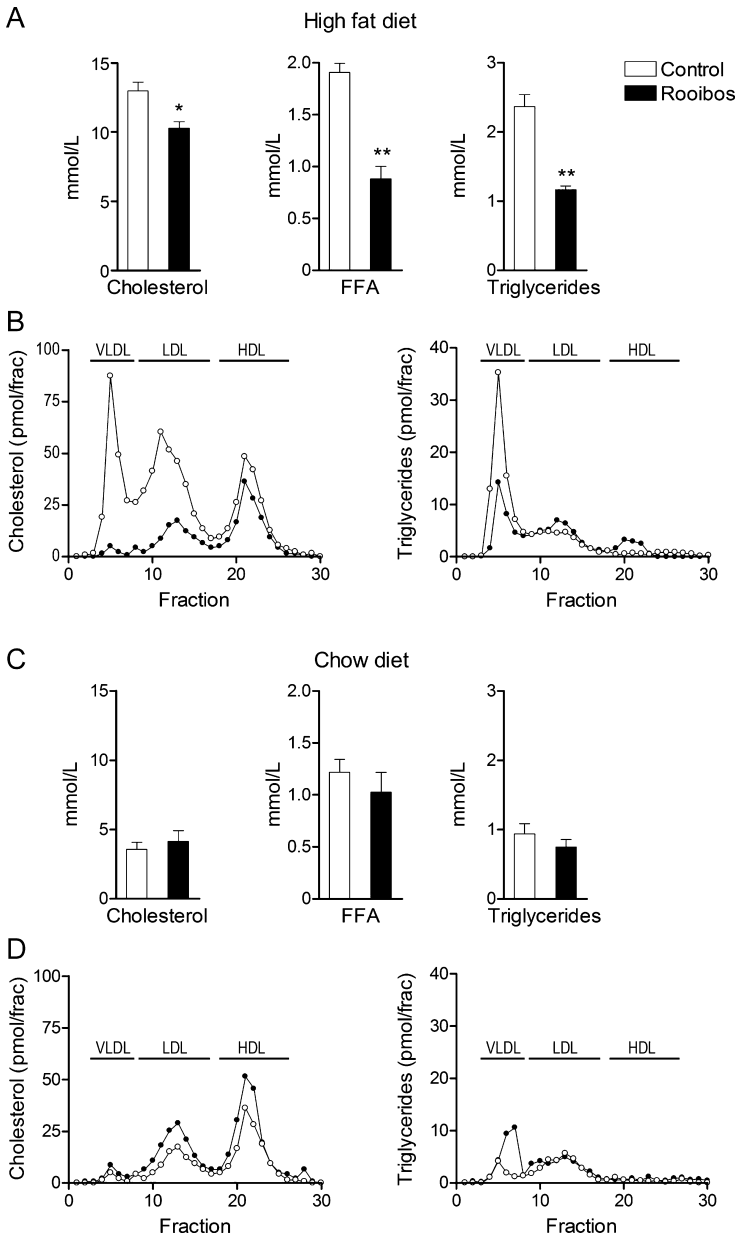


**Fig. 7.** The rooibos extract elicited a hypolipemic effect in mice fed on HF (A) with significant changes in lipoprotein distribution (B). Such effects were not observed in the CD dietary group (C and D). Open bars and circles represent controls, and closed bars and circles represent rooibos-treated animals. $^{*}p < 0.05$ and $^{**}p < 0.01$ for differences between control and rooibos treatment.

not be ruled out from our design, this seems unlikely because none of the beneficial effects were observed in metabolically normal mice. In this particular hyperlipemic model, which involves well-documented metabolic disturbances, we found that rooibos extract significantly affected body weight, the development of liver steatosis, the accumulation of triglycerides in adipocytes and the course of hyperlipidemia. These effects were not related to changes in insulin resistance and were most evident in animals fed

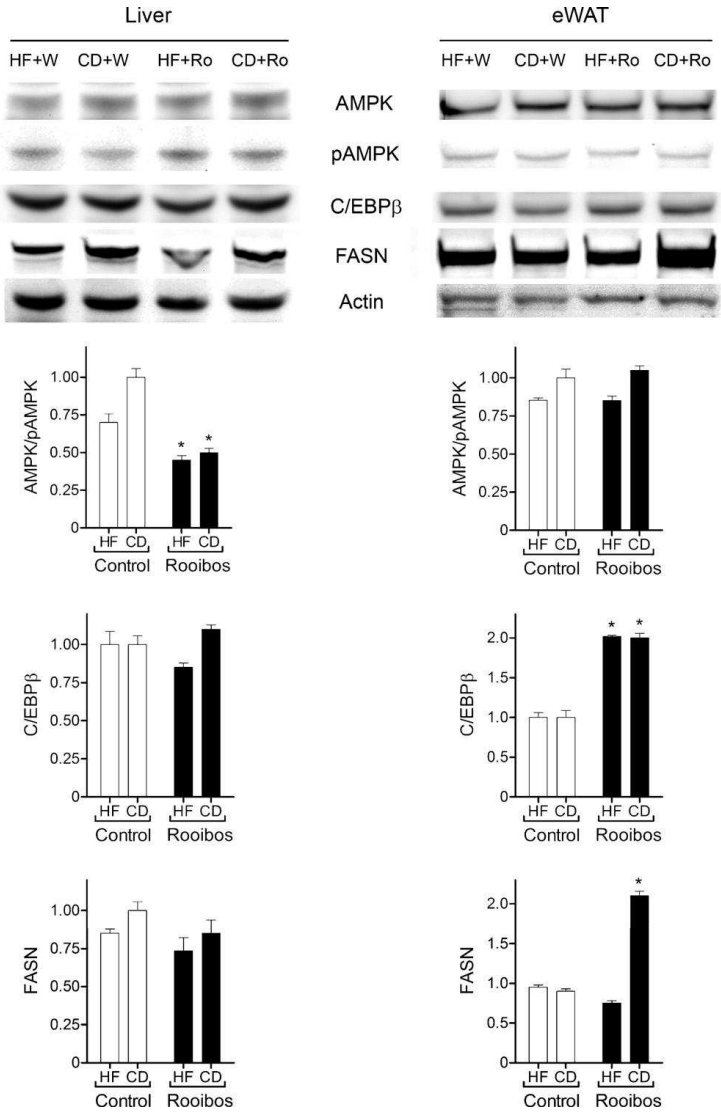


**Fig. 8.** The different expression of candidate proteins in the liver (left) and epididymal WAT (right) from all groups studied. The rooibos extract activated AMPK in both dietary groups by increasing pAMPK expression in the liver. In the eWAT, rooibos affected mainly C/EBPβ protein expression in both dietary groups, but the increased expression of FASN was only evident in the CD dietary group. $^{*}p < 0.05$ for differences between control and rooibos treatment.

a Western-type diet. That plants, and particularly fruits and vegetables, may benefit human health is widely recognized (Joshipura et al. 2001), but the role of dietary polyphenols, although suggestive, remains unclear. There is currently little serious effort to develop plant-derived molecules for therapeutic purposes. There is certainly no enthusiasm for promoting the ingestion of complex mixtures of polyphenols and testing their possible health benefits in humans, probably because patentability is clearly limited. In addition, polyphenols seem to interact with numerous targets and multiple deregulated signaling pathways (Johnson et al. 2010), raising doubts regarding their safety and ease of administration. For instance, resveratrol modulates more than twenty enzymes and receptors (Baur and Sinclair 2006). Green tea polyphenols are also an example of this notion (Khan et al. 2006; Johnson et al. 2010), and curcumin, a much simpler compound, may influence more than 60 molecular targets (Goel et al. 2008). Due to the recent increasing interest in the consumption of natural products, certain beverages containing polyphenols are widely distributed, proving

*ex juvantibus* that they are generally nontoxic and may provide a wide range of health benefits in humans. One of these examples is rooibos, which is now consumed in Western societies as a plant infusion and therefore as a complex mixture of molecules. Most of its components are recognized as having antioxidant properties (Bramati et al. 2002), although it is unlikely that *in vivo* antioxidant effects explain the alleged health benefits. On the other hand, it is documented that the polyphenols contained in the rooibos extract, mainly aspalathin, orientin and rutin, are effectively absorbed (Boyle et al. 2000; Courts and Williamson 2009; Kreuz et al. 2008; Li et al. 2008).

The effects we describe are certainly the result of intense modulation of the overall management of cellular energy. It is interesting to note that these effects are negligible in normal mice and only evident in a model in which metabolism is seriously disturbed (Rodríguez-Sanabria et al. 2010). Moreover, these effects are more intense in mice fed a clearly unhealthy diet similar to that recently adopted by humans. Our findings regarding the body weight and food intake of animals supplemented with aqueous rooibos extract may seem counterintuitive. Our interpretation is based on the fact that the liver and the WAT are the two primary lipid storage organs in mammals. Although obesity is characterized by an increase in adipocyte number and/or size (White and Stephens 2009), in the absence of aberrant diets, rooibos extract promotes lipid accumulation in adipose tissue and probably breaks down stored fat in the liver, thus preventing lipotoxicity in this non-adipose organ. Rooibos extract, in the presence of excessive caloric intake provided by a high-fat diet, protects the liver from lipid storage without increasing the accumulation of fat in adipose tissue. The effects we describe in the liver may be relevant because liver steatosis is highly prevalent in adults and children and may progress to more serious conditions (Alkhouri et al. 2009).

Obviously, impaired fat absorption as an explanation for such a net reduction in fat storage cannot be ruled out based on our data, but our findings clearly suggest that rooibos extract may elicit similar effects to those described for resveratrol, i.e., increasing metabolic rate and mitochondrial biogenesis (Marques et al. 2009). Interestingly, we did not observe a hypoglycemic effect or a change in insulin sensitivity as previously described in diabetic mice (Kawano et al. 2009), but we partially confirmed the hepatoprotective and anti-lipogenic effects reported in rats and other mouse models (Ulicna et al. 2003; Hoffmann-Bohm et al. 1992; Janbaz et al. 2002; Odbayar et al. 2006). It is also possible that the previously reported anti-inflammatory effects of rooibos extract (Baba et al. 2009) may play a role in the significant decrease in macrophage recruitment that we observed. Moreover, polyphenols selectively decrease macrophage chemotaxis via a reduction in MCP-1 expression (Beltran-Debon et al. 2010).

Additionally, we found a strong hypolipidemic effect that, again, was only observed in mice fed a HF diet, which may partially confirm the hypocholesterolemic effect observed in similar models (Ziaee et al. 2009). It is of note, however, that we also observed a significant decrease in serum triglyceride and free fatty acid concentrations, highlighting an overall effect on the management of fat by both the liver and adipose tissue. In this regard, we found significant activation of AMPK in the liver but not in adipose tissue. The activation of this master metabolic regulating enzyme is relevant and a novel finding. Activated AMPK switches cells from active ATP consumption (e.g., fatty acid and cholesterol biosynthesis) to active ATP production (e.g., fatty acid and glucose oxidation) (Carling 2004). Recent studies in mice deficient in the catalytic subunit of AMPK may also explain the responses of FASN and C/EBP in this model and suggest that AMPK is the central target for the metabolic effects of polyphenols (Um et al. 2010).

Our data also blaze new trails to further investigate a more general mechanism of action for ingested polyphenols. For instance, it is extremely suggestive of the recent finding that autophagy regulates adipose mass and differentiation in mice (Singh et al. 2009), a phenomenon that enables adaptation to stress, maintains metabolism and promotes cellular viability as well as fitness (Sasaki et al. 2010). Polyphenols have well documented antioxidant activities and provide other therapeutic targets (Ulrich-Merzenich et al. 2009). We confirm in this study previous findings suggesting more beneficial effects of the whole extract than, probably, individual, isolated compounds. We also support a change in paradigm in this field and the search for possible synergies may constitute an appropriate research effort (Wagner and Ulrich-Merzenich 2009).

We add Rooibos extract and its metabolic effects to such effort and suggest the combination with other bioactive extracts in order to attenuate the metabolic abnormalities associated to human diseases.

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# ESTUDIO 4

## The aqueous extract of *Hibiscus sabdariffa* calices modulates the production of monocyte chemoattractant protein-1 in humans.

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# The aqueous extract of *Hibiscus sabdariffa* calices modulates the production of monocyte chemoattractant protein-1 in humans

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ABSTRACT

Diet supplementation and/or modulation is an important strategy to significantly improve human health. The search of plants as additional sources of bioactive phenolic compounds is relevant in this context. The aqueous extract of *Hibiscus sabdariffa* is rich in anthocyanins and other phenolic compounds including hydroxycitric and chlorogenic acids. Using this extract we have shown an effective protection of cultured peripheral blood mononuclear cells from the cellular death induced by $H_2O_2$ and a significant role in the production of inflammatory cytokines. *In vitro*, the extract promotes the production of IL-6 and IL-8 and decreases the concentration of MCP-1 in supernatants in a dose-dependent manner. In humans, the ingestion of an acute dose of the extract (10 g) was well tolerated and decreased plasma MCP-1 concentrations significantly without further effects on other cytokines. This effect was not due to a concomitant increase in the antioxidant capacity of plasma. Instead, its mechanisms probably involve a direct inhibition of inflammatory and/or metabolic pathways responsible for MCP-1 production, and may be relevant in inflammatory and chronic conditions in which the role of MCP-1 is well established. If beneficial effects are confirmed in patients, *Hibiscus sabdariffa* could be considered a valuable traditional herbal medicine for the treatment of chronic inflammatory diseases with the advantage of being devoid of caloric value or potential alcohol toxicity.


## Introduction

Anthocyanins are responsible for the attractive bright colors of some fruits and vegetables consumed in Western-type diets where they represent the most abundantly ingested flavonoids. Moreover, dietary supplementation with commercially available anthocyanin-rich extracts, especially from berries and grapes is currently advised by some investigators (Jing et al. 2008). However, the relationship between their chemical structures and corresponding biological activity and consequent putative health benefits is poorly understood (Jing et al. 2008). It is plausible that their effects are not uniquely elicited by their anthocyanin profiles, which may vary greatly, and that other accompanying phenolic compounds are involved. It is also possible that the combined actions of naturally occurring compounds may be more beneficial than the observed in isolated compounds. Therefore, research into plants that may be directly incorporated into normal diets may represent an alternative strategy.

The flowers of *Hibiscus sabdariffa* are rich in anthocyanins (Segura-Carretero et al. 2008) and are normally consumed throughout the world. Their extracts exert significant effects in animal and *in vitro* models (Chen et al. 2003; Haji Faraji et al. 1999; Hou et al. 2005; Lin et al. 2005; Mojiminiyi et al. 2007; Onyenekwe et al. 1999; Tseng et al. 1997; Tseng et al. 2000) but studies in humans are limited to their diuretic effect (Herrera-Arellano et al. 2004). We hypothesize that monocyte chemoattractant protein-1 (MCP-1), an emergent biomarker in the evaluation of inflammatory diseases, is involved in some of the potential benefits ascribed to this plant (Gonzalez-Quesada et al. 2009; Marsillach et al. 2005; Rollins 1996; Rull et al. 2007). In this study, we have extended our previous (Segura-Carretero et al. 2008) characterization of the phenolic compounds present in the *Hibiscus sabdariffa* extract (HSE) and, with the rationale that the

*Abbreviations:* DAD, diode array detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; HSE, *Hibiscus sabdariffa* extract; IT, ion trap (analyzer); MCP-1, monocyte chemoattractant protein-1; MS, mass spectrometry; ORAC, oxygen radical absorbance capacity; PBMCs, peripheral blood mononuclear cells; TOF, time of flight (analyzer); UV, ultraviolet

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HSE may reduce oxidative stress and inflammation (Kao et al. 2009; Marsillach et al. 2009), we have also explored the *in vivo* and *in vitro* effects in the production of selected cytokines.

