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Introducción

Introduction

1. INTRODUCCIÓN

El estrés oxidativo ha sido implicado en el desarrollo de numerosas enfermedades crónicas y en el proceso de envejecimiento (Halliwell y Whiteman 2004). El término estrés oxidativo hace referencia al desequilibrio entre la generación de especies oxidantes (radicales libres y otras especies reactivas) y la capacidad de los sistemas de defensa antioxidante del organismo para hacer frente a la agresión oxidativa y sus efectos adversos (Mayne 2003). La dieta humana contiene diferentes antioxidantes naturales que pueden contribuir al refuerzo de las defensas naturales del organismo. Así, los efectos beneficiosos derivados del consumo de dietas ricas en alimentos vegetales han sido atribuidos principalmente a las vitaminas antioxidantes, los carotenoides y, particularmente, a los compuestos fenólicos presentes en estos alimentos (Lampe 1999; Prior 2003). Por este motivo, distintas organizaciones internacionales recomiendan el consumo de al menos 5 raciones diarias de frutas y/o verduras con la finalidad de aportar una ingesta adecuada de antioxidantes naturales y mejorar el estado de salud general (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997).

Debido a que la prevención de enfermedades crónicas constituye una mejor estrategia que su tratamiento, reducir el riesgo de enfermedades cardiovasculares o el cáncer se ha convertido en una materia de gran interés para profesionales de la salud, científicos y también para la industria alimentaria (Liu 2003). Por esta razón, muchos alimentos funcionales son diseñados actualmente con el objetivo de proporcionar una ingesta elevada de antioxidantes y reducir el riesgo de enfermedades asociadas al estrés oxidativo. El término alimento funcional se refiere a un alimento o ingrediente capaz de mejorar la salud y/o reducir el riesgo de enfermedad (Rafter 2002). Además se trata de alimentos a consumir dentro de una dieta normal para conseguir efectos beneficiosos que van más allá de los requerimientos nutricionales tradicionales (Roberfroid 2002).

Entre las fuentes habituales de antioxidantes de la dieta, las frutas rojas y bayas son ricas en compuestos fenólicos, principalmente flavonoides, los cuales se caracterizan por su actividad anticarcinogénica, antiinflamatoria, antiaterogénica, antimicrobiana y antioxidante (Robards et al., 1999; Moure et al., 2001). Además, estos compuestos permanecen en productos procesados de frutas tales como zumos, mermeladas, jaleas, etc. (Heinonen et al., 1998a; Häkkinen et al., 2000; Zafrilla et al., 2001). Por esta razón, el uso de estas frutas como ingredientes podría ser una buena estrategia para la obtención de alimentos funcionales ricos en compuestos fenólicos y capaces de aportar beneficios a la salud del consumidor. No obstante, para que un alimento sea aceptado como funcional, sus efectos

beneficiosos deben ser avalados experimentalmente mediante estudios de intervención en humanos (Roberfroid 2002; Aggett et al., 2005), que pongan de manifiesto los efectos reales de este producto para la salud humana.

1.1. ESTRUCTURA DE LA TESIS Y OBJETIVOS

El objetivo principal de la presente investigación fue la evaluación de la eficacia antioxidante de productos ricos en compuestos fenólicos elaborados a partir de frutas rojas y bayas, con la finalidad de obtener evidencias científicas que puedan contribuir en el futuro a su autorización como alimentos funcionales en el ámbito de la Unión Europea.

Descripción de los productos

Para el desarrollo de la investigación, el Departamento de Investigación y Desarrollo de Hero España S.A. elaboró dos productos ricos en compuestos fenólicos y con una alta actividad antioxidante, empleando en su formulación zumos concentrados comerciales de uva (26%), cereza (2%), frambuesa (1%), zarzamora (0.6%) y grosella (0.6%). La selección de los zumos concentrados se realizó en base a los datos existentes en la literatura científica sobre la actividad antioxidante total de las frutas empleadas, con el objetivo de obtener un producto final con una actividad antioxidante total similar o superior a la aportada por las cinco raciones diarias recomendadas de frutas y verduras. Los productos evaluados se elaboraron en dos formatos comerciales **(Figura 1)**; un postre gelificado y un zumo. En ambos casos el ingrediente principal fue el agua la cual fue mezclada con los zumos seleccionados en las proporciones indicadas anteriormente. La única diferencia fue la adición de pectina en el caso del postre para conseguir la consistencia gelatinosa.

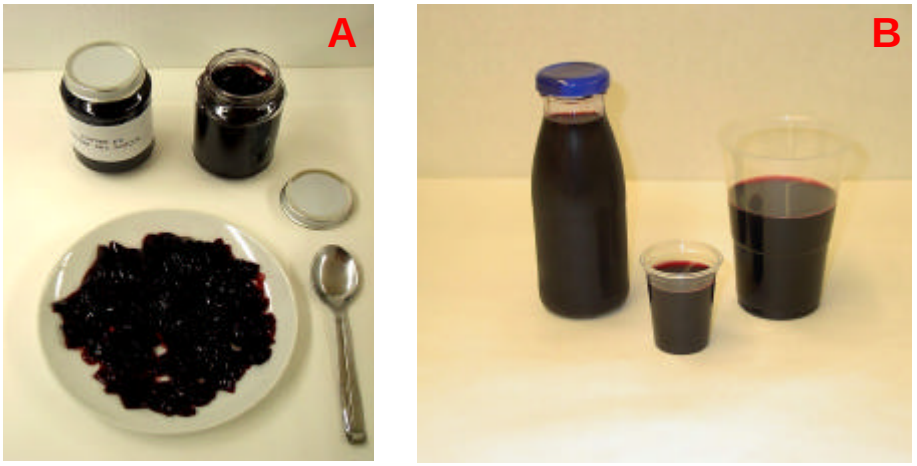


Figura 1. Imágenes del postre gelificado (A) y del zumo de frutas (B) empleados en el presente estudio.

Diseño experimental

Tal y como consta en la sección de Resultados y Discusión, la presente tesis está estructurada en 6 experiencias cada una de las cuales corresponde a un trabajo publicado, en fase de revisión por una revista científica o a un manuscrito pendiente de ser enviado para su evaluación. Además, se ha incluido una sección en la cual se realiza una discusión general de los resultados obtenidos en esta investigación. La fase experimental comprende tres etapas fundamentales. La primera consistente en la caracterización de los productos elaborados en términos de su composición nutricional, actividad antioxidante total y sus niveles de sustancias antioxidantes, y las siguientes etapas encaminadas a la evaluación de la eficacia antioxidante de los productos en modelos de células en cultivo e *in vivo* tras la suplementación en humanos.

1. Caracterización de los productos elaborados

Ambos productos fueron analizados con el objetivo de conocer su composición química proximal, mediante la aplicación de los métodos de la Asociación de Analistas Químicos Oficiales (AOAC internacional). Así, se determinaron el contenido en humedad, grasa, proteína, fibra, cenizas e hidratos de carbono del postre y el zumo. La actividad antioxidante total de los productos se evaluó mediante pruebas espectrofotométricas basadas en la captación de radicales libres y en la capacidad reductora de las muestras. El contenido en vitamina C de los productos fue analizado por cromatografía líquida de alta resolución (HPLC). El análisis de compuestos fenólicos totales se realizó por colorimetría, mientras que la determinación de los principales grupos de compuestos fenólicos se realizó por HPLC. En el caso del postre de frutas se realizó un estudio de envejecimiento (Estudio 1) a lo largo de un año de almacenamiento a diferentes temperaturas (8, 21 y 30ºC). Se evaluaron cambios en la actividad antioxidante total, vitamina C, compuestos fenólicos y color del postre de frutas tras 3, 6, 9 y 12 meses de almacenamiento.