## Materials and methods

### *Characterization and identification of phenolic compounds*

Sun-dried calices of *Hibiscus sabdariffa* were obtained from Guerle (Senegal). For analytical measurements, powdered plant material (650 g) was heated in 5 l of distilled water and the mixture was kept boiling for 5 min.

The infusion was filtered, cooled at room temperature, centrifuged and the supernatant was lyophilized and stored at 4 °C until use. The amount of lyophilized *Hibiscus sabdariffa* extract (HSE) obtained was 136 g (4.8:1).

For the HPLC analysis, 0.25 g of HSE were mixed with 5 ml of ultrapure water at room temperature obtaining a solution of 50 g/l freshly prepared for each analysis. The aqueous extract was stirred in a vortex for 10 min until diluted, filtered with single-use filter unit and directly injected into the HPLC system RRLC 1200 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a binary pump and a Zorbax Eclipse Plus C18 4.6 × 150 mm, 1.8 μm column. The compounds of the aqueous extract of *Hibiscus sabdariffa* were separated by the C18 column at room temperature at a flow rate of 0.5 ml/min and the injection volume was 10 μl. The system was equipped with DAD and coupled to a TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) with an orthogonal electrospray interface (ESI G1607A; Agilent Technologies). UV–visible spectrophotometry delimited the class of phenolic compounds and the accurate mass measurements on the TOF spectrometer enabled the identification of the compounds in the extract. Finally, the fragmentation pattern obtained in MS/MS experiments performed with an Esquire 2000 ion trap (IT)-mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) confirmed the proposed structures. Two optimized gradient programs were run in order to separate and identify the different families of compounds present in HSE. Polyphenols were successfully identified and analyzed with a gradient optimized for a negative ionization polarity mode while anthocyanins were identified with a gradient optimized for a positive ionization polarity mode.

### *In vitro studies*

Freshly prepared for each experiment, 100 mg of extract were dissolved in 10 ml of RPMI medium, centrifuged at 1500*g* for 10 min and the supernatant filtered (22 μm). Heparinized whole blood was obtained from healthy volunteers and peripheral blood mononuclear cells (PBMCs) immediately separated using conventional Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Separated cells were washed twice with D-PBS (Gibco-Invitrogen, Carlsbad, CA, USA) followed by resuspension in 3 ml of pre-warmed (37 °C, 30 min) RPMI 1640, 1% penicillin/streptomycin and 10% fetal bovine serum (Sigma-Aldrich Inc, St. Louis, USA). Viable PBMCs ($1 \times 10^6$) were treated with HSE according to the following protocols: (i) PBMCs were incubated simultaneously with HSE and 50 μM hydrogen peroxide ($H_2O_2$) at 37 °C in a humidified atmosphere of 5% $CO_2$ for 20 h; (ii) alternatively, after a pre-incubation of PBMCs with HSE for 24 h and subsequent washing with D-PBS, cells were incubated with 50 μM $H_2O_2$ alone under the same conditions for an additional 20 h. For each sample, the supernatants were kept at −80 °C until further assays. Apoptosis of cells was immediately determined by flow cytometry using the annexin V-FITC Apoptosis Detection Kit (MBL International, Woburn, MA, USA) following the manufacturer's instructions in an EPICS-MCL-XL flow cytometer (Beckman-Coulter, Fullerton, CA, USA).

### *Main characteristics of human volunteers*

Ten ostensibly healthy adult volunteers (5 males and 5 females) between 23 and 50 years of age were recruited from the hospital staff for the study. Each participant gave written fully informed consent to participate in the study and the study procedures were approved and monitored by the Ethics Committee of the Hospital Universitari de Sant Joan de Reus. Participants were instructed to avoid the ingestion of polyphenol-rich foods or beverages (i.e. coffee, tea, juice, oil, chocolate, fruits and vegetables) for at least 2 days and to fast overnight before the experiments. Samples of freeze-dried HSE (10 g) dissolved in tap water were freshly prepared. The HSE was consumed by the participants, after which only water was allowed to be ingested for the following 3 h. Venous blood was obtained in EDTA tubes at 0, 1.5 and 3 h and plasma stored at −80 °C until further assays.

### *Other laboratory measurements and statistical analyses*

Oxygen radical absorbance capacity (ORAC) measurements were performed as previously described by Ou et al. 2001 with minor modifications. Trolox (Sigma-Aldrich, Steinheim, Germany) was used as a standard and results were expressed as Trolox equivalents in micromoles per milliliter or gram. Assays were performed in a Synergy HT Multi-Mode microplate reader (BioTek instruments, Vermont, USA) in flat-bottom black-opaque microplates (Nunc, Langenselbold, Germany). The reaction was monitored for 3 h at intervals of 1 min. Interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) concentrations were measured in supernatants and plasma aliquots using a multiplex cytometric bead-based assay (FlowCytomix Multiplex, BenderMedsystems, Austria).

Differences between two or more groups were compared using non-parametric tests. Differences were considered statistically significant when $p < 0.05$. All statistical analyses were carried out with the Statistical Package for Social Science version 17.0 (SPSS, Chicago, Illinois, USA).

## Results

### *Hibiscus sabdariffa extract composition*

The HSE antioxidant effect of all prepared extracts had similar values of ORAC of approximately 360 TE/g (μmol Trolox per 1 g of HSE). The identified and characterized compounds are shown in Table 1. Representative base peak chromatograms in both negative and positive polarity modes are also depicted in Fig. 1. The most representative anthocyanins present in the extract, delphinidin-3-sambubioside and cyanidin-3-sambubioside, are also accompanied by significant amounts of other phenolic compounds. Among them, the most abundant were hydroxycitric, hibiscus and chlorogenic acids, which may all have additional properties to be considered. Due to the unavailability of commercial standards for the identified compounds a first approach for quantification was measured and expressed in terms of equivalents of a compound with similar UV–vis absorption characteristics and chemical structure. Thus, the content of anthocyanidins, as delphinidin equivalents was 0.45% (w/w, peaks 15 and 16) and the flavonols, as quercetin equivalents

**Table 1**
Mass Spectral Data obtained in the *Hibiscus sabdariffa* extract and identified by ESI-microTOF-IT.

| Compoud | RT | Molecular formula | *m/z*, experimental | *m/z*, calculated | UV (nm) | MS/MS fragments | Proposed compound |
|---|---|---|---|---|---|---|---|
| 1 | 3.20 | $C_6H_7O_8$ | 207.0140 | 207.0146 | – | 189, 127 | Hydroxycitric acid |
| 2 | 3.42 | $C_6H_5O_7$ | 189.0035 | 189.0041 | – | 127 | Hibiscus acid |
| 3 | 5.20 | $C_{16}H_{17}O_9$ | 353.0891 | 353.0878 | 297<br>324 | 191, 179, 135 | Chlorogenic acid isomer I |
| 4 | 7.10 | $C_{16}H_{17}O_9$ | 353.0872 | 353.0878 | 297<br>324 | 191 | Chlorogenic acid |
| 5 | 7.60 | $C_{16}H_{17}O_9$ | 353.0871 | 353.0878 | 297<br>324 | 191, 179, 135 | Chlorogenic acid isomer II |
| 6 | 10.00 | $C_{26}H_{27}O_{17}$ | 611.1271 | 611.1254 | 352 | 317 | Myricetin 3-arabinogalactose |
| 7 | 12.60 | $C_{26}H_{27}O_{16}$ | 595.1309 | 595.1305 | 345 | 463, 445, 301 | Quercetin 3-sambubioside |
| 8 | 13.40 | $C_{16}H_{15}O_8$ | 335.0768 | 335.0772 | 296<br>326 | 161, 135 | 5-*O*-Caffeoylshikimic acid |
| 9 | 14.50 | $C_{27}H_{29}O_{16}$ | 609.1462 | 609.1461 | 255<br>353 | 301 | Quercetin 3-rutinoside |
| 10 | 16.00 | $C_{21}H_{19}O_{12}$ | 463.0873 | 463.0882 | 253<br>356 | 301 | Quercetin 3-glucoside |
| 11 | 17.50 | $C_{27}H_{29}O_{15}$ | 593.1512 | 593.1512 | 265<br>350 | 285 | Kaempferol 3-*O*-rutinoside |
| 12 | 26.70 | $C_{18}H_{19}NO_4$ | 312.1234 | 312.1241 | 286<br>316 | 178, 135 | N-Feruloyltyramine |
| 13 | 27.60 | $C_{30}H_{25}O_{13}$ | 593.1312 | 593.1301 | – | 447, 285 | Kaempferol 3-(*p*-coumarylglucoside) |
| 14 | 28.40 | $C_{15}H_9O_7$ | 301.0339 | 301.0354 | 253<br>372 | 179, 151 | Quercetin |
| 15 | 12.71 | $C_{26}H_{29}O_{16}$ | 597.1446 | 597.1450 | 520<br>280 | 303 | Delphinidin-3-sambubioside |
| 16 | 14.00 | $C_{26}H_{29}O_{15}$ | 581.1493 | 581.1501 | 520<br>280 | 287 | Cyanidin 3-sambubioside |

Numbers designing compounds correspond to peaks as depicted in Fig. 1.

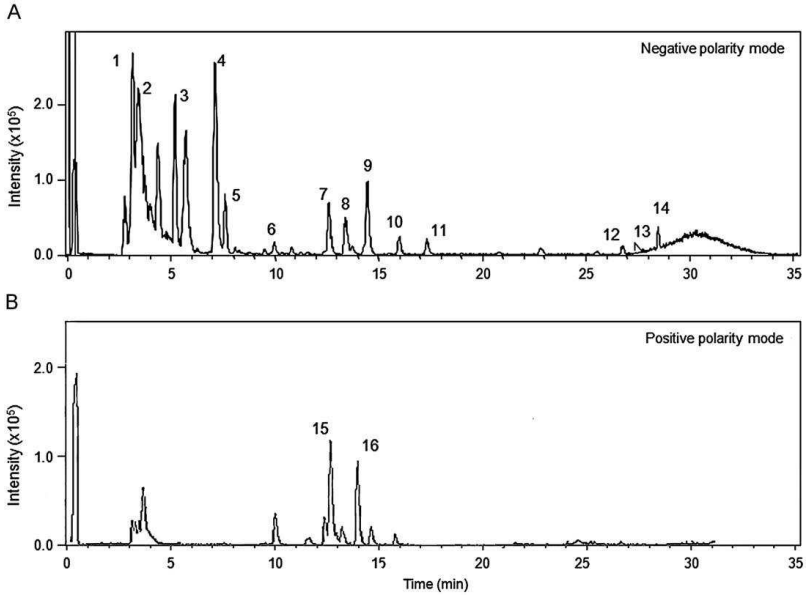


**Fig. 1.** Representative base peak chromatograms obtained in negative (A) and positive (B) polarity modes at the two optimized gradient programs. Peaks and mass spectral data of the proposed compounds are shown in Table 1.

were 0.07% (w/w, peaks 6, 7, 9, 10, 11, 13 and 14). Phenolic acid and N-feruloyltyramine were quantitated as chlorogenic equivalents (0.6%, w/w, peaks 3, 4, 5, 8 and 12), and organic acids as cafeic acid (4% w/w, peaks 1, 2).

*The in vitro effects of Hibiscus sabdariffa extract*

To characterize our model of cell apoptosis on freshly isolated PBMCs, we first assayed the possible cytotoxicity of HSE. When

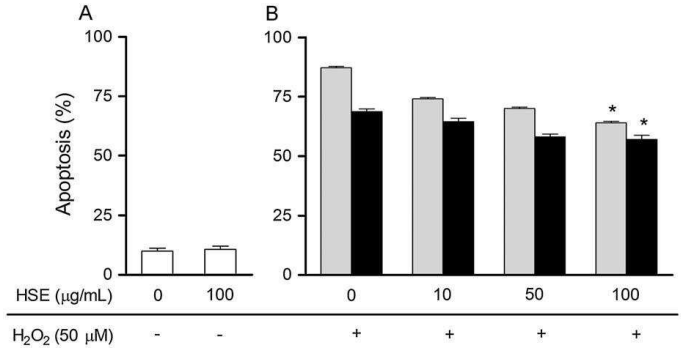


**Fig. 2.** *Hibiscus sabdariffa* extract do not show cytotoxic effects in cultured PBMCs (control, white bars) and prevents $H_2O_2$-induced cell death in co-incubation and pre-incubation models (grey and black bars, respectively). Bars represent mean ± SEM.

cells were exposed to incremental doses of HSE (up to 2000 μg/ml) for 24 h, the viability of treated PBMCs was not different from that of untreated cells. Since all tested HSE doses resulted in greater than 95% cell viability, we decided to limit our experiments to HSE concentrations of 10, 50 and 100 μg/ml. Cell death was induced by the addition of $H_2O_2$ (Fig. 2). When PBMCs were co-incubated simultaneously with HSE and $H_2O_2$ we observed a dose-dependent resistance to cell death which could be interpreted as the direct action of antioxidant compounds on the $H_2O_2$. However, if cells were pre-incubated with HSE and the supernatant was free of HSE before the addition of $H_2O_2$ (see Methods), this effect remained and was significantly greater than that observed with a co-incubation. We then measured MCP-1, IL-6 and IL-8 in the supernatants of cells using the pre-incubation model and found that the concentrations of these cytokines were low in the absence of $H_2O_2$ (MCP-1 = 345 ± 17 pg/ml, IL-8 = 665 ± 32 pg/ml and IL-6 was undetectable in most of the measurements). When cell death was induced by $H_2O_2$ the production and secretion of IL-6 and IL-8 increased considerably; this increase was higher when high doses of HSE were incorporated into the cells, indicating that the inflammatory reaction was facilitated by the addition of HSE. Surprisingly, the supernatant concentration of MCP-1 was also augmented in response to the addition of $H_2O_2$ but the effect of HSE on these cells was the opposite: MCP-1 values were decreased with the addition of the highest dose of HSE (Fig. 3).

*The effects of Hibiscus sabdariffa extract on humans*

To translate the above results to humans, HSE was orally administered to healthy volunteers. We found that IL-6 and IL-8 were not detectable before the administration of HSE in our participants and remained so throughout the experiment. This confirmed that the participants were free of inflammatory conditions that may interfere with the interpretation of the results and that the ingestion of HSE does not have further effects in the absence of inflammatory stimuli. However, MCP-1 was readily observed in circulating plasma before the experiment and decreased in all participants up to a mean 17% lower value at the 1.5 h time-point. This trend was significant at the 3 h time-point ($p < 0.05$; 23.2%). We did not observe significant differences between males and females hence the combined results are presented. Finally, this effect was not correlated with a putative antioxidant effect in plasma of the ingestion of HSE, at least as assessed by the ORAC method (Fig. 4).

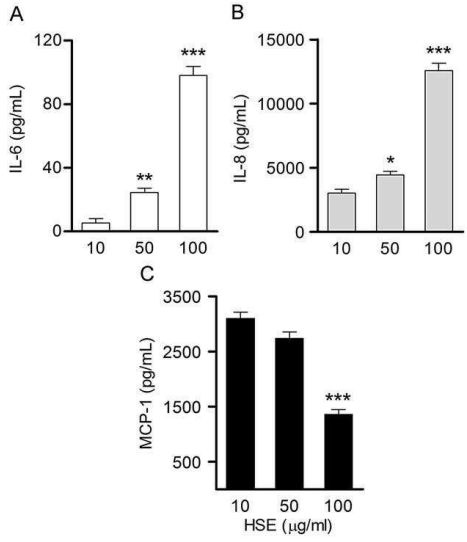


**Fig. 3.** Influence of pre-incubation with HSE at concentrations of 10, 50 or 100 μg/ml on the supernatant concentrations of IL-6, IL-8 and MCP-1 in cultured PBMCs after the addition of 50 μM $H_2O_2$. Values represent mean ± SEM (*$p < 0.03$; **$p < 0.01$; ***$p < 0.001$).