2. Evaluación in vitro de la eficacia antioxidante en cultivos celulares

Los modelos de células en cultivo son hoy en día una herramienta útil en la evaluación de la eficacia de fármacos y sustancias de distinto origen. En la presente tesis se han empleado dos líneas celulares humanas con el objetivo de evaluar la capacidad de los antioxidantes presentes en la muestra para modular procesos a nivel celular. Para facilitar la extracción de las sustancias antioxidantes de la muestra, en los estudios realizados en cultivos celulares se emplearon muestras del zumo de frutas. En los ensayos se emplearon la línea celular de carcinoma hepático HepG2 y la línea de células de leucemia mieloide U937.

Mediante el empleo de la línea celular HepG2 (Estudio 2) se evaluó el efecto de la incubación con extractos del zumo experimental sobre la proliferación celular, con el objetivo de conocer las dosis de compuestos fenólicos capaces de inhibir significativamente el crecimiento de las células tumorales en cultivo. Posteriormente, empleando dosis no tóxicas de extracto, se realizó un estudio en el cual se evaluó la capacidad de estas dosis subtóxicas para proteger las células frente al estrés oxidativo inducido por agentes oxidantes como el tert-butil hidroperóxido (tB-OOH) y el peróxido de hidrógeno. Así, se determinó la protección ejercida por el zumo de frutas frente a la muerte celular y peroxidación lipídica inducidas por ambos agentes oxidantes. Además, se evaluó la capacidad de los extractos para modular la activación de los sistemas enzimáticos endógenos glutatión peroxidasa y glutatión-*s*-transferasa tras la exposición de las células al tB-OOH.

La línea celular U937 (Estudio 3) se empleó en un estudio encaminado a evaluar el efecto protector de los extractos del zumo frente al estrés oxidativo causado por el peróxido orgánico tB-OOH. Para ello, tras la exposición al tB-OOH, se evaluó la capacidad del zumo para prevenir la muerte celular, el daño al ADN, la generación intracelular de especies reactivas del oxígeno y la apertura de poros en la membrana mitocondrial. Este último proceso se considera uno de los eventos finales que conducen a la muerte celular.

3. Evaluación in vivo del efecto de la ingesta de los productos

Cuando se evalúa la eficacia de un alimento rico en antioxidantes naturales, es necesario aportar pruebas de sus efectos beneficiosos en estudios en humanos. En la presente investigación se llevaron a cabo dos estudios de intervención en humanos con el objetivo de evaluar el efecto del consumo de los productos experimentales sobre el estado antioxidante total de los individuos y sobre determinados biomarcadores del daño oxidativo.

El primero de estos estudios de intervención se llevó a cabo con un grupo de ancianos residentes en un centro del Instituto de Servicios Sociales de la Región de Murcia. Como parte de este estudio se realizó una valoración del estado antioxidante y nutricional de los ancianos (Estudio 4), con el objetivo de conocer la adecuación de su dieta habitual, así como la relación de la ingesta de antioxidantes de la dieta con los niveles de antioxidantes y la capacidad antioxidante total del plasma/suero de los individuos. Para ello, se recopiló información a diario sobre el consumo de alimentos mediante encuestas dietéticas y la información se procesó con un programa informático a fin de evaluar la adecuación de la ingesta de nutrientes de acuerdo a las recomendaciones establecidas para este grupo de

población. Se determinaron en plasma/suero la capacidad antioxidante total sérica y los niveles séricos y plasmáticos de ácido úrico, albúmina, bilirrubina, a-tocoferol, retinol, β-caroteno, vitamina C y coenzima Q.

Para el desarrollo del estudio de intervención (Estudio 5) se empleó el postre gelificado de frutas, al tratarse de un producto diseñado principalmente para este colectivo de la población. Durante el mismo, los individuos siguieron la dieta habitual del centro y consumieron a diario durante 2 semanas una ración de 200 g del postre de frutas. Para la comparación de resultados se establecieron dos grupos control, el primero formado por ancianos del centro y el segundo por un grupo de individuos sanos de menor edad y ajenos al centro. Se evaluaron al inicio y al final del estudio la capacidad antioxidante total sérica, los niveles de diferentes antioxidantes séricos y plasmáticos (ácido úrico, albúmina, bilirrubina, a-tocoferol, retinol, β-caroteno, vitamina C, coenzima Q), la susceptibilidad a la oxidación de las lipoproteínas plasmáticas de baja densidad (LDL) y el daño al ADN de linfocitos.

El segundo estudio de intervención (Estudio 6) se realizó en un grupo de individuos sanos. El objetivo de dicho estudio fue conocer el efecto a corto plazo de la ingesta del zumo experimental rico en compuestos fenólicos y obtener evidencias de la absorción y excreción urinaria de estas sustancias antioxidantes. Para ello, los individuos siguieron durante 2 días una dieta pobre en antioxidantes naturales, evitando el consumo de frutas, verduras y bebidas como el vino, té o café. Tras este periodo de depleción, los individuos consumieron 400 mL del zumo en ayunas y se tomaron muestras de sangre antes de la ingesta y tras 1, 2, 4 y 6 horas. Se tomaron además muestras de orina antes y tras 1, 2, 4, 6 y 24 horas de la ingesta. Se analizaron en suero la capacidad antioxidante total, ácido úrico, vitamina C, compuestos fenólicos totales, productos de peroxidación lipídica y productos de la oxidación proteica. En orina se determinaron los fenoles totales y la capacidad antioxidante total como indicadores de la biodisponibilidad de sustancias antioxidantes del zumo.

Objetivos

De acuerdo a lo anteriormente expuesto, los objetivos específicos de la presente investigación fueron:

1. Evaluar la composición nutricional y las propiedades antioxidantes de productos experimentales elaborados a partir de uvas, cerezas y bayas, y ricos en compuestos fenólicos antioxidantes.

2. Estudiar mediante ensayos *in vitro* en cultivos celulares el efecto de un zumo con alto contenido en compuestos fenólicos sobre la proliferación celular y su capacidad para modular los sistemas de defensa antioxidante de las células en cultivo.

3. Conocer, mediante estudios de intervención con humanos, el efecto del consumo de los productos elaborados sobre el estado antioxidante total de las personas, así como su capacidad para modificar marcadores del daño oxidativo a biomoléculas.

1. INTRODUCTION

Oxidative stress has been associated with the development of several chronic diseases and with the aging process (Halliwell y Whiteman 2004). The term oxidative stress denotes an imbalance between the production of oxidants (free radicals and other reactive species) and the ability of the organism's natural antioxidant defences to cope with these oxidants and prevent adverse effects (Mayne 2003). The human diet contains an array of natural antioxidants that may contribute to the endogenous antioxidant defence system. Antioxidant vitamins, carotenoids, and especially the phenolics found in fruits and vegetables, have been proposed as the major bioactive compounds providing the health benefits associated with diets rich in plant-foods (Lampe 1999; Prior 2003). For this reason, several international organizations have recommended increasing the consumption of fruits and vegetables to five or more daily servings, in order to provide a desirable intake of antioxidants and to improve human health (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997).

Since the prevention of chronic diseases is a more effective strategy than their treatment, reducing the risk of diseases such as cardiovascular disease and cancer is a subject of great interest for doctors, scientists in general, consumers and the food industry (Liu 2003). For this reason, many functional foods are nowadays aimed at boosting intakes of antioxidants in order to reduce the risk of chronic disease linked to oxidative stress. Functional foods refer to foodstuffs or ingredients that improve overall health and/or reduce the risk of disease, and are food products to be taken as part of the usual in order to have beneficial effects that go beyond what are known as traditional nutritional effects (Rafter 2002; Roberfroid 2002).