## Discussion

Searching for conventional risk factors of chronic diseases is a limited strategy that has consistently failed. An alternative strategy is to focus on the overall population through simple dietary modifications, given that scientific evidence increasingly supports the view that alterations in diet have strong effects on the lifetime health status. However, funding clinical trials to demonstrate the effectiveness of dietary polyphenols is complicated by the fact that these agents have limited, if any, intellectual property protection. Some investigators, as well as the food industry, are focusing on individual elements in an effort to circumvent this limitation. Our approach, however, was to examine the effects of the whole extract. Our compositional study

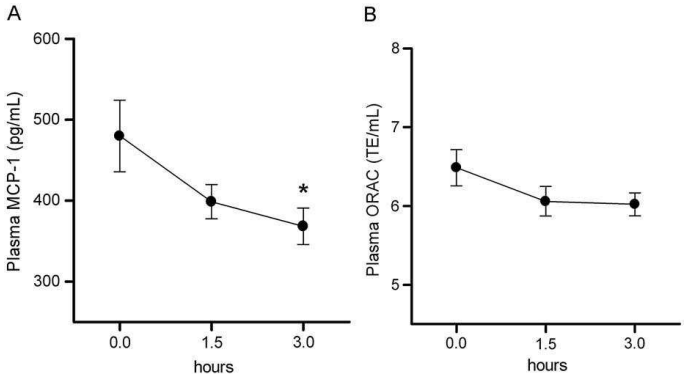


**Fig. 4.** Plasma MCP-1 concentration (A) and plasma ORAC (B) measured before and after the ingestion of HSE (10 g). Values represent mean ± SEM (*$p < 0.05$).

clearly demonstrates that *Hibiscus sabdariffa* contains numerous polyphenols with biological activities, including anthocyanins, hydroxycitric acid, hibiscus acid and chlorogenic acid, all of which are currently marketed as supplemental products for weight loss and other conditions (Van Gaal et al. 2004). Our approach may cause cumbersome interpretation of results but facilitates the oral administration of the extract when needed. Plants that are widely used in folk medicine are immediate candidates for research, including *Hibiscus sabdariffa*, which is traditionally used to treat hypertension and hepatic disorders (Haji Faraji et al. 1999; Tseng et al. 1997). Our *in vitro* studies show that PBMCs are effectively protected from $H_2O_2$-induced cell death when HSE is added to the experimental model. The combined results of the co-incubation and pre-incubation models indicate that the components responsible for these effects readily pass through the cell membrane and may accumulate in the cell and induce potent defensive mechanisms. The biological significance of the interplay between reactive oxygen species and certain inflammatory cytokines remains elusive. We found a significant secretion of IL-6 and IL-8 by PBMCs in response to oxidative stress that is further accentuated by the incorporation of HSE. IL-6 plays many biological roles but in this context it may be produced in order to maintain metabolic homeostasis during the altered metabolic demand experimentally induced by the addition of $H_2O_2$ (Nielsen et al. 2007; Tous et al. 2006). Additionally, IL-6 may also protect cells from $H_2O_2$-induced apoptosis by inhibiting the cell death signaling JNK pathway (Lin et al. 2001). IL-8 not only promotes the inflammatory response but also acts to suppress apoptosis mainly through the IL-8RA in an oxygen tension manner. The presence of IL-8 suppresses the apoptotic function of Fas-FasL interactions (Leuenroth et al. 1998). Conversely, some experiments suggest that MCP-1 prevents apoptosis in several models and enhances the survival of cells (Morimoto et al. 2008; Reid et al. 1999). Consequently, the observed decrease in MCP-1 in our experimental model could indicate a pro-apoptotic effect indicating that the regulation of apoptosis by cytokines depends on the balance of opposite effects elicited by concurrent molecules. It may also depend on the characteristics of the cell and the inducing factor, as exemplified by recent data (Kao et al. 2009) in which polyphenols from *Hibiscus sabdariffa* prevented the lipopolysaccharide-induced inflammation in macrophages.

In humans, we did not observe significant effects in IL-6 and IL-8 production after the ingestion of high doses of HSE, but there was a significant decrease in plasma MCP-1. Although further studies are needed, this phenomenon would likely be more dramatic if patients with inflammatory conditions were tested. It remains to be ascertained whether this effect is safe, maintained throughout long periods of treatment and due to either decreased production or increased clearance. Additional effort may be relevant because considerable research has been already focused on inhibiting the MCP-1 production to prevent inflammatory and chronic diseases through adenovirus-mediated anti-monocyte chemoattractant gene therapy and the injection of mutant MCP-1 produced by deletion of the N-terminal amino acids 2 to 8 (7ND) (Dawson et al. 2003; Kitamoto et al. 2003). Although the pharmacokinetics of the HSE remains to be established, the potent effect observed in our study suggests an immediate absorption and significant circulating concentrations of a biologically active compound. Anthocyanidin-3-glycosides are strong candidates as previously indicated by data obtained with a red wine anthocyanin extract or alcoholic drinks containing polyphenols (Blanco-Colio et al. 2007; Frank et al. 2005; Garcia-Alonso et al. 2008). But the role of other compounds or a combined effect cannot be disregarded. Furthermore, our results demonstrate that the decrease in plasma MCP-1 concentration is not due to a concomitant increase in the antioxidant capacity of plasma. Instead, the possible mechanisms may involve a direct inhibition of inflammatory and/or metabolic pathways responsible for the MCP-1 production (Hashimoto et al. 2009).

In conclusion, we have further characterized the HSE and shown that it is rich in important compounds with proven biological activities and that it effectively protects PBMCs from the cellular death induced by $H_2O_2$ modulating the production of inflammatory cytokines. In humans, HSE elicits a significant decrease in MCP-1 plasma concentration, suggesting that *Hibiscus sabdariffa* may be a valuable traditional herbal medicine for the treatment of chronic inflammatory diseases with the advantage of being devoid of caloric value or potential alcohol toxicity.

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## RESULTADOS PRELIMINARES

# ESTUDIO I

## El tratamiento con un extracto acuoso de *Hibiscus sabdariffa* en un modelo animal de hiperlipémia sometido de forma crónica a una dieta rica en grasa previene la esteatosis hepática y la hiperlipémia.

**Resultados preliminares.**

Los resultados presentados a continuación se observaron en ratones deficientes en el receptor de las lipoproteínas de baja densidad ($LDLr^{-/-}$). Este modelo presenta importantes alteraciones metabólicas que en algunos aspectos son equiparables al síndrome metabólico en humanos[184]. El *background* genético de estos animales es C57BL/6J y fueron obtenidos de Jackson Laboratories (EE.UU.).

## Materiales y métodos

### Estudios animales

El número de animales utilizados (n=32) lo determinamos basándonos en estudios previos[185]. Los animales fueron estabulados en un ciclo de 12 h de luz/oscuridad a una temperatura controlada de 25ºC y alimentados *ad libitum* con agua y dieta comercial (Harlan, Barcelona). A las 10 semanas de edad, los animales se dividieron en dos grupos. El grupo alimentado con dieta normal (chow diet, CD, n=16) continuó con la misma dieta (3% grasa y 0,03% de colesterol) y el segundo grupo (n=16) fue alimentado con una dieta rica en grasa suplementada con colesterol (high fat diet, HF, 20% aceite de palma, 0,25% de colesterol). Cada uno de estos grupos dietéticos fue dividido a su vez en dos grupos de tratamiento (n=8). El grupo control recibió agua como único aporte líquido, mientras que el otro recibió un extracto vegetal acuoso de 10 g/L de *Hibiscus sabdariffa*. Para prevenir la degradación de los extractos, estos se renovaban cada dos días. El peso y la ingesta de los animales se controlaron semanalmente y los tratamientos duraron 14 semanas. Pasado este periodo, los animales fueron sacrificados utilizando una sobredosis de ketamina y xilacina.

**Recogida de muestras y determinaciones analíticas**

Las muestras de sangre y los tejidos se procesaron siguiendo los protocolos de nuestro laboratorio publicados anteriormente[186]. En las muestras de suero se analizaron la concentración de glucosa, colesterol, triglicéridos, ácidos grasos libres y bilirrubina y las actividades enzimáticas AST y ALT, utilizando al analizador automático Synchron LXi 725 (Beckman Coulter, IZASA, Barcelona). El test de tolerancia a la glucosa se llevó a cabo siguiendo protocolos descritos anteriormente[186].

**Histología**

La esteatosis hepática se evaluó cualitativamente en una escala de 0 a 3, donde 0 representa la ausencia total de esteatosis y 3 el mayor grado de esteatosis (>66%).

**Proteómica (*Western blot*)**



Mediante inmunodetección por *Western Blot*, se analizó la expresión de diversas proteínas en el hígado y el tejido adiposo epididimal. Los tejidos fueron homogeneizados en tampón de lisis (5 ml/g; 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% CHAPS, 1 mM Pefabloc y 1% cóctel inhibidor de fosfatasas nº 2, Sigma-Aldrich Inc., Steinheim, Alemania) utilizando el homogenizador Precellys 24 (Bertin Technologies, Francia). Las muestras fueron deslipidadas utilizando metanol/dietileter (3:7). Las proteínas se cuantificaron utilizando el 2D Quant kit (GE Healthcare, Piscataway, NJ, EE.UU.) y la electroforesis se realizó utilizando geles de gradiente de poliacrilamida Bis-Tris NuPage 4-12% (Invitrogen, Barcelona). Las proteínas fueron transferidas a membranas de nitrocelulosa utilizando el sistema de transferencia iBlot (Invitrogen). Los anticuerpos de detección utilizados fueron rabbit anti-AMPK (2532, Cell Signaling Tech., Danvers, MA, EE.UU.), rabbit anti-pAMPK (2531, Cell Signaling Tech.), rabbit anti-C/EBPβ (Δ198, St. Cruz Biotech., Heidelberg, Alemania), rabbit anti-FAS (3180, Cell

Signaling Tech.) y rabbit anti-actin (H-300, St. Cruz Biotech.). El anticuerpo secundario utilizado fue goat anti-rabbit-HRP (Dako, Glostrup, Dinamarca). Para la detección quimioluminiscente se utilizó el kit ECL Advance Western Blotting Detection kit (Amersham, GE Healthcare, Barcelona), y el revelado se realizó en el sistema VersaDoc (Bio-Rad, Madrid).

**Análisis estadísticos**

Todos los análisis estadísticos se llevaron a cabo utilizando el software *Statistical Package for Social Sciences* (SPSS, Chicago, IL, EE.UU.).

## Resultados

Se realizó un control semanal del peso de los animales y de la ingesta de alimentos. Tanto en los animales que recibieron dieta rica en grasa (*High-fat diet*), como los que recibieron dieta normal (*Chow diet*), la administración del extracto vegetal redujo significativamente el aumento de peso de los ratones.



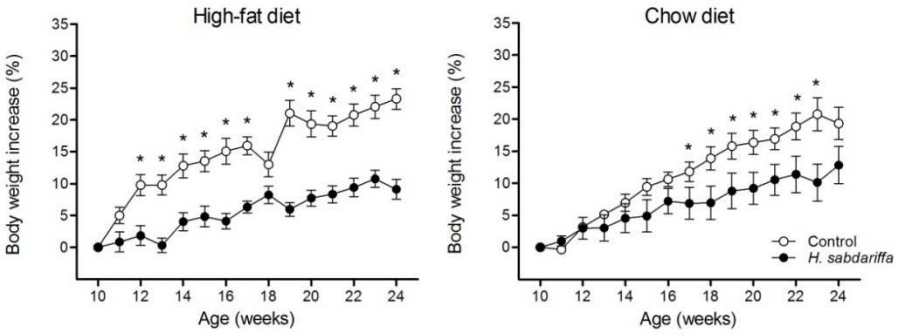



**Figura 1.** Incremento de peso de los animales alimentados con dieta rica en grasa (*High-fat diet*) y dieta normal (*Chow diet*) y tratados con *H. sabdariffa* (puntos negros) o con agua (puntos blancos).

Esta reducción del aumento de peso producida por el extracto vegetal, podía atribuirse a una disminución en la ingesta de alimentos. En cambio, el análisis de este parámetro mostró que los animales comieron prácticamente lo mismo, incluso, los animales alimentados con la dieta rica en grasa y tratados con el extracto de *H. sabdariffa* comieron más.

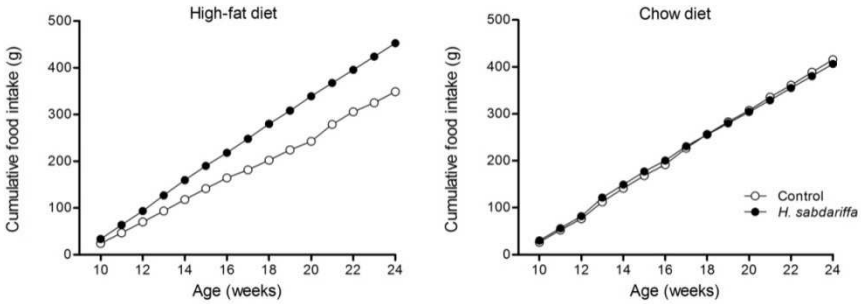



**Figura 2.** Ingesta de los animales alimentados con dieta rica en grasa (*High-fat diet*) y dieta normal (*Chow diet*) y tratados con *H. sabdariffa* (puntos negros) o con agua (puntos blancos).



Se realizó un análisis bioquímico en los sueros de los ratones con el fin de conocer algunos parámetros de interés metabólico y de función hepática de los ratones.

En ambos tratamientos dietéticos, la concentración de colesterol plasmática aumentó en los animales tratados con *H. sabdariffa*, siendo significativa en los animales alimentados con dieta normal (*Chow diet*). Por el contrario, la concentración de triglicéridos disminuyó en ambos grupos dietéticos en los animales tratados con *H. sabdariffa*, aunque este descenso solamente fue significativo en los animales alimentados con dieta rica en grasa (*High-fat diet*). La concentración de ácidos grasos libres (NEFA) disminuyó significativamente en ambos grupos dietéticos en los animales tratados con el extracto vegetal.

El análisis de los marcadores de función hepática, bilirrubina y actividad AST, solamente mostró diferencias significativas en los animales tratados con el extracto de *H. sabdariffa* y alimentados con dieta rica en grasa.

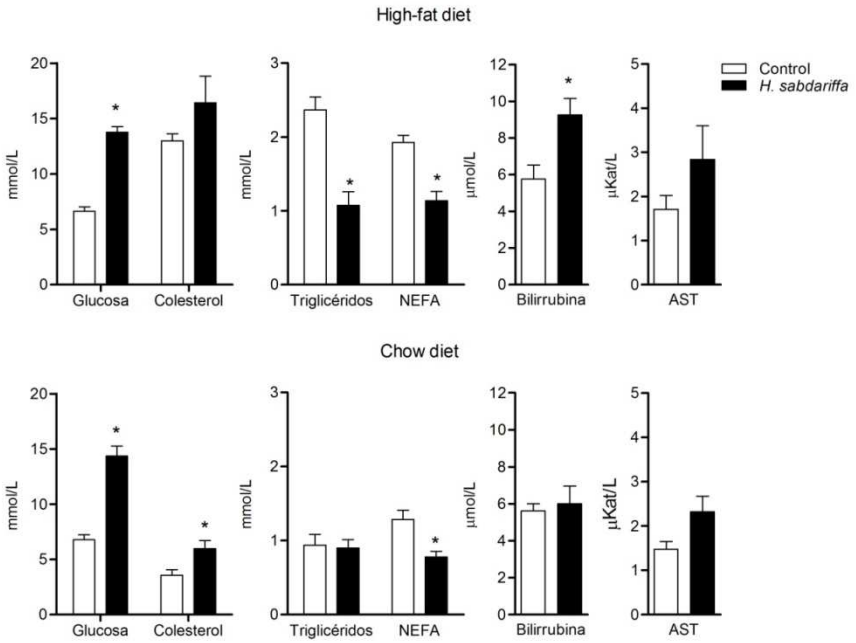



**Figura 3.** Concentraciones séricas de diferentes parámetros bioquímicos. NEFA – ácidos grasos libres, del inglés *non-esterified fatty acids*. AST – Aspartato aminotransferasa. * $p < 0.05$

La concentración sérica de glucosa en los animales tratados con *H. sabdariffa* mostró un aumento significativo en estos animales (Figura 3). Este dato no corresponde al observado en los test de tolerancia a la glucosa, donde la concentración basal de glucosa era la misma tanto en animales tratados con *H. sabdariffa* como en los no tratados. Creemos que el aumento observado en el análisis bioquímico se debe a un fallo en el diseño experimental. Antes de ser sacrificados, los animales se dejaron en ayunas durante 8 horas. En este periodo de tiempo, se les retiró la comida pero no la bebida, por lo que los animales tratados con *H. sabdariffa* continuaron ingiriendo el extracto. Este extracto

contiene una fracción de polisacáridos los cuales pueden ser los responsables del aumento de glucosa observado.