Among the most common dietary sources of natural antioxidants, grapes and berries are rich in phenolic compounds, particularly flavonoids, which are valued for their role in anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial and antioxidant activities (Robards et al., 1999; Moure et al., 2001). Furthermore, most of these compounds remain present in berry and fruit products (Heinonen et al., 1998a; Häkkinen et al., 2000; Zafrilla et al., 2001). In this context, the use of these fruits as ingredients could be a feasible strategy for the development of phenolic-rich functional foods providing potential nutritional benefits. However, important to the acceptance of health claims is the necessity to generate enough scientific evidence to support the efficacy of the claim. Consequently, any effects have to be scientifically demonstrated with human intervention studies (Roberfroid, 2002; Aggett et al., 2005), showing the beneficial effects of this functional product to human health.

1.1. PLANNING OF THE THESIS AND OBJECTIVES

The present research has focused on the evaluation of the antioxidant effectiveness of products made from grapes, cherries and berries. For that purpose, both *in vitro* and *in vivo* studies were carried out in order to generate scientific evidence supporting their potential health benefits.

Description of the test products

The test products were a jellified dessert and a juice (see Figure in page 2) prepared by the Research and Development Department of Hero Spain S.A. Both products were formulated and designed with the objective of yielding a high content of phenolic compounds and to reach per serving an average antioxidant capacity of at least 5 servings of fruits and vegetables. In both products, the major ingredient was water, which was mixed with commercially available concentrated juices of grape (26%), cherry (2%), raspberry (1%), blackberry (0.6%) and blackcurrant (0.6%).

Experimental design

The present thesis work consists of 6 experiences, each one corresponding to a published work, a paper on review process or a manuscript to be submitted to a journal. Our experimental approach can be briefly summarized as follows:

1. Assessment of the nutritional and antioxidant properties of the products.

Proximate composition, total phenolic, the main groups of phenolic, vitamin C and total antioxidant activity were evaluated in both products. In addition, in the case of the dessert, a one-year storage trial was carried out involving different temperatures (8, 21 and 30 ºC) to assess the stability of the antioxidant properties of the dessert during storage (Study 1).

2. Evaluation of the antioxidant effectiveness in vitro in cell culture assays.

For the *in vitro* cell culture assays, the test product in a juice format was used to facilitate sample preparation. The human hepatoma cell line HepG2 and the human myeloid leukaemia cell line U937 were used. By using the HepG2 cell line (Study 2), we first evaluated the antiproliferative activity of the phenolic-rich juice. Once the juice doses capable of affecting cell growth were characterized, we tested the ability of sub-toxic concentrations of the juice to afford protection against induced oxidative stress. For that, juice pre-treated cells were challenged with either *tert*-butyl hydroperoxide or hydrogen peroxide. After the treatments, cell viability, lipid peroxidation and the activity of glutathione-related enzymes were evaluated. Similarly, by using the U937 cell line (Study 3) we evaluated the ability of the

juice to protect cells from *tert*-butyl hydroperoxide-induced cell death, DNA damage, intracellular reactive oxygen species generation, and mitochondrial permeability transition pore opening.

3. Evaluation of the antioxidant activity in vivo in human intervention studies.

In the present research, we carried out two human intervention studies with the aim of evaluating the effect of the intake of the test products on the individual's total antioxidant status and on certain biomarkers of oxidative damage.

The first study was carried out in a group of institutionalized elderly people and involved the intake of the test product in the dessert format. As part of the study, we evaluated the antioxidant and nutritional status of the elderly (Study 4), in order to estimate the adequacy of their diet, and any relationship between the intake of antioxidants from the diet and the plasma/serum antioxidant levels and total antioxidant capacity. For the intervention study (Study 5), subjects consumed daily 200 g of the jellified dessert for up to 2 weeks, and the total antioxidant capacity, plasma/serum levels of antioxidants, the susceptibility of low-density lipoproteins to oxidation and the peripheral lymphocyte DNA damage were evaluated at the beginning and end of the study.

The second human intervention study (Study 6) was carried out in a group of healthy subjects who consumed a single oral dose of the test product in a juice format. The aim of this study was to evaluate the short-term effect of the intake of the phenolic-rich juice on the total antioxidant status and biomarkers of oxidative damage, and to give evidence of the absorption and excretion of phenolic compounds. After a 48-hours washout period, fasting subjects consumed 400 mL of the juice and blood and urine samples were taken. Serum total antioxidant capacity, uric acid, vitamin C, lipid-bound phenolic compounds, lipid peroxidation products and protein carbonyl content were analyzed before and 1, 2, 4 and 6 hours post-intake. Urinary total phenolic compounds and total antioxidant capacity were analyzed before and 1, 2, 4, 6 and 24 hours post-intake, as indicators of urinary excretion of antioxidants from the juice.

Objectives

1. To assess the proximate composition and antioxidant properties of experimental products made from grapes, cherries and berries, and rich in antioxidant phenolic compounds.

2. To study the ability of a phenolic-rich juice to modulate cell proliferation and the activity of the cellular endogenous antioxidant defence system, in cultured human cells.

3. To evaluate the effect of the consumption of the experimental products on the total antioxidant status and biomarkers of oxidative damage in human subjects.

2

Revisión Bibliográfica

Review

2.1. EL ESTRES OXIDATIVO EN LA SALUD HUMANA

El oxígeno es esencial para bs organismos vivos. Sin embargo, la generación de especies reactivas del oxígeno (ROS) y radicales libres (RL) es inevitable en el metabolismo aeróbico. Estas especies oxidantes provocan daños acumulativos en moléculas fundamentales para el funcionamiento del organismo, tales como proteínas, lípidos y ADN. No obstante, el organismo tiene sus propios mecanismos de defensa para hacer frente a la acción de las especies oxidantes. En determinadas situaciones las defensas antioxidantes pueden verse desbordadas por la excesiva generación de ROS. Este desequilibrio entre especies oxidantes y antioxidantes se conoce como estrés oxidativo, el cual está asociado a numerosas enfermedades y al proceso normal de envejecimiento (Lee et al., 2004).

La dieta juega un papel importante en la prevención de enfermedades relacionadas con el estrés oxidativo, fundamentalmente a través del aporte de compuestos bioactivos de origen vegetal. Entre ellos, las vitaminas hidrosolubles y liposolubles, carotenoides y una gran variedad de compuestos fenólicos, cuya actividad antioxidante y potenciales efectos beneficiosos están siendo ampliamente investigados en los últimos años (Lampe 1999; Prior 2003). Así, las evidencias epidemiológicas que asocian el consumo de vegetales y frutas con una menor incidencia de enfermedades crónicas, junto con la mayor preocupación de los consumidores por mantener un estado de salud adecuado, está llevando a las industrias alimentarias a diseñar alimentos funcionales que supongan un aporte extra de estos antioxidantes naturales.

2.1.1. Especies oxidantes. Especies reactivas del oxígeno y radicales libres.

Una especie oxidante es aquella capaz de aceptar electrones de modo que va a generar un desequilibrio electrónico en las moléculas vecinas. El término antioxidante hace referencia a cualquier sustancia que, estando presente a una concentración más baja comparada con la de un sustrato oxidable, es capaz de retrasar o prevenir la oxidación de dicho sustrato. Los radicales libres se definen como especies químicas que poseen uno o más electrones desapareados, lo cual las hace altamente inestables y reactivas. Para estabilizarse reaccionarán rápidamente con moléculas adyacentes mediante reacciones de oxido-reducción. El termino especies reactivas del oxígeno **(Tabla 1)** es un término colectivo que incluye radicales libres y ciertas especies no radicales que son oxidantes y/o se convierten fácilmente en radicales libres, como por ejemplo HClO, HBrO, O_3, $ONOO^-$, $^1O_2$, o H_2O_2 (Halliwell y Whiteman 2004).