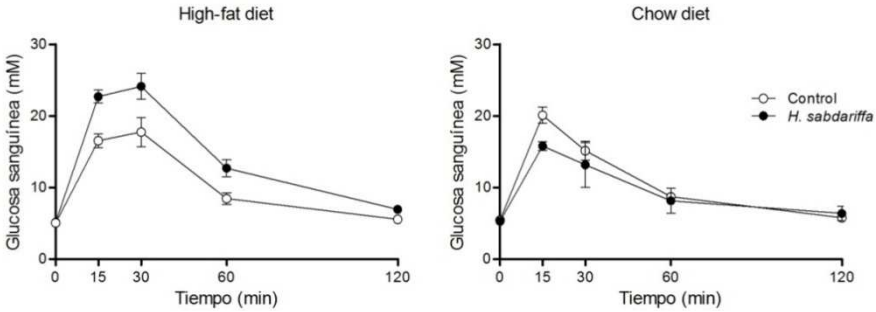



**Figura 4.** Curvas de glucosa de los animales alimentados con dieta rica en grasa (*High-fat diet*) y dieta normal (*Chow diet*) y tratados con *H. sabdariffa* (puntos negros) o con agua (puntos blancos).

El análisis de la esteatosis hepática reveló que el tratamiento con el extracto de *H. sabdariffa* evitaba la acumulación intrahepática de lípidos. En ambos grupos dietéticos, la incidencia de esteatosis hepática fue menor, pero los resultados más significativos, de los cuales se presentan imágenes histológicas en la figura siguiente, se obtuvieron en los animales alimentados con la dieta rica en grasa y tratados con el extracto de *H. sabdariffa*.



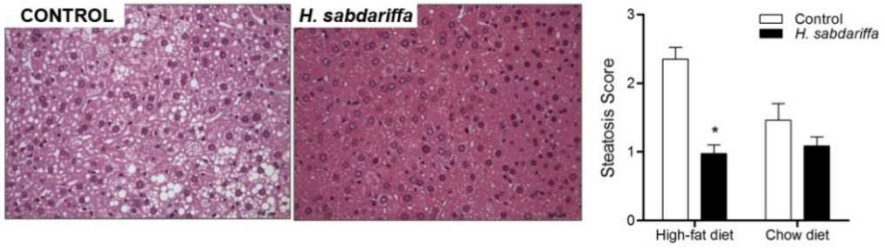



**Figura 5.** Fotografías de cortes histológicos hepáticos de animales alimentados con la dieta rica en grasa y tratados con agua (Control) o con *H. sabdariffa*. El gráfico muestra la valoración cualitativa de la esteatosis en los grupos estudiados. * $p < 0.05$.

Mediante *western blot* se analizó la expresión hepática de uno de los enzimas claves en el control de metabolismo energético, la AMPK. La forma activa de este enzima se presenta de forma fosforilada, por lo que se analizó la AMPK total, y la AMPK fosforilada. En la figura inferior, se observa como en ambos grupos dietéticos, la cantidad hepática de AMPK fosforilada (pAMPK) es ligeramente mayor en aquellos animales que fueron tratados con *H. sabdariffa*.

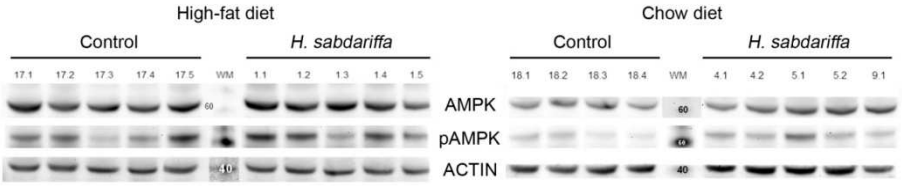



**Figura 6.** Análisis de la expresión en homogenados de tejido hepático mediante *western blot* de AMPK y AMPK fosforilada (pAMPK). Como control se utilizó la Actina para comprobar que en todos los casos se había utilizado la misma cantidad de muestra.

# ESTUDIO II

## Metabolomic study of liver composition of animals treated with *Hibiscus sabdariffa* and *Aspalathus linearis*.

### Study description and results

## I. Purpose of Experiment

The goal of this study was to characterize alterations in liver metabolism associated with preventive intervention of liver steatosis by treatment with *Hibiscus sabdariffa* (Hb) and *Aspalathus linearis* (Roo) plant extracts.

## II. Experimental design

Liver samples utilized for this study were collected from C57BL/6J background mice deficient in low density lipoproteins receptor (LDLr KO).

Dietary and treatment group identification:

HF - high fat diet (20% palm oil + 0.25% cholesterol)

CD - normal chow diet

Hb - treatment with Hb (*Hibiscus sabdariffa* extract)

Roo - treatment with Roo (*Aspalathus linearis* extract)



Global biochemical profiles were compared across the following sample groups.

| **Treatment Groups** | | | |
|---|---|---|---|
| Group | Animals | Group ID | Description |
| 1 | 6 | CD+C | LDLr KO + Water + Chow Diet |
| 2 | 6 | CD+Hb | LDLr KO + Hb + Chow Diet |
| 3 | 6 | HF+C | LDLr KO + Water + HF diet+0.25% Cholesterol |
| 4 | 6 | HF+Hb | LDLr KO + Hb + HF diet+0.25% Cholesterol |
| 5 | 6 | HF+Roo | LDLr KO + Roo + HF diet+0.25% Cholesterol |

## III. Summary of Procedure

At the time of analysis samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on the GC/MS and LC/MS/MS platforms. Also included were several technical replicate samples created from a homogeneous pool containing a small amount of all study samples.

## IV. Data Quality: Instrument and Process Variability

| QC Sample | Measurement | Median RSD |
|---|---|---|
| Internal Standards | Instrument Variability | 4 % |
| Endogenous Biochemicals | Total Process Variability | 12 % |



Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled client samples. Values for instrument and process variability meet Metabolon's acceptance criteria as shown in the table above.

## V. Metabolite Summary and Significantly Altered Biochemicals

The mView product specification includes all detectable compounds of known identity (named biochemicals). The present dataset comprises a total **499 biochemicals**, **272 named** and **227 unnamed** compounds (with the Hb study sample set exhibiting 495 total compounds, 272 named and 223 unnamed). Following log transformation and imputation with minimum observed values for each compound, contrasts resulting from the two-way ANOVA were used to identify biochemicals that differed significantly between study diet/treatment/gene knockout groups. A summary of the numbers of biochemicals that achieved statistical significance ($p \leq 0.05$), as well as those approaching significance ($0.05 < p < 0.1$), is shown below. When analyzing 493-499 compounds, we would expect to see 25 compounds meeting the $p \leq 0.05$ cut-off by random chance. For the primary Hb sample set, the two-way ANOVA identified biochemicals exhibiting a significant diet main effect, treatment main effect or diet:treatment interaction.

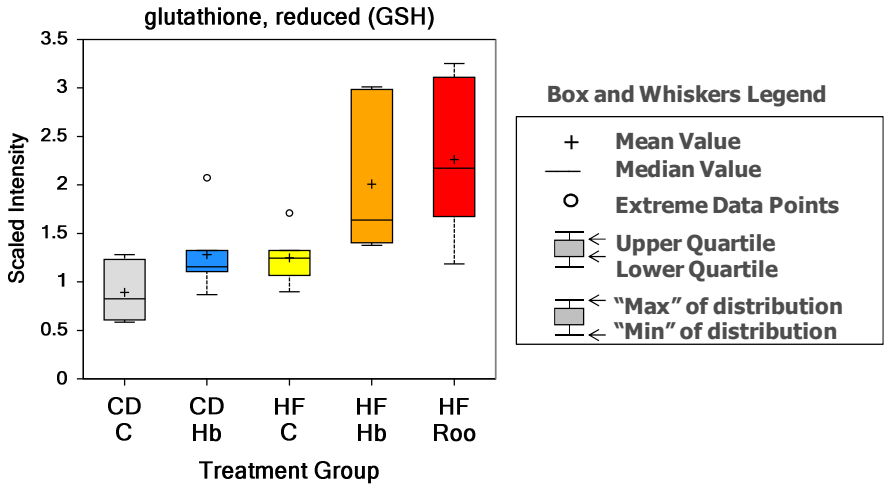


An estimate of the false discovery rate (*q*-value) is calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies; as *q*-values were reasonable for $p \leq 0.05$, no *q*-value cutoff was established for this study.

| Statistical Comparisons - Significantly Altered Biochemicals<br>Hb Treatment Study | | | | | | |
|---|---|---|---|---|---|---|
| ANOVA Contrasts | CD+Hb / CD+C | HF+C / CD+C | HF+Hb / HF+C | HF+Hb / CD+C | HF+Roo / HF+C | HF+Roo / HF+Hb |
| Total biochemicals $p \leq 0.05$ | 103 | 73 | 226 | 227 | 128 | 228 |
| Biochemicals (↑\|↓) | 50\|53 | 38\|35 | 43\|183 | 51\|176 | 98\|30 | 215\|13 |
| Total biochemicals $0.05 < p < 0.10$ | 39 | 32 | 32 | 41 | 36 | 35 |

| Biochemicals (↑↓) | 18\|21 | 17\|15 | 5\|27 | 14\|27 | 28\|8 | 31\|4 |
|---|---|---|---|---|---|---|
| Two-Way ANOVA | Diet Main Effect | | Treatment Main Effect | | Diet:Treatment Interaction | |
| Total biochemicals $p \leq 0.05$ | 151 | | 214 | | 105 | |
| Total biochemicals $0.05 < p < 0.10$ | 32 | | 40 | | 47 | |

We have also included in the electronic deliverables, a file with data for each biochemical displayed as box plots like that shown in the example figure below.






## VI. Biochemical Summary

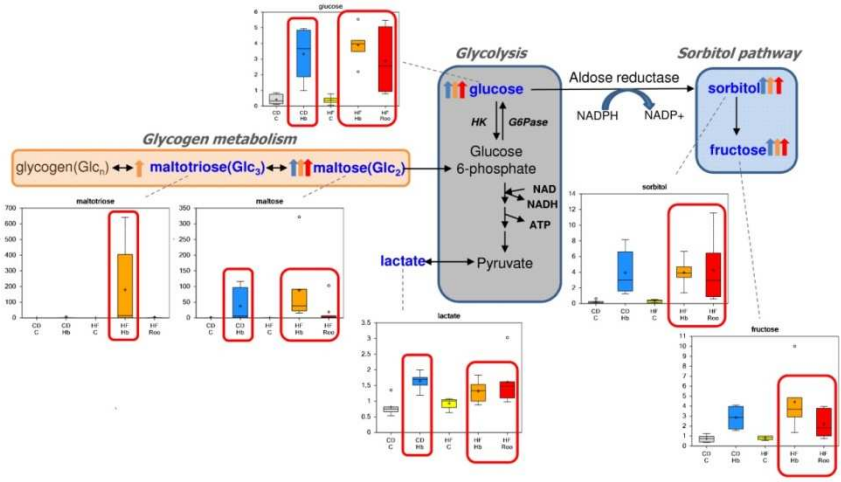
This study was conducted in order to identify differences in the metabolic profiles of LDLr knockout mice under normal or high fat diets. Numerous studies have been conducted with LDLr knockout mice which have reported diet-induced weight gain and glucose intolerance in response to a high fat diet as well as modulating aspects of energy metabolism. In addition to the LDLr knockout and dietary differences, we were also interested in metabolic changes induced by

supplementation with either hibiscus (Hb) or rooibos (Roo) plant extracts in conjunction with liver steatosis. Previous studies with either Hb or Roo have suggested that phenolic compounds present in these extracts may impact oxidative stress, inflammation, hyperglycemia and even altered liver function. For this study, liver samples from LDLr knockout mice maintained under normal or high fat diets were further treated with control ($H_2O$), Hb, or Roo extracts with groups consisting of 6 biological replicates each. Equivalent amounts of liver tissue were extracted for each sample and, therefore, no additional normalization of the biochemical data was required.

Datasets provided in the mView product can be quite large and contain a great deal of information. To provide an initial focus for further consideration, a few observations are offered below from a cursory view of the data.

A number of carbohydrate-related metabolites were found to be significantly and consistently higher with plant extracts (either Hb or Roo) regardless of diet (high fat (HF) or control (CD)). In particular, **maltose** was nearly 300-fold higher in CD+Hb than in the CD+$H_2O$, 600-fold higher in HF+Hb than in HF+$H_2O$ or CD+$H_2O$, and 130-fold greater in the HF+Roo diet compared to the HF control samples. Previous studies have observed that plant extract formulations may often be reconstituted in maltose which would be consistent with these findings. In this case, the plant extracts in this experiment were derived solely from freshly ground plant material, indicating that the Hb and Roo extracts may be both extremely high in maltose which may be impacting the liver. In addition to this, the higher level of maltose may have caused the liver to compensate possibly first through gluconeogenesis (generating excess **glucose**) and then secondly through the sorbitol pathway resulting in the significantly higher levels of **sorbitol** and **fructose**. **Lactate** levels were also higher in all extract-treated groups and may indicate an elevated level of glycolysis. These are important observations consistent between the extract-treated samples regardless of diet that may

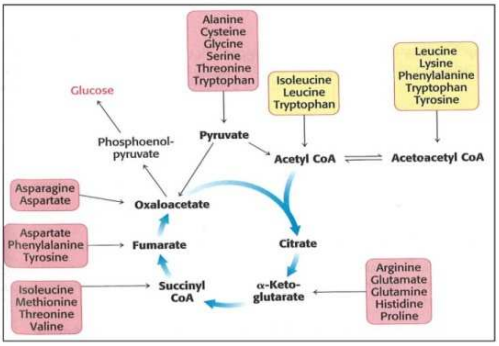
suggest that the formulation process is contributing to differences observed in the liver.



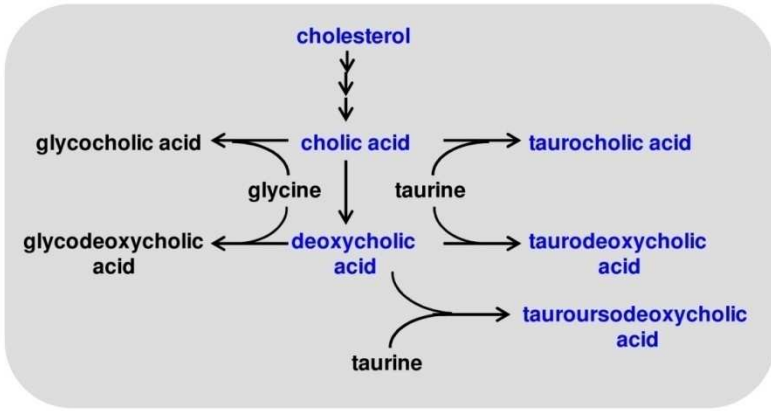


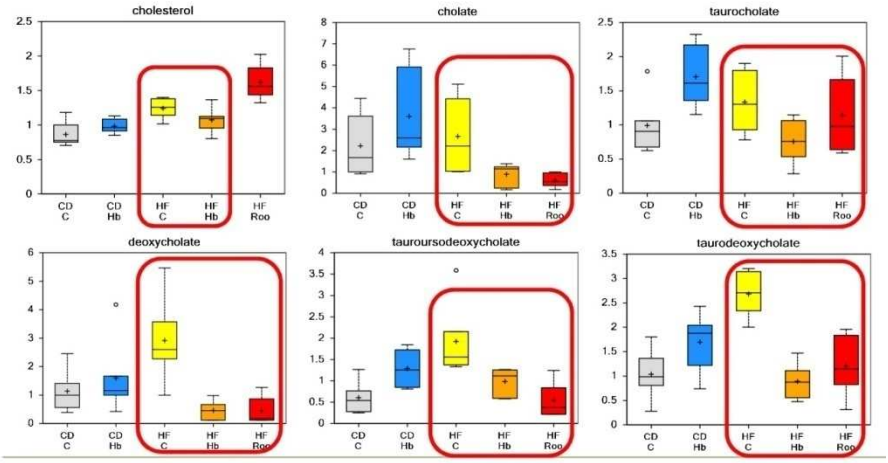

While the HF diet or plant extracts alone did not significantly affect the levels of amino acids in comparison to the control diet, the combination of HF+Hb did result in significant lower levels of nearly all **amino acids**. This decrease may be an indication of reduced uptake from the diet or potentially represent increased amino acid metabolism for energy purposes in response to hibiscus extract. However, this seems unlikely given the abundance of glucose observed under these conditions. In contrast to this, the HF+Roo diet resulted in slightly higher levels of free amino acids in contrast to the HF+C diet. These results are intriguing and if they are indicative of increased amino acid utilization, these results may be consistent with reports in the literature which have suggested that diets fortified with essential and in particular ketogenic amino acids may prevent hepatic steatosis in mouse models of dietary induced obesity.

| BIOCHEMICAL NAME | CD Hb CD C | HF C CD C | HF Hb HF C | HF Hb CD C | HF Roo HF C | HF Roo HF Hb |
|---|---|---|---|---|---|---|
| glycine | 0.91 | 0.95 | 0.58 | 0.56 | 1.28 | 2.19 |
| serine | 0.87 | 0.90 | 0.62 | 0.56 | 1.24 | 1.98 |
| threonine | 0.93 | 0.97 | 0.75 | 0.72 | 1.03 | 1.38 |
| alanine | 1.17 | 0.94 | 0.82 | **0.77** | **1.30** | 1.58 |
| aspartate | 0.67 | 0.83 | 0.55 | 0.46 | 1.24 | 2.23 |
| asparagine | 1.11 | 0.97 | 0.68 | 0.67 | 1.17 | 1.71 |
| glutamate | 0.98 | 1.11 | 0.75 | 0.83 | 0.99 | 1.32 |
| glutamine | 0.96 | **0.87** | 1.28 | 1.11 | 1.32 | 1.03 |
| histidine | 0.76 | 0.98 | 0.47 | 0.46 | 0.97 | 2.06 |
| lysine | **0.86** | 0.90 | 0.68 | 0.61 | 1.04 | 1.53 |
| phenylalanine | 0.91 | 0.97 | 0.75 | 0.73 | 1.13 | 1.52 |
| tyrosine | 0.85 | 0.96 | 0.71 | 0.68 | 0.97 | 1.36 |
| tryptophan | 0.86 | 0.94 | 0.73 | 0.68 | **1.25** | 1.72 |
| isoleucine | **0.85** | 0.94 | 0.74 | 0.70 | 1.07 | 1.44 |
| leucine | 0.89 | 0.98 | 0.72 | 0.71 | 1.06 | 1.48 |
| valine | 0.85 | 0.92 | 0.70 | 0.65 | 1.11 | 1.58 |
| cysteine | 1.46 | 0.97 | 1.03 | 1.00 | 1.69 | 1.64 |
| methionine | **0.79** | 0.92 | 0.65 | 0.59 | 1.16 | 1.79 |
| arginine | 1.68 | 1.31 | 1.13 | 1.48 | 1.15 | 1.02 |
| proline | 0.92 | 0.87 | 0.70 | 0.61 | 1.12 | 1.59 |




The HF diet resulted in greater levels of bile acids in the liver (many that were statistically significant compared to control), which may be an indication of decreased liver function (consistent with steatosis). It is interesting to note that there was a significant reduction in the levels of bile acids (**cholate, taurocholate, deoxycholate, taurodeoxycholate, 6-beta-hydrolithocholate, beta-muricholate** and **taurourso-deoxycholate)** in livers maintained on HF diet when Hb extract was also co-administered. The HF+Roo samples also showed a similar result (lower bile acids) in relation to the HF control samples. The significant decreases in bile acids associated with Hb or Roo extract may be suggestive of improved liver function. This would be consistent with previously reported results from this client, who found that dietary-induced liver steatosis was prevented by the Roo extract.

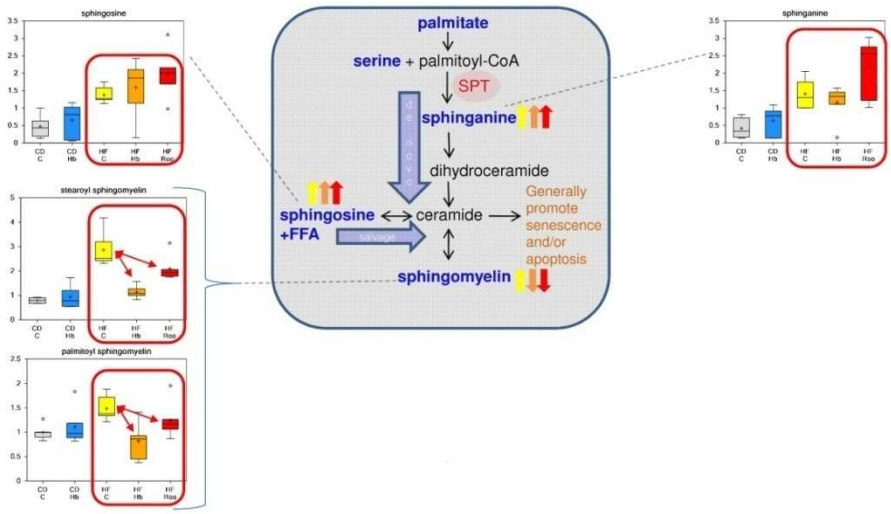




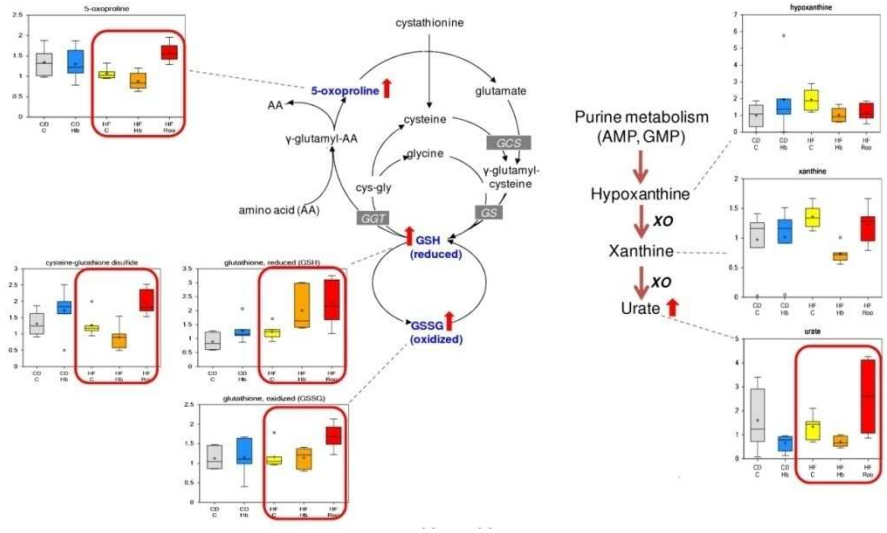

With respect to lipid levels in the liver, several **long-chain fatty acids** and **glycerol** were found to be significantly lower in the HF+Hb liver samples in contrast to the HF control diet. While the liver is the primary tissue for *de novo* synthesis of fatty acids, the changes observed here may be indicative of reduced lipolysis. However, this is difficult to confirm since monoacyl- or diacyl-glycerols were not observed. By comparison, sphingolipids including **sphinganine** (a ceramide precursor) and **sphingosine** and **sphingomyelin** (ceramide catabolites) were higher in mice reared on the HF diet compared to CD. Ceramide itself was not detectable on our platform because of its physical characteristics. The

inclusion of plant extracts in the diet generated interesting changes in the levels of liver sphingolipids. Though not statistically significant, the levels of sphinganine and sphingosine in HF+plant extract-treated animals trended upward compared to HF diet alone. In contrast, **stearoyl sphingomyelin** and **palmitoyl sphingomyelin** were lower in livers from HF+plant extract-treated animals, returning to levels that mimicked the CD. These results may suggest that either plant extract in combination with a high fat diet may cause preferential catabolism of ceramide to sphingosine instead of sphingomyelin. Since elevated levels of ceramides have been recognized as a factor contributing to impaired insulin signaling in non-adipose tissue – events that are strongly associated with liver steatosis – altered ceramide catabolism in association with these plant extracts may also impact liver steatosis, perhaps in a favorable manner.

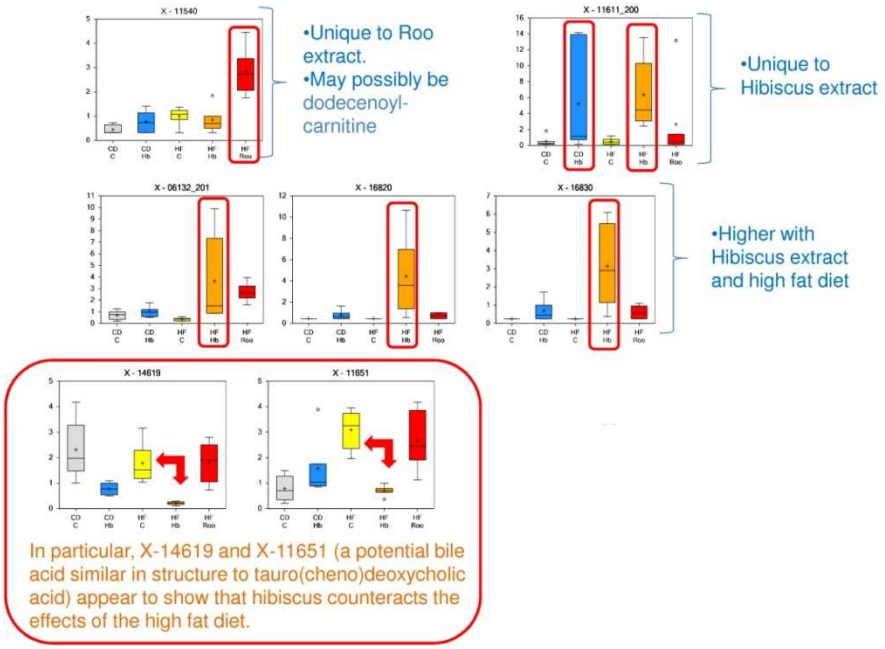



One of the most consistent differences associated with HF+Roo samples were the significant differences in glutathione related metabolites. Both **oxidized and reduced glutathione** levels were found to be significantly higher in the HF+Roo samples in relation to the HF+$H_2O$ controls. **5-oxoproline,** a degradation product of glutathione, was also higher in the HF+Roo samples in relation to the HF controls suggesting increased glutathione turnover. In addition, **cysteine-**

**glutathione disulfide**, which is formed during oxidative reactions with glutathione, was higher in the HF+Roo samples when compared to HF controls. Elevated levels of cysteine-glutathione disulfide are often an indicator of an oxidative environment or of increased oxidative stress. Moreover, **urate** may be generated during purine metabolism through the activity of xanthine oxidase – an enzyme whose activity is increased under conditions of oxidative stress. Urate levels were nearly 4-fold higher in the HF+Roo samples in relation to the HF controls, providing further support for conditions of an increased oxidative environment or oxidative stress with Roo treatment. Finally, additional antioxidants including **alpha-tocopherol** and **ascorbate** were generally greater in the HF+Roo samples compared to control and may suggest a buildup of antioxidants to compensate for elevated oxidative stress.



Data derived from structurally unnamed biochemicals were also provided with this dataset, resulting in the identification of an additional 227 compounds. Many of these unnamed metabolites may serve as potential biomarkers of Hb- or Roo-treatment in relation to a high fat diet. Of particular interest are **X-14619** and **X-11651** (a potential bile acid with structural similarities to tauro(cheno)deoxycholic acid), whose levels were significantly reduced upon addition of Hb to the high fat diet. X-11651 behaves similar to that of the bile acids discussed previously but X-14619 may show an amelioration of the effects of the high fat diet with the Hb extract and may be worth pursuing further. Additional unnamed compounds of interest included **X-11540** (possibly dodecenoyl-carnitine) which was uniquely higher within the HF+Roo samples as well as **X-11611_200** which was unique to both HF and CD with hibiscus. **X-06132_201, X-16820,** and **X-16830** showed signs of being significantly higher within only the HF+Hb samples.



In conclusion, there were a number of significant differences identified in the LDLr knockout mouse liver with respect to dietary changes as well as following treatment with plant extracts, which may have favorable impacts on liver steatosis. Unique findings with respect to the plant extracts showed that the Hb extract in particular may have had a positive impact on liver steatosis, possibly increasing metabolism of amino acids and decreased levels of bile acids. By comparison, treatment with the Roo extract often generated overlapping effects to that seen after treatment with Hb. Additionally, the biochemical data provided in this study may suggest that Roo treatment generates a more oxidative environment in the liver or perhaps lead to conditions of increased oxidative stress.

# DISCUSIÓN Y CONCLUSIONES

# 1. DISCUSIÓN

La obesidad es una de las enfermedades más frecuentes en todo el mundo. Tanto es así que la Organización Mundial de la Salud la califica de pandemia[63], siendo particularmente alarmante el incremento en la edad infantil. Esta enfermedad multifactorial se asocia con el desarrollo de otras patologías como la diabetes, la hipertensión y alteraciones inflamatorias. La tasa de obesidad, del orden del 15% en Europa y superior al 30% en Norteamérica, justifica los esfuerzos que la comunidad científica destina a investigar las causas de esta enfermedad, entre las que se encuentran componentes genéticos, endocrinos y factores ambientales como hábitos dietéticos inadecuados y modelos sedentarios de actividad física[60].

Desgraciadamente, la búsqueda de factores de riesgo convencionales para las enfermedades crónicas es una estrategia limitada que no proporciona una solución eficaz para el tratamiento de la enfermedad. Una estrategia alternativa podría centrarse en la población general a través de simples modificaciones en la dieta, dado que la evidencia científica apoya cada vez más la opinión de que estas intervenciones dietéticas tienen importantes efectos sobre el estado de salud a largo plazo. Sin embargo, la financiación de ensayos clínicos para demostrar la eficacia de los polifenoles en la dieta se complica por el hecho de conseguir extractos naturales siempre con la misma composición. La industria alimentaria, con el fin de evitar esta limitación, centra sus investigaciones en los efectos de los elementos individuales.