Tabla 1. Nomenclatura de las principales especies reactivas del oxígeno (ROS)[1]

Radicales		**No radicales**	
Hidroxilo	$^{\bullet}OH$	Peróxidos orgánicos	ROOH
Alcoxilo	$RO^{\bullet}$	Oxígeno singlete	$^1O_2$
Hidroperoxilo	$HOO^{\bullet}$	Peróxido de hidrógeno	H_2O_2
Superóxido	$O_2^{\bullet-}$	Ácido hipocloroso	HClO
Peroxilo	$ROO^{\bullet}$	Ácido nitroso	HNO_2
Óxido nítrico	$NO^{\bullet}$	Catión nitrilo	NO_2^+
Dióxido de nitrógeno	$NO_2^{\bullet}$	Peroxinitrito	$ONOO^-$
		Ácido peroxinitroso	ONOOH
		Alquil peroxinitritos	ROONO
		Ozono	O_3
		Ácido hipobromoso	HBrO

[1]Modificada de Halliwell y Whiteman (2004)

2.1.2. Generación *in vivo* de especies reactivas y mecanismos de defensa antioxidante.

Aunque ciertas condiciones ambientales favorecen la formación de especies reactivas y radicales libres, los procesos fisiológicos normales del organismo generan *per se* cierta tasa de estas sustancias oxidantes (Beckman y Ames 1998; Abuja y Albertini 2001; Fang et al., 2002; Lee et al., 2004). Las principales fuentes endógenas de especies oxidantes y radicales libres en el organismo son:

- Generación de especies reactivas durante la respiración mitocondrial. En la formación de ATP a través del metabolismo oxidativo y transporte electrónico mitocondrial el O_2 se reduce normalmente hasta agua siguiendo la vía tetravalente. Así, el O_2 se reduce en 4 etapas en cada una de las cuales se transfiere un electrón. Sin embargo, el transporte electrónico mitocondrial es imperfecto y la reducción monoelectrónica del oxígeno genera $O_2^{\bullet-}$. La dismutación espontánea y enzimática del $O_2^{\bullet-}$ da lugar a la formación de H_2O_2, y tanto el $O_2^{\bullet-}$ como el H_2O_2, pueden generar radicales $^{\bullet}OH$ a través de reacciones de Fenton catalizadas por metales de transición. De este modo, se estima que en

condiciones fisiológicas normales entre el 1 y el 3% del oxígeno consumido por el organismo no llega a formar agua y acaba generando radicales libres y ROS.

- Reacciones en cadena de peroxidación lipídica. Los radicales libres y otras especies reactivas son capaces de extraer un átomo de hidrógeno de un ácido graso saturado generando un radical lipídico ($R^{\bullet}$), el cual puede reaccionar con O_2 dando lugar a un radical peroxilo ($ROO^{\bullet}$). Los radicales peroxilo contribuyen a la propagación de las reacciones en cadena de peroxidación lipídica sustrayendo átomos de hidrógeno de otros ácidos grasos insaturados cercanos, generando así nuevos radicales lipídicos ($R^{\bullet}$) y hidroperóxidos lipídicos (ROOH). Estos últimos se descomponen en radicales alcoxilo ($RO^{\bullet}$) y peroxilo ($ROO^{\bullet}$) contribuyendo a la propagación del daño oxidativo. Esta descomposición se ve facilitada por la exposición a luz ultravioleta o por la presencia de iones metálicos, a través de reacciones de Fenton. Así, metales de transición tales como el hierro y el cobre son capaces de acelerar la iniciación y propagación del proceso de peroxidación lipídica por el siguiente mecanismo (Lee et al., 2004):

$$RH + Metal^{n+} \longrightarrow R^{\bullet} + Metal^{(n-1)+}$$

Los metales pueden descomponer los hidroperóxidos lipídicos generando radicales peroxilo y alcoxilo, acelerando las reacciones en cadena.

$$ROOH + Fe^{3+} \text{ o } Cu^{2+} \longrightarrow ROO^{\bullet} + H^{+} + Fe^{2+} \text{ o } Cu^{+}$$

$$ROOH + Fe^{2+} \text{ o } Cu^{+} \longrightarrow RO^{\bullet} + OH^{-} + Fe^{3+} \text{ o } Cu^{2+}$$

Los metales están implicados en la generación del oxígeno singlete, pudiendo además reaccionar con peróxido de hidrógeno para generar radicales hidroxilo.

$$Fe^{2+} + 2O_2 \longrightarrow O_2^{\bullet -} + {}^1O_2 + Fe^{3+}$$

$$Metal^{n+} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^{-} + Metal^{(n-1)+}$$

Existen además estímulos exógenos que incrementan la generación de especies oxidantes. Entre ellos cabe destacar diversos tipos de radiación, contaminantes ambientales, la actividad física intensa, la metabolización de fármacos, el humo del tabaco,

la acción de células del sistema inmunológico, o dietas deficientes en antioxidantes (Beckman y Ames 1998).

Es evidente que los procesos que generan especies reactivas y radicales libres *in vivo* son muy diversos. Para contrarrestar los efectos nocivos de las especies reactivas el organismo posee sus propios mecanismos de defensa antioxidante, integrados por sistemas enzimáticos y no enzimáticos, los cuales quedan recogidos en la **Tabla 2**.

Tabla 2: Principales sistemas de defensa antioxidante del organismo[1].

SISTEMA	**FUNCIÓN**
Enzimas	
Superóxido dismutasa	Eliminación de radical superóxido
Catalasa	Eliminación de hidroperóxidos (e.j. H_2O_2)
Glutatión peroxidasa (GPx)	Eliminación de hidroperóxidos
Glutatión reductasa (GRed)	Redución de glutatión oxidado
Glutatión-*s*-transferasa (GST)	Eliminación de peróxidos lipídicos
Metionina sulfóxido reductasa	Reparación de residuos oxidados de metionina
Peroxidasa	Descomposición de peróxido de hidrógeno y peróxidos lipídicos
Antioxidantes del plasma/suero	
Ácido úrico	Captador de oxígeno singlete y radicales libres
Albúmina	Actividad peroxidasa en presencia de GSH
Bilirrubina	Captación de radicales peroxilo
Glutatión reducido (GSH)	Sustrato para la acción de los enzimas GPx y GST y captador de radicales libres
Ubiquinol (Coenzima Q)	Captador de radicales libres
Antioxidantes de la dieta	
Ácido ascórbico	Reacción con superóxido, oxígeno singlete y radical peroxilo. Regeneración de tocoferoles
Tocoferoles	Protección de membranas lipídicas. Bloqueo de la cadena de reacciones de peroxidación.
Carotenoides	Desactivación del oxigeno singlete. Bloqueo de la cadena de reacciones de peroxidación
Compuestos fenólicos	Captación de radicales libres y actividad quelante de metales

[1]Basado en Beckman y Ames (1998), Fang et al. (2002) y Lee et al. (2004)

2.1.3. Estrés oxidativo y enfermedad.

El término estrés oxidativo hace referencia a un desequilibrio entre la generación de especies oxidantes y los sistemas de defensa antioxidante de un organismo. Este desequilibrio en favor de los oxidantes conduce a un potencial daño oxidativo sobre biomoléculas. Según Halliwell y Whiteman (2004), el estrés oxidativo puede originarse por dos motivos fundamentales:

I. Disminución de los niveles de antioxidantes debido a mutaciones que afectan la actividad de los enzimas antioxidantes, o a toxinas que causan depleción de las defensas antioxidantes. Por ejemplo, muchos xenobióticos son metabolizados mediante su conjugación con GSH, así, en altas dosis pueden disminuir los niveles de GSH y causar estrés oxidativo incluso si el xenobiótico en si mismo no es un generador de especies reactivas. Dietas inadecuadas que aportan niveles bajos de minerales (Fe, Zn, Se, Mg, Cu) y/o antioxidantes pueden causar también estrés oxidativo.