Nuestro enfoque, sin embargo, examina la actividad biológica del extracto completo. Algunos de los compuestos presentes en los extractos estudiados poseen numerosas actividades biológicas, como las antocianinas, el ácido hidroxicítrico, el ácido hibiscus y el ácido clorogénico, los cuales se comercializan como suplementos dietéticos destinados a reducir el sobrepeso y otras afecciones[187] . Una de las desventajas de nuestra estrategia es que la

interpretación de los resultados puede convertirse en una tarea complicada debido a las múltiples dianas metabólicas de los compuestos polifenólicos[46-48]. Además, este planteamiento condena la incorporación del extracto como suplemento dietético antes que como fármaco. Las plantas que son ampliamente utilizadas en la medicina popular son candidatas inmediatas a la investigación, como ocurren en el caso de *Hibiscus sabdariffa* y *Aspalathus linearis*. La primera se utiliza tradicionalmente para tratar la hipertensión y los trastornos hepáticos[146,188], mientras que a la segunda se le atribuyen efectos beneficiosos en el tratamiento de alergias, asma y problemas dermatológicos[158].

Las técnicas analíticas desarrolladas proporcionan una herramienta eficaz en la caracterización de los compuestos polifenólicos presentes en extractos acuosos vegetales. El objetivo era desarrollar un método cualitativo muy simple para separar y caracterizar simultáneamente los compuestos presentes en ambos extractos. En el caso de *H. sabdariffa* se describieron dos compuestos por primera vez, el *5-O-caffeoylshikimic acid* y la *N_feruloyltyramina* (Estudio 1; pág.47). Además sabemos que los compuestos mayoritarios presentes en este extracto son el ácido hibiscus, el ácido hidroxicítrico, el ácido clorogénico y las dos antocianinas (Material suplementario; pág. 147). También en el caso del extracto de *A. linearis* se describieron nuevos compuestos. Concretamente los once siguientes: *patuletin 7-glucoside*, *esculin*, *safflomin A*, *carlinoside*, *isocarlinoside*, *neocarlinoside*, *2"-O-β-arabinopyranosylorientin*, *vicenin-2*, *eriodictyol-5,3' di-O-glucoside*, *quercetin-3-O-arabinoglucoside* y *scoparin* (Estudio 2; pág. 57).



En los estudios llevados a cabo en ratones, los extractos vegetales de *H. sabdariffa* y *A. linearis* muestran una alta actividad biológica, aunque su comportamiento difiere en algunos aspectos. En ambos casos, los efectos producidos por los extractos parecen ser mayores cuanto mayor es la agresión externa, que en estos estudios, es el consumo de una dieta hipercalórica. Ambos extractos previenen el incremento de peso en los animales alimentados con la dieta normal, pero solamente *H. sabdariffa* consigue hacerlo en los animales

alimentados con una dieta rica en grasa. Los responsables de este efecto pueden ser el ácido hidroxicítrico[189,190] y su isómero, el ácido hibiscus, ya que su concentración es muy alta en los extractos de *H. sabdariffa* (ver Material suplementario; pág. 147).

Ambos extractos muestran una gran actividad hipolipemiante, tanto a nivel sérico como hepático (Estudio 3; pág. 71 y Estudio I; pág. 95). La diferencia más importante la observamos en los niveles plasmáticos de colesterol. Mientras que *A. linearis* disminuye la concentración plasmática de colesterol, los animales tratados con *H. sabdariffa* muestran una concentración mayor respecto al control. Esto podría ser debido a un aumento de la excreción de colesterol por parte del hígado. Los resultados obtenidos en el análisis metabolómico de los hígados de los animales alimentados con una dieta rica en grasa, muestran un contenido de colesterol intrahepático menor en los animales tratados con *H. sabdariffa,* mientras que en los tratados con *A. linearis* la acumulación de colesterol es mayor, comparados con el control (Estudio II; pág. 105). Además, estos últimos muestran una concentración plasmática de VLDL y LDL menor. En ambos casos, los resultados encajan con la concentración plasmática de colesterol observada.



Por otro lado, la administración de dieta rica en grasa en los animales control produce un incremento en la concentración hepática de ácidos biliares, lo que puede indicar una alteración en la función hepática[191]. Tanto el tratamiento con *H. sabdariffa* como con *A. linearis* reduce significativamente la concentración de ácidos biliares en el hígado, sugiriendo una mejora en la función hepática (Estudio II; pág. 105). Estos resultados se correlacionan además con la esteatosis observada

Los efectos observados son independientes de cambios en la resistencia a la insulina, como muestran los test de tolerancia a la glucosa realizados en los animales de experimentación. Sin embargo, el tratamiento con *H. sabdariffa* produce un incremento significativo en la glucosa plasmática. En el hígado, el

contenido de glucosa es mucho mayor en los animales tratados con los extractos vegetales. Particularmente, la concentración de maltosa es significativamente mayor. Estos resultados sugieren que ambas plantas son ricas en este azúcar (o en polisacáridos cuya digestión produzca este disacárido), hecho que podría afectar particularmente al hígado. La alta concentración de maltosa puede activar mecanismos compensatorios; por un lado, vía gluconeogénesis generando un exceso de glucosa, y por otro lado, produciendo sorbitol y fructosa por la vía del sorbitol (Estudio II; pág. 105). Este alto contenido en glucosa parece activar parcialmente la glicolisis, como revela la alta concentración de lactato observada en los animales tratados con los extractos vegetales (Estudio II; pág. 105). Una posible estrategia, en la que ya hemos empezado a trabajar para evitar estas alteraciones en las concentraciones hepáticas y plasmáticas de glucosa, es eliminar los polisacáridos de los extractos vegetales, obteniendo así un extracto mucho más concentrado.

Las alteraciones observadas en el metabolismo de la glucosa y de los lípidos, apuntan a un enzima clave en la regulación del metabolismo energético, la AMPK. Cuando las reservas energéticas disminuyen, AMPK activa vías catabólicas como la β-oxidación de los ácidos grasos[192,193]. Además, inhibe rutas biosintéticas como la síntesis de ácidos grasos o la gluconeogénesis[192,193]. Los dos extractos estudiados activan la AMPK en el hígado. Este hecho podría bloquear la síntesis de ácidos grasos, promoviendo su oxidación, lo que explicaría la reducción de los depósitos intrahepáticos de lípidos. Lo que no explica es el aumento de glucosa intrahepática, aunque podría deberse a que los hepatocitos transforman la maltosa en glucosa para dar salida a las grandes cantidades ingeridas de este disacárido. Por otro lado, como se especifica en el Estudio I del apartado de Resutados Preliminares, los animales se dejaron en ayunas antes del sacrificio, pero no se les retiró el extracto vegetal. Este error en el diseño del experimento explicaría la alta concentración plasmática y hepática de glucosa, y la ausencia en estos animales de un estado de resistencia a la insulina.

Aunque los niveles altos de glucosa en sangre son un factor que no se debe menospreciar, los datos obtenidos muestran que la administración continuada de extractos ricos en polifenoles puede ayudar en el control de las enfermedades metabólicas.

En humanos, nuestros estudios *in vitro* muestran en células mononucleares de sangre periférica (PBMCs) una protección efectiva ante la muerte celular inducida por $H_2O_2$. Los resultados indican que los componentes responsables de estos efectos pasan fácilmente a través de la membrana de la célula y pueden acumularse en ellas e inducir potentes mecanismos de defensa. La importancia biológica de la interacción entre las especies reactivas de oxígeno y ciertas citoquinas inflamatorias sigue siendo difícil de explicar. Hemos encontrado una secreción significativa de IL-6 e IL-8 por parte de los PBMCs en respuesta al estrés oxidativo que se acentúa aún más por la incorporación del extracto, lo que sugiere un efecto xenohormético. IL-6 desempeña muchas funciones biológicas, pero en este contexto, su producción puede deberse a la demanda metabólica inducida experimentalmente por la adición de $H_2O_2$[194,195]. Además, la IL-6 también puede proteger las células contra la apoptosis inducida por $H_2O_2$ al inhibir la muerte celular por la vía de señalización de JNK[196]. IL-8 no sólo promueve la respuesta inflamatoria, sino que también actúa para suprimir la apoptosis, principalmente a través de la IL-8RA en situación de estrés oxidativo. La presencia de IL-8 suprime la función de la apoptosis en las interacciones Fas-FasL[197]. Por el contrario, algunos experimentos sugieren que la MCP-1 previene la apoptosis en varios modelos y aumenta la supervivencia de las células[198,199]. En nuestros resultados, la disminución observada de MCP-1 en nuestro modelo experimental podría indicar un efecto proapoptótico. En la regulación de la apoptosis pueden concurrir diferentes vías y moléculas de señalización, y en nuestro caso, el resultado de esta regulación depende del equilibrio de los efectos opuestos provocados por citoquinas concurrentes. También puede depender de las

características de la célula y el factor de inducción, como se ejemplifica en datos recientes[144] en los que los polifenoles de *H. sabdariffa* impiden la inflamación inducida por lipopolisacáridos en macrófagos.

Los efectos observados en voluntarios sanos fueron diferentes después de la ingestión de dosis altas de *H. sabdariffa*. La concentración plasmática de MCP-1 disminuye significativamente y no se observa ningún efecto en la concentración de IL-6 e IL-8. Estos estudios deben ampliarse en pacientes con un elevado grado de inflamación, como pacientes diagnosticados de síndrome metabólico u obesidad mórbida, donde los efectos podrían ser más importantes. Queda por determinar si el tratamiento es seguro y si sus efectos se mantienen cuando es administrado de forma crónica. Así pues, puede ser relevante la investigación enfocada a la inhibición de la producción de MCP-1 para prevenir enfermedades inflamatorias crónicas mediante el uso de estos extractos. Aunque no poseemos datos propios en humanos de la farmacocinética de estos extractos, el potente efecto observado sugiere una absorción inmediata y significativa de compuestos biológicamente activos. Existen datos previos acerca de la absorción de las antocianidinas del *H. sabdariffa*[200]. Además, los glucósidos de estas antocianidinas son fuertes candidatos según lo indicado previamente por datos obtenidos con antocianinas del vino tinto purificadas[201,202]. Aun así, el papel de otros compuestos o un efecto combinado no puede ser ignorado. Los flavonoides poseen una gran actividad antioxidante y tradicionalmente se ha atribuido los efectos beneficiosos que producen a esta capacidad antioxidante[49,50]. Sin embargo, nuestros resultados demuestran que la disminución en el plasma de la concentración de MCP-1 no se debe a un aumento concomitante en la capacidad antioxidante del plasma. En cambio, los posibles mecanismos pueden involucrar una inhibición directa de las vías inflamatorias y metabólicas responsables de la producción de MCP-1[203].

En resumen, los resultados obtenidos muestran que los extractos vegetales ricos en polifenoles pueden producir beneficios para la salud humana. Además cuentan con la ventaja de carecer de valor calórico y de la toxicidad potencial del alcohol. En los animales de experimentación, el metabolismo de los lípidos y la glucosa se ven claramente alterados a consecuencia del consumo crónico de estos extractos, reduciendo los lípidos plasmáticos, bloqueando el desarrollo de esteatosis hepática, y todo ello independientemente a cambios en la resistencia a la insulina. En los seres humanos, *H. sabdariffa* provoca una disminución significativa en la concentración plasmática de MCP-1, lo que sugiere que esta planta puede ser un valioso complemento de la dieta.

# 2. CONCLUSIONES

- La metodología desarrollada para HPLC acoplada a ESI-TOF-MS y ESI-IT-$MS^2$ es una técnica analítica potente para la separación y caracterización de los compuestos polifenólicos contenidos en extractos vegetales acuosos.

- Los efectos biológicos observados en modelos animales de experimentación parecen ser más importantes cuanto mayor es la agresión externa (en este caso, el consumo de una dieta hipercalórica).

- Los extractos vegetales, administrados de forma crónica, previenen trastornos en el metabolismo lipídico como la hiperlipémia y la esteatosis hepática en modelos animales de experimentación alimentados con una dieta rica en grasa.

- Los efectos biológicos observados en estos animales son independientes de cambios en la resistencia a la insulina, y sugieren una activación de la AMPK como uno de los posibles mecanismos responsables de dichos efectos.



- En humanos, la administración aguda de una dosis alta de polifenoles disminuye la concentración plasmática de MCP-1 en unas horas, sugiriendo una absorción inmediata y significativa de compuestos bioactivos.

- La gran actividad antiinflamatória de estos compuestos observada en humanos es independientemente de un aumento en la capacidad antioxidante del plasma y propone la utilización de extractos acuosos vegetales en el tratamiento de enfermedades inflamatorias.

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# Quantification of the polyphenolic fraction and *in vitro* antioxidant and *in vivo* anti-hyperlipemic activities of *Hibiscus sabdariffa* aqueous extract

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ABSTRACT

In the present study the quantification of the polyphenolic fraction, anthocyanins and other polar compounds, the antioxidant capacity and the anti-hyperlipemic action of the aqueous extract of *Hibiscus sabdariffa* has been achieved. Seventeen compounds were successfully quantified either by HPLC-DAD or HPLC-ESI-TOF-MS. Six of them were directly quantified by their corresponding standards, whereas the rest were indirectly quantified as equivalents using standards of similar compounds. The antioxidant capacity have also been estimated by comparing different assays, i,e, Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), and measurement of thiobarbituric acid reacting substances (TBARS). *H. sabdariffa* showed high reducing capacity in FRAP assay and significant capability to scavenge peroxyl radicals in the ORAC assay. Nevertheless, the extract exhibited poor efficacy to inhibit peroxyl radicals in lipid systems. The plant extract also exhibited the capacity to decrease serum triglyceride concentration on hyperlipemic mouse model.



## 1. Introduction

The *Hibiscus sabdariffa* L. (family: *Malvaceae*), usually named bissap, karkade or roselle is a tropical plant commonly used as local soft drink. It is highly appreciated all over the world for the particular sensation of freshness conveyed. Traditionally, it has been used effectively against hypertension, inflammation, and liver disorders (Wang et al., 2000). Previous studies showed that *H. sabdariffa* possesses anti-tumoral, anti-oxidant and anti-hyperlipemic activities (Chen et al., 2003; Hou, Tong, Terahara, Luo, & Fujii, 2005; Kao et al., 2009; Lin, Huang et al., 2007; Lin, Lin et al., 2007; Tseng et al., 1997, 2000). Recently, it was reported that the extract of *H. sabdariffa* inhibited the LDL oxidation and lowered serum triglycerides, cholesterol and LDL-cholesterol in animal models (Chen et al., 2003; Lin, Huang et al., 2007; Lin, Lin et al., 2007). Histological examination revealed that it could reduce foam cell formation and inhibit VSMC proliferation and migration, suggesting the anti-atherosclerotic effect of *H. sabdariffa*. In addition, studies on humans show the anti-hypertensive and anti-inflammatory effects of *H. sabdariffa* consumption (Beltrán-Debón et al., 2010; Herrera-Arellano, Flores-Romero, Chavez-Soto, & Tortoriello, 2004). The brilliant red color and unique flavor make it a valuable food product. The anthocyanin pigments that create the color (Tsai & Ou, 1996) are responsible for the wide range of coloring in many foods. The *H. sabdariffa* petals are potentially a good source of antioxidant agents as anthocyanins (Segura-Carretero et al., 2008). Overall, there is now increasing evidence that antioxidants in the human diet are of major benefit for health and well-being. The antioxidant properties of *H. sabdariffa* and other hibiscus species have been widely studied (Büyükbalci & El, 2008; Oboh & Rocha, 2008; Vankar & Srivastava, 2008).

In this work we focused on quantifying the phenolic fraction, anthocyanins and other polar compounds in the aqueous extract of *H. sabdariffa*. This quantification was achieved using two detection systems, i.e. RP-HPLC coupled to DAD or RP-HPLC coupled to ESI-TOF-MS. Recently, the qualitative characterization of the compounds present in the aqueous extract of *H. sabdariffa* was carried out successfully (Rodríguez-Medina et al., 2009). These methods were also suitable for the quantification of these substances. To evaluate the antioxidant capacities of foods, numerous *in vitro* methods have been developed and reviewed. However, there has not been a consensus for the preferred method. ORAC (Oxygen Radical Absorbance Capacity),

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TBARS (Thiobarbituric Acid Reactive Substances), TEAC (Trolox Equivalent Antioxidant Capacity) and FRAP (Ferric Reducing Antioxidant Power) assays are among the more popular methods that have been used (Wua et al., 2004). Advantages and disadvantages of these methods have been fully discussed in several reviews (Frankel & Meyer, 2000; Prior, Wu, & Schaich, 2005; Sánchez-Moreno, 2002; Strube, Haenen, Van Den Berg, & Bast, 1997). In this work we have studied the antioxidant capacity of *H. Sabdariffa* aqueous extract using four different methods TEAC and FRAP (based on electron transference) vs. TBARS and ORAC (based on hydrogen atom transference) (Huang, Ou, & Prior, 2005). To further assess *H. sabdariffa* aqueous extract bioactivity, it was administered as sole drinking fluid to mice fed with a high fat-high cholesterol diet in order to assay its anti-hyperlipemic effects.

## 2. Materials and methods

### 2.1. Chemicals and reagents

All chemicals were of analytical HPLC reagent grade and used as received. Formic acid and acetonitrile used for preparing mobile phases were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Solvents were previously filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). The standards, for the calibration curves, chlorogenic acid, quercetin 3-rutinoside, quercetin 3-glucoside, kaempferol 3-O-rutinoside and kaempferol 3-(*p*-coumarylglucoside), quercetin, 4-hydroxycoumarin and delphinidin-3-sambubioside were purchased either from Fluka, Extrasynthese (Genay Cedex, France) or Polyphenols (Polyphenols Laboratories AS, Hanaveien Sandnes, Norway). The stock solutions containing these analytes were prepared in methanol (Lab-Scan). The reagents to measure the antioxidant capacity, EYPC (egg yolk phosphatidylcholine), AAPH (2,2′-Azobis (2-methyl-propionamine) dihydrochloride), BHT (butylhydroxytoluene), TEP (1,1′,3,3′-tetraethoxypropane), SDS (Sodium Dodecyl Sulfate), TBA (thiobarbituric acid), TPTZ (Tripyridyltriazine), ABTS (2,2`-azinobis (3-ethylbenzothiazoline-6-sulphonate), fluorescein, Trolox and ferric sulfate were purchased from Sigma-Aldrich. Sodium acetate, ferric chloride, sodium chloride, hydrochloric acid, sulphuric acid, acetic acid, chloroform, ethanol, TRIS (Tris(hydroxymethyl)aminomethane) were purchased from Panreac (Barcelona, Spain).

### 2.2. Sample preparation

The *H. sabdariffa* plant was originally from the village of Guerle in Senegal. It was kindly provided by Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan (Reus, Tarragona, Spain). The dry calices from the plant were manually mill grounded and mixed with ultrapure water, up to a concentration of 25 g/l, stirred it in vortex until dissolved, filtered with units of single use Filters Millex (Millipore, Bedford, MA, USA) and directly injected into the HPLC system for the direct and indirect quantifications. On the other hand, the aqueous extract of *H. sabdariffa* for the different antioxidant capacity determinations was conveniently diluted in order to comply with the working range of each spectrophotometric method.

### 2.3. Instrumentation

HPLC analyses were performed with a RRLC 1200 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump with Zorbax Eclipse Plus $C_{18}$ 4.6×150 mm, 1.8 μm column. Prefilters were used as precolumn, RRLC in-line filters, 4.6 mm, 0.2 μm supplied by Agilent Technologies. The mobile phase flow rate was 0.5 ml $min^{-1}$. HPLC was equipped with DAD and coupled to a TOF mass spectrometer equipped with an orthogonal electrospray interface ESI (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative mode and positive mode of ionization. (MS-Instrument: microTOF, ESI-TOF mass spectrometer) (Bruker Daltonik GmbH, Bremen, Germany). Fluorescence (ORAC and TBARS) and absorbance (FRAP and TEAC) measures were carried out on a spectrofluorimeter Polarstar Omega (BMG Labtechnologies, GMBH; Offenburg, Germany) (Thermostated at 37 °C for the ORAC assay).

### 2.4. Chromatografic, UV and spectrophotometric conditions

The compounds of the aqueous extract of *H. sabdariffa* were separated by the C18 column at room temperature at a flow rate of 0.5 ml/min and the injection volume was 10 μl for both gradient elution programs. The use of the prefilters as guard column also provided some protection against decomposition and blocking of the working column. The linear gradient used for the analysis, separation and identification of the polyphenols, hydroxycitric acid and its lactone (gradient program 1) was as follow: Mobile phases: A: water/ACN (acetonitrile) 90:10 (1% HCOOH) and B: ACN. The linear gradient elution program was run as stated: 0 min, 5% (B); 20 min 20% (B); 25 min 40% (B); 30 min 5% (B); 35 min, isocratic of B 5%. Anthocyanins, due to their acid–base equilibrium, needed lower pH to be resolved, and a different chromatographic method (gradient program 2) was employed. Solvents that constituted the mobile phases were: A, water (10% HCOOH) and B, ACN. The applied elution conditions were: 0 min, 0% (B); 13 min 20% (B); 20 min 30% (B); 25 min 80% (B); 30 min 0% (B); 35 min, isocratic of B 0%. The DAD coupled to the HPLC system was set in a spectrum range starting at 190 nm and ending at 950 nm. The excitation and emission wavelengths were 485 and 520 nm respectively for the ORAC assay, whereas these sets were 500 and 530 nm for the TBARS determination. The absorbance wavelength for FRAP and TEAC assays were 593 and 734 nm respectively.

### 2.5. ESI-TOF-MS conditions

TOF-MS transfer parameters were optimized by direct infusion experiments with Tunning mix (Agilent Technologies). The trigger time was set to 53 s (50 s for setting transfer time and 3 s for pre-pulse storage time), corresponding to a mass range of 50–1000 *m/z*. The other optimum values of the ESI-MS parameters were capillary, 4500 V gas heater temperature, 200 °C; drying gas flow, 7 l/min; nebulizing gas pressure, 1.5 bar and the spectra rate was 1 Hz. At this stage the use of a splitter was required to the coupling with the MS detector as the flow which arrived to the ESI-micro-TOF detector had to be 0.25 ml/min in order to obtain reproducible results. The TOF mass spectrometer was equipped with an ESI interface operating in both, negative and positive, polarity modes. To tune the detector to optimal conditions calibration was performed with sodium formate clusters (5 mM sodium hydroxide in water/isopropanol 1/1 (v/v), with 0.2% (v/v) of formic and acetic acids) in quadratic + high precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior the polyphenol identification. The accurate mass data for the molecular ions were processed using the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formula by using the GenerateMolecularFormula™ editor. The GenerateFormula™editor uses the sigmaFit™algorithm, CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue™) for increased confidence in the suggested molecular form (Bruker Daltonik, Technical Note 008; Rodríguez-Medina et al., 2009). The use of isotopic abundance patterns as a single further constraint removes >95% of false candidates. This orthogonal filter can condense

several thousand candidates down to only a small number of molecular formulas.

### 2.6. Antioxidant capacity assays

The Trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the ABTS radical cation by antioxidants, was based on method previously described by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993) and Re et al. (1999) and performed as in Laporta, Pérez-Fons, Mallavia, Caturla, and Micol (2007). The quantitative evaluation of the antioxidant capacity of the compounds against lipid peroxidation was determined through thiobarbituric acid reactive substances (TBARS) assay. Small unilamellar vesicles (SUVs) were prepared as previously described (Caturla, Vera-Samper, Villalaín, Reyes-Mateo, & Micol, 2003). Then, TBARS assay was performed as in Laporta et al. (2007). To assay the capacity of the extracts to scavenge peroxyl radicals a validated ORAC method, which uses fluorescein (FL) as the fluorescent probe ($ORAC_{FL}$), was utilized (Ou, Hampsch-Woodill, & Prior, 2001) and performed as in Laporta et al. (2007). The final ORAC values were calculated by using a regression equation between the Trolox concentration and the net area of the FL decay curve (area under curve, AUC) as previously described (Ou et al., 2001). The ferric reducing antioxidant power (FRAP) was performed according to Al-Duais, Müller, Böhm, and Jetschke (2009). Ferrous sulfate solutions (0–300 μM) were used for calibration.

### 2.7. Hypolipemic effect of H. sabdariffa

Male $LDLr^{-/-}$ mice (n = 32) were purchased from the Jackson Laboratory (Bar Harbor, Me). In accordance with our institutional guidelines, animals were housed under standard conditions and given a commercial mouse diet (14% Protein Rodent Maintenance diet, Harlan, Barcelona, Spain). At 10-weeks of age, animals were distributed into two dietary experimental groups (n = 16/group). One group was feed with the same maintenance diet (Chow diet, 3% fat and 0.03% cholesterol, w/w) and the other group was feed with a Western-type diet (High fat diet, 20% fat and 0.25% cholesterol, w/w). Each dietary group was divided into two groups (n = 8). One of them consumed water during the study while the second group consumed only *H. sabdariffa* aqueous extract at final concentration of 10 g/l. Plant calyxes were grounded and heated until boil in tap water. Infusion was then cooled down to room temperature and filtered to discard insoluble fraction. Extract was prepared freshly every two days and liquid in both groups was administered *ad libitum*. Animals were sacrificed at 24-weeks of age. The size of the experiment was planned according to previous data (Joven et al., 2007) and the animals were randomly assigned into experimental groups. Autoanalyser Synchron LXi 725 system (Beckman Coulter, IZASA, Barcelona, Spain) was used to determine cholesterol and triglyceride serum concentrations. SPSS/PC + 15.0 (SPSS, Chicago, IL) software was used and differences between groups were analyzed using U Mann–Whitney test with the level of significance set at $p < 0.05$.

## 3. Results and discussion

### 3.1. HPLC-UV and HPLC-ESI-TOF-MS quantification

#### 3.1.1. Sensitivity and repeatability

The sensitivity of the method was studied by defining the limits of detection (LODs) and limits of quantification (LOQs) for individual compounds in standard solutions for the UV spectra measured at the optimum wavelength. Four different wavelengths were considered at 325, 312, 350, 370 and 520 nm for the different compounds, except for the determination of the hydroxycitric acid and its lactone, the hibiscus acid in which the MS detection, based in the extract ion chromatogram, was used to measured the area peaks as the two above compounds did not present a measurable absorbance. Table 1 indicates the way the areas were obtained for the different analytes in terms of the detection system used in order to obtain the calibration curves, as well as molecular formula, *m/z* and UV–vis absorption bands of each compound from *H. sabdariffa* aqueous extract. Table 2 summarizes the analytical parameters for the different compounds present in the aqueous extract of *H. sabdariffa*. Seven standards were available and the rest of the compounds were expressed as equivalent of those, except for the hydroxycitric and hibiscus acids which were expressed as equivalents of caffeic acid (not present in the aqueous extract of *H. sabdariffa*, but used as standard for the quantification of these two compounds). Calibration curves were obtained for each standard with high linearity ($r^2 > 0.99$) by plotting the standard concentration as a function of the peak area obtained from HPLC–UV and HPLC–ESI-TOF-MS analyses. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated according to IUPAC recommendation (Currie, 1995). Intraday and interday precisions were developed to evaluate the repeatability of HPLC–UV and HPLC-ESI-TOF-MS methods. A sample of the aqueous extract was injected ($n = 2$) on the same day (intraday precision) for 3 consecutive days (interday precision, $n = 6$). The intraday repeatability of peak area, expressed as the RSD, varied between the interval of 0.16% and 4.6% whereas the interday repeatability was between 0.87% and 6.12%.

**Table 1**
Molecular formula, $[M—H]^-$, UV–vis absorption bands and used technique for the quantification of each compound in *H. sabdariffa* aqueous extract.

| Compound | Molecular formula | $[M—H]^-$ | UV–vis (nm) | Quantification technique |
|---|---|---|---|---|
| Hydroxycitric acid | $C_6H_8O_8$ | 207.0140 | – | MS-TOF (m/z 207) |
| Hibiscicus acid | $C_6H_6O_7$ | 189.0035 | – | MS-TOF (m/z 189) |
| Chlorogenic acid (isomer I) | $C_{16}H_{18}O_9$ | 353.0891 | 297, 324 | DAD-UV (325 nm) |
| Chlorogenic acid | $C_{16}H_{18}O_9$ | 353.0872 | 297, 324 | DAD-UV (325 nm) |
| Chlorogenic acid (isomer II) | $C_{16}H_{18}O_9$ | 353.0871 | 297, 324 | DAD-UV (325 nm) |
| Myricetin-3-arabinogalactose | $C_{26}H_{28}O_{17}$ | 611.1271 | 352 | DAD-UV (350 nm) |
| Quercetin-3-sambubioside | $C_{26}H_{28}O_{16}$ | 595.1309 | 345 | DAD-UV (350 nm) |
| 5-O-Caffeoylshikimic acid | $C_{16}H_{16}O_8$ | 335.0768 | 296, 326 | DAD-UV (325 nm) |
| Quercetin-3-rutinoside | $C_{27}H_{30}O_{16}$ | 609.1462 | 255, 353 | DAD-UV (350 nm) |
| Quercetin-3-glucoside | $C_{21}H_{20}O_{12}$ | 463.0873 | 253, 356 | DAD-UV (350 nm) |
| Kaempferol-3-O-rutinoside | $C_{27}H_{30}O_{15}$ | 593.1512 | 265, 350 | DAD-UV (350 nm) |
| N-Feruloyltyramine | $C_{18}H_{20}NO_4$ | 312.1234 | 286, 316 | DAD-UV (325 nm) |
| Kaempferol-3-(*p*-coumarylglucoside) | $C_{30}H_{26}O_{13}$ | 593.1312 | 312 | DAD-UV (312 nm) |
| Quercetin | $C15H_{10}O_7$ | 301.0339 | 253, 372 | DAD-UV (370 nm) |
| 7-Hydroxycoumarin | $C_9H_6O_3$ | 161.0244 | 300 | DAD-UV (300 nm) |
| Delphinidin-3-sambubioside | $C_{26}H_{30}O_{16}$ | 595.1446 | 280, 520 | DAD-UV (520 nm) |
| Cyanidin-3-sambubioside | $C_{26}H_{30}O_{15}$ | 579.1493 | 280, 520 | DAD-UV (520 nm) |

**Table 2**
Calibration and standard deviation data, where RSD is the relative standard deviation, LOD is the limit of detection and LOQ is the limit of quantification. LOD and LOQ values were calculated for the available standards solely.