II. Incremento en la producción de especies reactivas a causa de la exposición a elevados niveles de O_2 u otras toxinas que en si mismas son especies reactivas (e.j. $NO_2^{\bullet}$) o son metabolizadas vía generación de especies reactivas (e.j. paraquat), o bien debido a la activación excesiva de los mecanismos naturales que generan especies reactivas, como por ejemplo la activación inapropiada de fagocitos en la inflamación crónica.

Debido a la acción oxidante de las especies reactivas sobre los lípidos de membrana, las proteínas celulares y los ácidos nucleicos (ADN, ARN), las especies reactivas han sido asociadas con numerosas enfermedades crónicas y con el proceso de envejecimiento **(Tabla 3)**, entre las que se encuentran dos de las mayores causas de mortalidad en las sociedades occidentales; el cáncer y las enfermedades cardiovasculares (Lee et al., 2004). El principal efecto de la oxidación de lípidos de membrana, a través de las reacciones en cadena de peroxidación lipídica, es la pérdida de fluidez de la membrana. Esto altera sus propiedades y funcionalidad, e incluso puede liberar proteínas ligadas a la membrana celular (Beckman y Ames 1998). Además, productos de la peroxidación lipídica (e.j. malondialdehido, 4-hidroxinonenal) son capaces de reaccionar con proteínas, fosfolípidos y ácidos nucleicos, causando así modificaciones estructurales en estas moléculas (Lee et al., 2004). En relación con las patologías

asociadas con el estrés oxidativo, la oxidación de las lipoproteínas plasmáticas de baja densidad (LDL) ha sido implicada en el desarrollo de la aterosclerosis (Abuja y Albertini 2001). El daño oxidativo a proteínas incluye la oxidación de grupos sulfhidrilo, reacciones de oxidación catalizadas por metales que inducen uniones entre residuos de aminoácidos en las cercanías de los sitios de unión con metales, reacción con aldehídos, uniones cruzadas entre proteínas y fragmentación de péptidos (Beckman y Ames 1998). Estas lesiones de las proteínas son importantes *in vivo* porque introducen modificaciones que pueden afectar a la función de receptores, enzimas, proteínas transportadoras, e incluso generar nuevos antígenos capaces de desencadenar la respuesta inmune. Además, las modificaciones proteicas podrían contribuir al daño secundario a otras biomoléculas, como por ejemplo inactivando los enzimas reparadores de ADN o alterando el funcionamiento de las ADN polimerasas durante la replicación del ADN (Halliwell y Whiteman 2004). Los ácidos nucleicos son susceptibles al daño oxidativo, el cual incluye modificaciones en las bases nitrogenadas, la formación de aductos entre bases y azúcares, uniones entre timina y tirosina, roturas de la hebra de ADN y enlaces cruzados con otras moléculas (Beckman y Ames 1998). Por ejemplo, los radicales hidroxilo oxidan los nucleósidos guanosina y timina a 8-hidroxi-2-desoxiguanosina y timinglicol, respectivamente, introduciendo cambios en el ADN. Si el estrés oxidativo es excesivo y los sistemas de reparación del ADN se pueden ver superados, estas modificaciones pueden conducir a la mutagénesis y carcinogénesis (Lee et al., 2004).

Tabla 3. Condiciones clínicas que implican especies reactivas y radicales libres[1]

TEJIDO/SISTEMA/ÓRGANO	**PATOLOGÍA**
Tracto gastrointestinal	Hepatitis, lesiones hepáticas
Ojos	Cataratas, daños en la retina
Piel	Dermatitis, pigmentos del envejecimiento
Sistema circulatorio	Aterosclerosis, ataques al corazón
Sistema respiratorio	Asma, hiperoxia
Sistema nervioso	Ictus, Parkinson, Alzheimer, demencia senil
Sistema endocrino	Diabetes, pancreatitis
Dientes	Periodontitis
Articulaciones	Artritis
Fallo multiorgánico	Cáncer

[1]Según Beckman y Ames (1998), Meydani (2000), Lee et al. (2004)

2.1.4. Envejecimiento y estrés oxidativo.

El envejecimiento se ha definido como la acumulación en el tiempo de diversos cambios que van en detrimento de la funcionalidad de las células y tejidos, resultando en un incremento del riesgo de enfermedad y muerte. El proceso de envejecimiento está influido por diversos factores, entre ellos el estilo de vida, condiciones ambientales y características genéticas. Las principales causas del envejecimiento han sido asociadas a la continua formación *in vivo* de especies reactivas y radicales libres, en lo que se conoce como teoría del envejecimiento por radicales libres. Esta teoría, que fue propuesta por Harman en 1956, considera que los radicales libres producen al azar un daño acumulativo en las macromoléculas biológicas, que conduce a una disminución de las funciones vitales y al envejecimiento. De este modo, a medida que se avanza en edad, se acumulan en el organismo productos de oxidación de lípidos, proteínas y ácidos nucleicos (Beckman y Ames 1998; Sastre et al., 2000; Harman 2003; Lee et al., 2004). El envejecimiento implica además un peor funcionamiento de los sistemas de defensa antioxidante del organismo. Esta situación hace a los individuos más vulnerables frente a condiciones patológicas asociadas al estrés oxidativo, haciendo necesario un aporte adecuado de sustancias antioxidantes para hacer frente a la agresión de los radicales libres (Meydani 2000).

2.1.5. Requerimientos nutricionales en ancianos.

El proceso de envejecimiento lleva asociada una serie de cambios fisiológicos y metabólicos que modifican los requerimientos nutricionales de las personas de edad avanzada (Ruíz-López et al., 2000; Russell 2000). Entre los cambios fisiológicos asociados al proceso de envejecimiento, destacan las disminuciones en la masa celular activa, consumo de oxígeno, tasa metabólica basal, agua corporal, función renal, masa ósea, actividad enzimática y hormonal, y de la respuesta inmune. Se producen además cambios en sistemas y órganos que conllevan una disminución en la ingesta de alimentos y absorción de determinados nutrientes. Entre ellos la disminución en la percepción por los sentidos, puede conducir a un menor consumo de alimentos a causa de una menor sensibilidad al olor y al sabor. Hay pérdidas en el número de papilas gustativas y en la percepción de los sabores dulce y salado. Además, los problemas dentales que dificultan la masticación y la disminución en la visión hacen la comida menos placentera, lo que contribuye a la disminución del apetito y aparición de anorexia (Ruíz-López et al., 2000).