| Analyte | RSD intraday | RSD interday | LOD (μg/ml) | LOQ (μg/ml) | Calibration range (μg/ml) | Calibration equations | $r^2$ |
|---|---|---|---|---|---|---|---|
| Hydroxycitric acid | 4.619 | 4.797 | – | – | 100–1000 | Y = 1214164.4 X + 649809.3 | 0.9958 |
| Hibiscicus acid | 3.607 | 3.626 | – | – | 100–1000 | Y = 1214164.4 X + 649809.3 | 0.9958 |
| Chlorogenic acid isomer I | 1.575 | 2.266 | – | – | 5–100 | y = 5268.2 X − 119.8 | 0.9951 |
| Chlorogenic acid | 0.707 | 2.011 | 0.235 | 0.782 | 5–100 | y = 5268.2 X − 119.8 | 0.9951 |
| Chlorogenic acid isomer II | 1.977 | 1.629 | – | – | 5–100 | y = 5268.2 X − 119.8 | 0.9951 |
| Myricetin 3-arabinogalactose | 1.714 | 4.384 | – | – | 0.5–10 | Y = 3737.3 X − 5.0 | 0.9993 |
| Quercetin 3-sambubioside | 0.601 | 1.939 | – | – | 0.5–10 | Y = 3737.3 X − 5.0 | 0.9993 |
| 5-O-Caffeoylshikimic acid | 0.788 | 4.037 | – | – | 0.5–10 | Y = 4313.0 X − 14.1 | 0.9958 |
| Quercetin 3-rutinoside | 1.197 | 0.876 | 0.116 | 0.386 | 5–100 | Y = 3750.4 + 26.0 | 0.9991 |
| Quercetin 3-glucoside | 1.93 | 1.501 | 0.102 | 0.341 | 0.5–10 | Y = 4642.0 X − 6.0 | 0.9996 |
| Kaempferol 3-O-rutinoside | 3.084 | 2.477 | 0.088 | 0.293 | 0.5–10 | Y = 4516.1 X − 4.6 | 0.9994 |
| N-Feruloyltyramine | 1.13 | 1.819 | – | – | 0.5–10 | Y = 4313.0 X − 14.1 | 0.9958 |
| Kaempferol 3-(p-coumarylglucoside) | 1.837 | 1.698 | 0.086 | 0.288 | 0.5–10 | Y = 6660.9 X − 19.4 | 0.9967 |
| Quercetin | 1.849 | 1.658 | 0.048 | 0.156 | 0.5–10 | Y = 8831.1 X − 22.0 | 0.9988 |
| 7-Hydroxycoumarin | 0.341 | 1.377 | – | – | 1–100 | Y = 7451.0 X − 74.2 | 0.9961 |
| Delphinidin 3-sambubioside | 0.658 | 6.128 | 0.184 | 0.612 | 1–100 | Y = 3418.9 X + 9.1 | 0.9983 |
| Cyanidin 3-sambubioside | 0.162 | 2.025 | – | – | 1–100 | Y = 3418.9 X + 9.1 | 0.9983 |

### 3.1.2. Calibration curves

In order to quantify the amount of each compound in the aqueous extract of *H. sabdariffa*, seven calibration curves were prepared with the seven standards available, chlorogenic acid, quercetin 3-rutinoside (rutin), quercetin 3-glucoside, kaempferol 3-O-rutinoside, kaempferol 3-(*p*-coumarylglucoside), quercetin, 4-hydroxycoumarin and delphinidin 3-sambubioside. The other compounds, for which no commercially standards were available, were tentatively quantified on basis of the other compounds bearing similar structures. 7-Hydroxycoumarin was quantified with 4-hydroxycoumarin. A calibration curve of caffeic acid was also prepared for the quantification of hydroxycitric acid and its lactone, the hibiscus acid. The ranges are also stated in Table 2, including the RSD values obtained for two replicates of each calibration point. The calibration plots indicate good correlation between peak areas and the analyte concentrations. All calibration curves showed good linearity in the studied range of concentration. Regression coefficients were higher than 0.99 for all the compounds and for the considered ranges. All the features of the proposed method are summarized in Tables 1 and 2.

### 3.1.3. Quantification of compounds in the aqueous extract of H. sabdariffa

A previous method optimized in our laboratory was applied to the quantification of the seventeen compounds qualitatively characterized and present in the aqueous extract of *H. sabdariffa* (Rodríguez-Medina et al., 2009). The concentration of the extract was set at 25 g/l in all the cases in order to fix in the considered working ranges. Two replicates of the extract in three consecutive days were carried out and the results, expressed in mg analyte/Kg of dry weighted extract (n = 6; value = X ± SD), are summarized in Table 3.

**Table 3**
Quantification of polyphenolic fraction from *H. sabdariffa* aqueous extract, expressed in ppm (m/m).

| Analyte | *Hibiscus sabdariffa* |
|---|---|
| Hydroxycitric acid | 8288.03 ± 397.63 |
| Hibiscicus acid | 31122.02 ± 1128.39 |
| Chlorogenic acid isomer I | 2755.15 ± 62.42 |
| Chlorogenic acid | 1923.72 ± 38.69 |
| Chlorogenic acid isomer II | 1041.19 ± 16.96 |
| Myricetin 3-arabinogalactose | 57.32 ± 2.51 |
| Quercetin 3-sambubioside | 304.02 ± 5.90 |
| 5-O-Caffeoylshikimic acid | 171.47 ± 6.92 |
| Quercetin 3-rutinoside | 495.70 ± 4.34 |
| Quercetin 3-glucoside | 143.74 ± 2.16 |
| Kaempferol 3-O-rutinoside | 91.86 ± 2.28 |
| N-Feruloyltyramine | 98.97 ± 1.80 |
| Kaempferol 3-(p-coumarylglucoside) | 28.37 ± 0.48 |
| Quercetin | 121.24 ± 2.01 |
| 7-Hydroxycoumarin | 1839.20 ± 25.34 |
| Delphinidin 3-sambubioside | 2701.21 ± 165.55 |
| Cyanidin 3-sambubioside | 1939.15 ± 39.27 |

### 3.2. Antioxidant capacity

Four different methods were used to evaluate the antioxidant capacity of the aqueous extract of *H. sabdariffa* utilized in this study. TEAC and FRAP methods are based on electron transfer mechanisms (ET), i.e. reducing capacity. On the contrary, TBARS and ORAC methods are based on hydrogen atom transfer (HAT) reactions, which scavenge the generation of peroxyl radicals through decomposition of azo compounds (Huang et al., 2005; Prior & Cao, 1999). Therefore, we tried to study deeply the performance of *H. sabdariffa* extract under these two different antioxidant mechanisms. *H. sabdariffa* aqueous extract was compared to another extract with proven antioxidant properties, i.e. olive leaf extract containing 25% oleuropein and also pomegranate extract (*Punica granatum* L.) which has a high content in anthocyanins (Alighourchi et al. 2008; Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000; Martin, Krueger, Rodríquez, Dreher, & Reed, 2008). Results are summarized in Table 4. As expected, *H. Sabdariffa* showed a different behavior in relation to olive leaf and pomegranate extracts depending on the antioxidant measurement considered.

TEAC value was higher for the olive leaf extract (0.92 vs. 0.16 mmol Trolox equivalent/g extract), indicating the lower reducing capacity of *H. sabdariffa* aqueous extract. However, the values obtained in the FRAP assay for *H. sabdariffa* aqueous extract doubled

**Table 4**
Results of antioxidant capacity assays.

| ASSAY | *Hibiscus sabdariffa* | Olive leaf | Pomegranate |
|---|---|---|---|
| TEAC[a] | 0.16 ± 0.005 | 0.92 ± 0.12[b] | 0.25 ± 0.02[c] |
| FRAP[d] | 2.31 ± 0.09 | 1.24 ± 0.02 | 1.68 ± 0.26[e] |
| TBARS ($IC_{50}$)[f] | 163.66 ± 5.28 | 7.00 ± 1.20[b] | 32.40 ± 1.70[g] |
| ORAC[h] | 2307 ± 10 | 4950 ± 300[i] | 3210 ± 11[e] |

[a] Expressed in mmol Trolox equivalent/g extract.
[b] Data obtained from Funes et al. (2009).
[c] Data obtained from Zhang, Wang, Lee, Henning, and Heber (2009).
[d] Expressed in mmol $FeSO_4$ equivalent/g extract.
[e] Data obtained from Madrigal-Carballo, Rodriguez, Krueger, Dreher, and Reed (2009).
[f] Expressed in mg/l of extract able to inhibit 50% of lipid peroxidation.
[g] Data obtained from Kulkarni, Aradhya, and Divakar (2004).
[h] Expressed in μmol Trolox equivalent/g extract.
[i] Data obtained from Laporta et al. (2007).

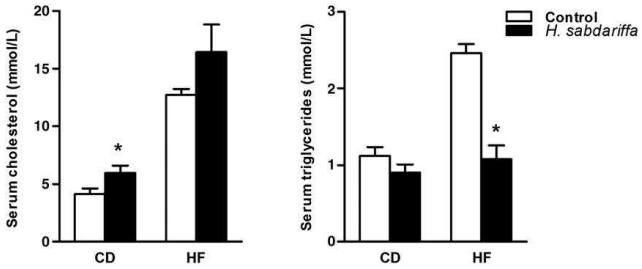


**Fig. 1.** Serum cholesterol and triglyceride concentrations were measured after treatment with *H. sabdariffa* extract. Cholesterol concentrations trend to increase in both dietary groups, but this difference was only significant in chow diet dietary group (CD). Serum triglyceride concentration was effectively reduced in both dietary groups, being significant in the high-fat diet (HF) fed animals. Differences are marked with * ($p<0.05$).

those obtained for olive leaf extract, although this was also an ET-based assay too. The high FRAP value of the *H. sabdariffa* aqueous extract could be explained through the reported efficacy of chlorogenic acid and derivatives as reductants (De Leonardis, Pizzella, & Macciola, 2008; Wu, 2007), which are the main compounds in *H. sabdariffa* aqueous extract (Table 3).

Moreover, *H. sabdariffa* aqueous extract exhibited a significant ORAC value and a very low potency in the TBARS assay as revealed by its elevated $IC_{50}$ value (concentration of extract able to inhibit 50% of lipid peroxidation). Although both methods take place through HAT-based mechanisms, ORAC measures the capacity of antioxidants to scavenge peroxyl radicals in a water environment, whereas TBARS assay determines the same capacity under a lipophilic environment, i.e. in the presence of a phospholipid membrane. Major antioxidant compounds in *H. sabdariffa* aqueous extract are chlorogenic acid derivatives and anthocyanins (delphinidin 3-sambubioside and cyanidin 3-sambubioside), which are highly hydrophilic antioxidants and hence these compounds may have worse interaction with lipid peroxyl radicals that are the main radical species generated in the TBARS assay. It has been reported that delphinidin and cyanidin, which lack methoxy group in their B ring, are less antioxidant in lipid systems than malvidin or peonidin due to their higher polarity (Kähkönen & Heinonen, 2003). In addition, glycosylated flavonoids such as anthocyanins showed less antioxidant potency than their corresponding aglycones in lipid systems, since glycosylation decreases the access of the compound to the lipid phase what reinforces our hypothesis (Fukumoto & Mazza, 2000; Kähkönen & Heinonen, 2003).

The comparison of the *H. sabdariffa* aqueous extract vs. Pomegranate extract showed the similarity between the action mechanisms of the antioxidant capacity in TEAC and ORAC assays. However, the low value in FRAP assay could be due to the absence of chlorogenic acid in pomegranate extract. The low value for pomegranate extract in TBARS assay was due to the presence of ellagic acid and ellagitannins, which exhibit a high antioxidant capacity to prevent the lipid peroxidation (Yu, Chang, Wu, & Chian, 2005).

### 3.3. Effects of H. sabdariffa extract on serum lipid concentrations

*H. sabdariffa* hypolipemic effects were tested on low density lipoprotein receptor deficient mice ($LDLr^{-/-}$). Lipid metabolism has been largely studied on this animal model since these animals develop hyperlipemia when feed with hypercaloric diets.

Cholesterol and triglycerides concentration were measured in both dietary groups (chow diet, CD; high fat diet, HF) after 14 weeks of treatment. Serum cholesterol concentration increased in both dietary groups compared to the control, but this difference was only significant in those animals fed with chow diet (Fig. 1A). The strongest effect was observed on serum triglyceride concentration when a high fat diet was fed. Serum triglycerides were reduced more than 50% in mice treated with *H. sabdariffa* infusion (Fig. 1B), compared to control. However, no change in serum triglycerides was observed in the chow diet group. Animals that consumed *H. sabdariffa* aqueous extract and were treated with high fat diet, showed a triglyceride concentration similar to that one observed in the chow diet group. Taken together, these results point to lipoprotein metabolism modulation by the *Hibiscus sabdariffa* extract. Triglyceride concentration reduction could be because a reduction in VLDL concentrations, the major triglyceride-containing lipoprotein. This effect could be observed in other polyphenol-rich plant extracts (Beltrán-Debón et al., 2010). On the other hand, the higher cholesterol concentration after *H. sabdariffa* treatment could be explained by an increase in LDL and HDL concentrations.

## 4. Concluding remarks

The proposed method exhibited excellent performance in the determination of the different families of phenolic compounds in *H. sabdariffa* plant material. The method used for the characterization of the aqueous extract of *H. sabdariffa* has been successfully validated and has proven to be suitable for quantification purposes. Using this method, seventeen main phenolic compounds in *H. sabdariffa* aqueous extract have been quantified. *H. sabdariffa* aqueous extract possesses a significant antioxidant capacity to reduce peroxyl radicals by hydrogen atom transfer (ORAC). Moreover, *H. sabdariffa* exhibited a stronger capacity to donate electrons (FRAP) in the presence of metals than the strong antioxidant olive leaf extract (25% oleuropein). In addition, *H. sabdariffa* aqueous extract showed hypolipemic properties in a hyperlipemic mouse model through the reduction of 50% of serum triglyceride concentration under hypercaloric diet for several weeks.

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**OTROS**

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## Publicaciones indexadas

1. García-Heredia A, Marsillach J, Aragonès G, Guardiola M, Rull A, **Beltrán-Debón R**, Folch A, Mackness B, Mackness M, Pedro-Botet J, Joven J, Camps J. Serum paraoxonase-3 concentration is associated with the severity of hepatic impairment in patients with chronic liver disease. Clin Biochem. 2011 Aug 10.

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2. Herranz-Lopez M, Barrajon-Catalan E, **Beltran-Debon R**, Joven J, Micol V. Stevia is a source for alternative sweeteners: potential medicinal effects. Agro Food Industry Hi-Tech. 2010 May; 21: 3. 38-42

## Participaciones en congresos

**79th Annual Congress of the European Atherosclerosis Society (EAS)**
Gothenburg 2011

**4th International Conference on Paraoxonases**
La Pineda 2010

## ABREVIATURAS

ABCA1 – ATP-binding cassette, member 1

ALT – alanino amino transferasa

AMPK – AMP-activated protein kinase

AST – aspartato aminotransferasa

ATP – adenosine triphosphate

CE – Capilar electrophoresis

Da – Dalton

EC – epicatechin

ECG – epicatechin gallate

ECGC – epigallocatechin gallate

EDTA – ethylenediaminetetraacetic acid

EGC – epigallocatechin

EGTA – ethylene glycol tetraacetic acid

ERK – elk-related tyrosine kinase



ESI – electrospray injection

FAS – TNF receptor superfamily member 6

FASL – Fas ligand

GLUT4 – insulin-responsive glucose transporter 4

HDL – high-density lipoprotein

HPLC – high performance liquid chromatography

ICAM-1 – intercellular adhesion molecule 1

IL-6 – interleucina-6

IL-8 – interleucina-8

IL-8RA – chemokine (C-X-C motif) receptor type 2 (CXCR2)

IL-10 – interleucina-10

IT – ion trap

JNK – c-Jun N-terminal kinase

LDL – low-density lipoprotein

LDLr$^{-/-}$ - low-density lipoprotein receptor deficient mice

LP – lipasa pancreática

LPS – lipopolisacárido

MCP-1 – monocyte chemoattractant protein-1

MS – mass spectrometry

*m/z* – relación masa/carga

NAD – nicotinamide adenine dinucleotide

NADPHox – nicotinamide adenine dinucleotide phosphate oxidase

NAFLD – non-alcoholic fatty liver disease

Nampt – nicotinamide phosphoribosyltransferase

NASH – non-alcoholic steatohepatitis

NEFA – non-esterified fatty acids

NF-κβ – nuclear factor κβ

pAMPK – phosphorylated AMP-activated protein kinase

PBMCs – peripheral blood mononuclear cells

PNLIP – pancreatic lipase

PPARγ – peroxisome proliferator-activated receptor gamma

SCD1 – stearoyl-Coenzyme A desaturase 1

SIRT1 – sirtuin 1

SREBP-1 – sterol regulatory element binding protein 1

TOF – Time of flight

VCAM-1 – vascular cell adhesion molecule 1

VLDL – very low-density lipoprotein