Si bien la función gastrointestinal se mantiene en cuanto a la digestión y absorción de macronutrientes, la capacidad de digestión y absorción de micronutrientes si se ve

comprometida, particularmente en los casos del ácido fólico, vitamina B_{12}, β-caroteno y de los minerales calcio y hierro (Russell 2001). El principal cambio en la fisiología gastrointestinal que afecta la biodisponibilidad de nutrientes es la gastritis atrófica, un trastorno frecuente en la edad adulta, que se caracteriza fundamentalmente por una disminución en la secreción gástrica (hipoclorhidria) que conduce a una mayor supervivencia bacteriana en el estómago y la parte proximal del intestino delgado (Russell 2000). Además, la secreción del factor intrínseco necesario para la absorción de vitamina B_{12} disminuye (Russell 2001). Así, los niveles reducidos de ácido dificultan la absorción de hierro férrico, calcio, ácido fólico, vitamina B_{12} y β-caroteno, que dependen en gran medida del pH. Aunque el pH parece ser un factor determinante, la malabsorción de calcio en ancianos se relaciona con cambios en el metabolismo de la vitamina D. Entre ellos; menor síntesis cutánea de vitamina D, menor absorción de vitamina D de la dieta, disminución de los receptores intestinales de vitamina D, y una menor conversión de 25-hidroxicalciferol a la forma activa 1,25-dihidroxicalciferol (Russell 2001). Por esta razón, los requerimientos de estos nutrientes están aumentados en ancianos (Ruíz-López et al., 2000) e incluso algunos autores recomiendan el uso de suplementos de calcio, vitamina D y vitamina B_{12} (Russell et al., 1999).

En cuanto a los requerimientos de vitaminas antioxidantes, merecen especial atención los de vitamina A. La ingesta diaria recomendada (RDA) para esta vitamina se considera adecuada y no es recomendable incrementarla ya que en los ancianos, la depuración de vitamina A es menos eficaz y puede dar problemas de intoxicación cuando se toma en forma de suplementos (Ruíz-López et al., 2000; Russell 2001). Por el contrario, en el caso de la vitamina E se ha recomendado el consumo de 5-8 raciones de frutas y vegetales junto a un suplemento de 200 UI/día en ancianos, con el fin de mejorar el estado inmunitario general y reducir el riesgo de enfermedad cardiovascular (Meydani 2000). En cuanto a la ingesta de vitamina C, no se ha descrito que una suplementación adicional tenga efectos beneficiosos sobre el envejecimiento, por este motivo la RDA habitual se considera adecuada (Ruíz-López et al., 2000).

Con el objetivo de obtener un aporte adecuado de fibra y antioxidantes naturales es recomendable, no sólo para la población anciana sino también para la población general, el aumentar la ingesta de frutas y verduras hasta al menos 5 raciones diarias (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997). Se recomienda además que la elección de estos alimentos se haga entre aquellas más coloreadas, que aportarán cantidades adecuadas de ácido fólico, vitaminas antioxidantes, provitaminas (carotenoides), y diversos compuestos bioactivos con actividad

antioxidante, fundamentalmente compuestos fenólicos (Russell et al., 1999). Sin embargo, incrementar la ingesta de frutas y verduras puede ser dificultoso en el caso de ancianos que presenten problemas de masticación. En este caso, sería más recomendable suministrar estos alimentos en el formato de zumos a través de los cuales, a la vez que sustancias antioxidantes, se aportarían líquidos al organismo. En la edad adulta, son característicos los cambios en la función renal que afectan al balance ingesta/excreción de nitrógeno, agua y electrolitos. Hay una menor sensación de sed y una disminución del agua corporal. Por ello es recomendable incrementar la ingesta de líquidos hasta aproximadamente 2 litros diarios (Russell et al., 1999).

Si en cualquier etapa de la vida la nutrición es un factor importante para mantener el estado de salud, los ancianos precisan una mayor atención por ser uno de los colectivos más vulnerables. El estudio del estado nutricional de este grupo de población aporta valiosa información sobre sus hábitos de vida, principales carencias y necesidades nutricionales específicas, permitiendo la planificación de estrategias preventivas encaminadas a mejorar su estado general de salud y bienestar.

2.2. COMPUESTOS FENÓLICOS Y DISEÑO DE ALIMENTOS FUNCIONALES.

Los compuestos fenólicos constituyen una de las principales clases de metabolitos secundarios de las plantas, donde desempeñan diversas funciones fisiológicas. Entre otras, intervienen en el crecimiento y reproducción de las plantas y en procesos defensivos frente a patógenos, predadores o radiación ultravioleta. Los compuestos fenólicos presentan un anillo benceno hidroxilado como elemento común en sus estructuras moleculares, las cuales pueden incluir grupos funcionales como ésteres, metil ésteres, glicósidos, etc. (Martínez-Valverde et al., 2000; Duthie y Crozier 2000). Aunque existe una gran variedad de compuestos fenólicos en las plantas (se conocen más de 8000), la mayor parte de ellos tienen como origen metabólico común la ruta del ácido siquímico y el metabolismo de los fenilpropanoides (Robards et al., 1999). Los fenilpropanoides simples poseen un esqueleto básico de 9 carbonos (C_6-C_3) y derivan de los aminoácidos fenilalanina y tirosina producidos en la ruta del ácido siquímico. Las distintas familias de compuestos fenólicos se caracterizan principalmente por el número de átomos de carbono de su esqueleto básico molecular.

- Ácidos cinámicos (C_6-C_3)
- Ácidos benzoicos (C_6-C_1 o C_6-C_2)
- Flavonoides (C_6-C_3-C_6)
- Proantocianidinas o taninos condensados ((C_6-C_3-C_6)$_n$)
- Estilbenos (C_6-C_2-C_6)
- Cumarinas (C_6-C_3)
- Lignanos (C_6-C_3-C_3-C_6)
- Ligninas ((C_6-C_3)$_n$)

Así, los compuestos fenólicos comprenden desde moléculas simples como los ácidos benzoicos hasta polímeros complejos como las ligninas. Dentro de cada familia, el número de compuestos fenólicos existentes será más o menos variado. Por ejemplo, se conocen más 4000 flavonoides diferentes, distribuidos en varias subfamilias. Los compuestos fenólicos están presentes en todo el reino vegetal y sus cantidades y tipos varían en función de la especie vegetal y variedad, parte de la planta considerada (frutos, semillas, hojas, tallos, etc.), horas de exposición solar, grado de madurez, condiciones de cultivo, procesado y almacenamiento, etc. En los alimentos, los compuestos fenólicos habitualmente se presentan conjugados con azúcares como la glucosa, galactosa, arabinosa, ramnosa, xilosa, o los ácidos glucurónico y galacturónico. También pueden unirse a ácidos carboxílicos, ácidos orgánicos, aminas y lípidos (Duthie et al., 2003).

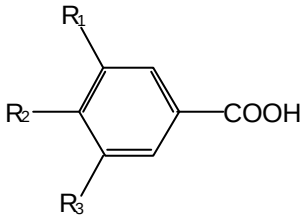
En la actualidad este grupo de compuestos de origen vegetal presenta gran interés nutricional por su contribución al mantenimiento de la salud humana. De hecho, desde 1990, varias organizaciones internacionales en el ámbito de la nutrición recomiendan un consumo diario de al menos 5 raciones de frutas y/o verduras para asegurar una adecuada ingesta de antioxidantes y prevenir enfermedades relacionadas con el estrés oxidativo (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997). Así, muchos de los efectos beneficiosas asociados al consumo de alimentos de origen vegetal se atribuyen en gran medida a los compuestos fenólicos (Manach et al., 2004). La actividad antioxidante de los compuestos fenólicos se atribuye a su facilidad para ceder átomos de hidrógeno de un grupo hidroxilo aromático a un radical libre y a la posibilidad de deslocalización de cargas en el sistema de dobles enlaces del anillo aromático (Duthie et al., 2003). Los compuestos fenólicos poseen además una estructura química ideal para captar iones metálicos (principalmente hierro y cobre) y por tanto para inhibir la formación de radicales libres a través de reacciones de Fenton (Rice-Evans et al., 1997). Además de las propiedades antioxidantes, a estos compuestos se les atribuyen actividades biológicas beneficiosas para salud. Entre estas destacan sus efectos vasodilatadores, anticarcinogénicos, antiinflamatorios, bactericidas, estimuladores de la respuesta inmune, antialérgicos, antivirales, efectos estrogénicos, o inhibidores de enzimas prooxidantes, como cicloxigenasa, lipooxigenasa y xantina oxidasa (Cao et al., 1997).

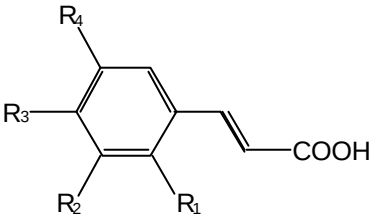
En este capítulo se describen brevemente los principales grupos de compuestos fenólicos presentes en las frutas rojas y bayas, que son el objeto del presente estudio. Se tratan además, aspectos relevantes de los compuestos fenólicos como su biodisponibilidad, metabolismo y actividad antioxidante, así como el posible empleo de las frutas rojas como ingredientes en el desarrollo de alimentos funcionales con alta actividad antioxidante y beneficios potenciales para la salud humana.

2.2.1 Las frutas rojas como fuentes de compuestos fenólicos

Las frutas, incluidas las bayas, constituyen una de las principales fuentes de compuestos fenólicos en la dieta. En ellas es frecuente encontrar derivados de los ácidos hidroxibenzoicos e hidroxicinámicos, antocianinas, flavonoles, catequinas, y taninos condensados e hidrolizables (Macheix et al., 1990). La mayoría de estos compuestos permanecen en productos elaborados a base de frutas y bayas (Heinonen et al., 1998a), como por ejemplo zumos, mermeladas, jaleas, gelatinas, etc. (Häkkinen et al., 2000; Zafrilla et al., 2001). A continuación se describen los principales grupos de compuestos fenólicos presentes en los alimentos vegetales.

Ácidos fenólicos. Los ácidos fenólicos **(Figura 1)** son abundantes en los alimentos. Los más frecuentes son el ácido cafeico, y en menor medida el ácido ferúlico, que se encuentra asociado a la fibra dietética mediante la formación de enlaces éster con la hemicelulosa. El ácido cafeico también se encuentra esterificado, principalmente con el ácido quínico, dando lugar al ácido clorogénico (ácido 5-cafeilquínico), que está presente en el café, y en muchas frutas y verduras (Scalbert y Williamson, 2000). Se pueden diferenciar dos grupos principales de ácidos fenólicos, los ácidos benzoicos y los ácidos cinámicos.





Ácidos benzoicos

Ácido	R_1	R_2	R_3
Gálico	OH	OH	OH
Protocatecuico	H	OH	OH
Vaníllico	H	OH	OCH_3
Siríngico	OCH_3	OH	OCH_3

Ácidos cinámicos

Ácido	R_1	R_2	R_3	R_4
Ferúlico	H	H	OH	OCH_3
p-cumárico	H	H	OH	H
Cafeico	H	H	OH	OH
Sinápico	H	OCH_3	OH	OCH_3

Figura 1. Estructura química de los principales ácidos fenólicos

Los ácidos benzoicos o derivados del ácido hidroxibenzoico, tienen una estructura básica C_6-C_1. Los principales son los ácidos gálico, salicílico, *p*-hidroxibenzoico, protocatécuico, vanílico y siríngico, estos cuatro últimos se consideran universales ya que forman parte

de las ligninas. Generalmente se presentan de forma conjugada en los vegetales, aunque pueden ser detectados en forma libre en algunas frutas o tras su liberación como consecuencia del procesado. El ácido gálico se puede encontrar conjugado como tal o como sus dímeros (ácido elágico), trímeros (ácido tergálico) o tetrámeros (ácido galágico), los dos últimos menos frecuentes. Los ácidos gálico y elágico son componentes esenciales de los taninos hidrolizables, como por ejemplo los elagitaninos de fresas, frambuesas y zarzamoras. Generalmente los contenidos en estos ácidos son bajos a excepción de las frutas rojas (Manach et al., 2004).

Los ácidos cinámicos o derivados del ácido hidroxicinámico, están ampliamente distribuidos como conjugados en materias vegetales, incluyendo muchos alimentos y bebidas. Entre ellas, las frutas rojas constituyen una fuente significativa de estos compuestos. Salvo en el caso de alimentos procesados, raramente se encuentran como ácidos libres y de forma predominante aparecen esterificados con ácido quínico, tartárico o glucosa. Los más comunes son los ácidos cafeico, ferúlico, sinápico y *p*-cumárico. Uno de los conjugados más frecuentes en frutas es el ácido clorogénico, que resulta de la esterificación de los ácidos cafeico y quínico. Así, el ácido cafeico, libre o esterificado, constituye el ácido fenólico más abundante en muchas frutas (Manach et al., 2004). Algunos contenidos en ácidos fenólicos de varias frutas rojas se muestran en la **Tabla 4**.

Tabla 4. Contenido en mg/Kg de ácidos fenólicos totales en frutas rojas.

Fruta	Ácidos benzoicos	Ácidos cinámicos
Zarzamora	80-270[1]	15[2]
Frambuesa	60-100[1]	3[2]
Cereza	2.5[2]	180-1150[1]
Grosella	40-130[1]	--
Fresa	20-90[1]	19-27
Arándano	--	2000-2200[1]

[1]Manach et al. (2004), [2]Heinonen et al. (1998b)

Taninos hidrolizables. Los taninos hidrolizables resultan de la esterificación de los ácidos gálico y elágico **(Figura 2)**. Se distinguen 2 grupos principales; los galotaninos, que son frecuentes en frutas como el mango, y los elagitaninos, característicos de frutas rojas como las fresas, frambuesas y zarzamoras (Clifford y Scalbert 2000).

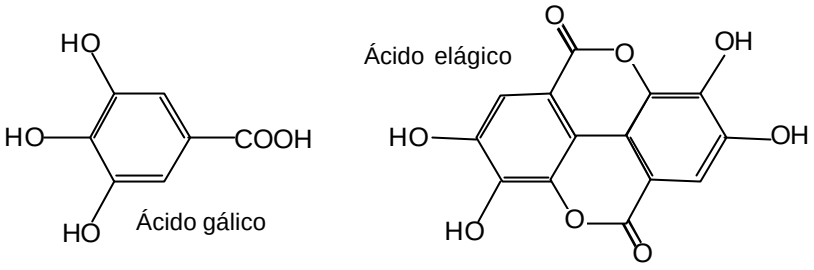


Figura 2. Estructura química de los ácidos gálico y elágico

Así, el zumo de frambuesa puede contener en torno a 70 mg/L de ácido elágico (Clifford y Scalbert 2000). En las zarzamoras, ácido elágico llega a constituir hasta el 88% de los compuestos fenólicos totales, con niveles comprendidos entre 207 y 244 mg/Kg de peso fresco (De Ancos et al., 2000). En frambuesas de 10 a 40 mg/Kg de peso fresco y en fresa 150 mg/Kg de peso fresco (Tomás-Barberán y Clifford 2000). El procesado incrementa el contenido en ácido elágico libre llegando en torno al doble del contenido presente en la fruta fresca. Así, la elaboración de mermelada de zarzamora incrementó los niveles de ácido elágico de 70 mg/Kg en la fruta fresca hasta los 160 mg/Kg en la mermelada. En el caso de la frambuesa el contenido inicial fue de 8 mg/Kg y tras el procesado llegó a 12 mg/Kg (Amakura et al., 2000).

Estilbenos. Los estilbenos tienen un esqueleto básico de 14 carbonos (C_6-C_2-C_6) y su distribución en alimentos vegetales no es muy amplia (Scalbert y Williamson 2000). Los estilbenos con mayor interés nutricional son el resveratrol (3, 5, 4'-trihidroxiestilbeno) y el piceido (resveratrol-3-O-β-D-glucósido), presentes en uvas y vinos **(Figura 3)**. El resveratrol se encuentra en uvas, zumos de uva y vinos, por lo que estos alimentos van a ser las principales fuentes de estilbenos en la dieta. El resveratrol muestra actividades antitumorales e inhibe reacciones que incrementan el riesgo de enfermedades coronarias (Manach et al., 2004). Al actuar como antioxidante es capaz de inhibir la oxidación de las LDL (Frankel et al., 1995). Un vino tinto puede contener 200 mg/L de resveratrol (Frankel

et al., 1995). Las uvas rojas de la variedad Napoleón, contienen 2.4 mg de estilbenos (resveratrol y piceido) por Kg de peso fresco (Cantos et al., 2000).

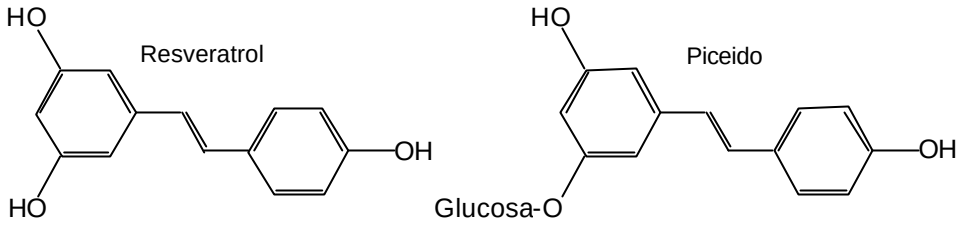
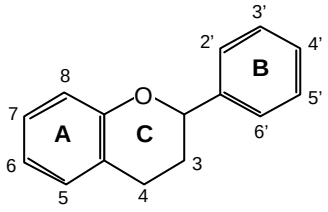


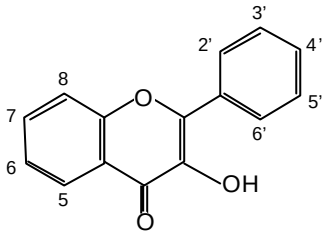
Figura 3. Estructura química de los estilbenos

Flavonoides. Los flavonoides constituyen el grupo de compuestos fenólicos más diverso y ampliamente distribuido en las plantas. Su estructura básica (flaván) consta de dos grupos fenilo (A y B) unidos por un puente de tres carbonos que forma un anillo heterocíclico oxigenado (anillo C) **(Figura 4)** (Manach et al., 2004). En función de los grados de oxidación e insaturación del anillo heterocíclico se pueden diferenciar varias clases de flavonoides y dentro de cada clase se pueden establecer diferencias en base a la naturaleza y número de los sustituyentes unidos a los anillos (Robards et al., 1999). Los flavonoides se encuentran a menudo hidroxilados en las posiciones 3, 5, 7, 3', 4' y 5'. La presencia o ausencia de un grupo hidroxilo unido a la posición 3 determina la subdivisión en las dos clases principales de flavonoides; los 3-hidroxiflavonoides (flavanoles, flavonoles, flavanonoles, flavan-3,4-dioles o leucoantocianidinas, antocianidinas, proantocianidinas o taninos condensados) y los flavonoides no hidroxilados en posición 3 (flavonas, isoflavonas, flavanonas).

La mayoría de los tejidos de la planta pueden sintetizar flavonoides, los cuales se presentan en forma de glicósidos solubles en agua en las hojas y frutas empleadas en la alimentación humana. Las agliconas de los flavonoles y flavonas no se encuentran en el vegetal fresco pero pueden presentarse como consecuencia del procesado. Los azúcares predominantemente se unen al núcleo del flavonoide mediante enlace β-glicosídico, preferentemente en posición 3, aunque las uniones se pueden producir en otras posiciones. Se han identificado más de 80 azúcares diferentes unidos a los flavonoides; monosacáridos, disacáridos, trisacáridos e incluso tetrasacáridos (Hollman y Arts 2000). Los azúcares más comunes son glucosa, galactosa, ramnosa, arabinosa, xilosa y ácido glucurónico (Manach et al., 2004).



Flaván



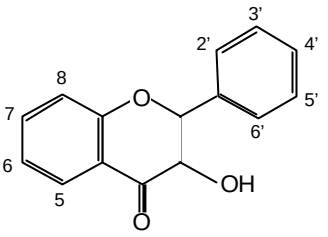
Flavonoles

Canferol 5=7=4'=OH

Quercetina 5=7=3'=4'=OH

Isoramnetina 5=7=4'=OH 3'=OCH$_3$

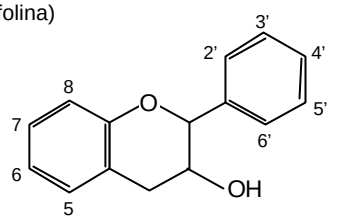
Miricetina 5=7=3'=4'=5'=OH



Flavanonoles

Dihidroquercetina 5=7=3'=4'=OH

(Taxifolina)

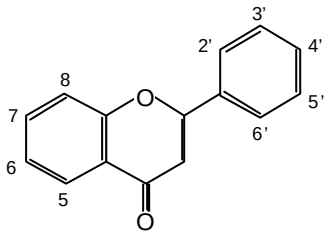


Flavanoles

Catequina 5=7=3'=4'=OH

Epicatequina 5=7=3'=4'=OH

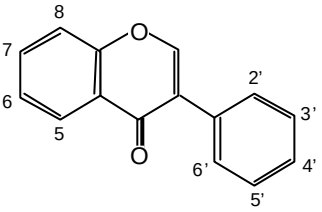
Epigalocatequina 5=7=3'=4'=5'=OH



Flavonas

Apigenina 5=7=4'=OH

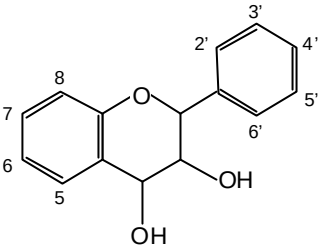
Luteolina 5=7=3'=4'=OH



Isoflavonas

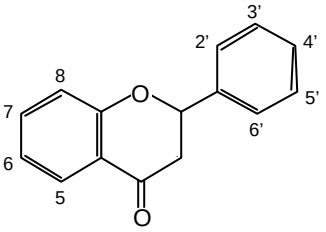
Daidzeina 7=4'=OH

Genisteina 5=7=4'=OH



Leucoantocianidinas

(Flaván-3,4- dioles)



Flavanonas

Naringenina 5=7=4'=OH

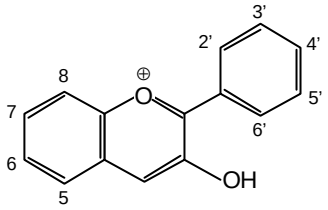
Hesperitina 5=7=3'=OH

Glicósidos de flavanonas

Prunina naringenina-7-glucósido

Naringina naringenina-7-neohesperidósido

Hesperidina hesperitina-7-rutinósido



Antocianidinas

Pelargonidina 5=7=4'=OH

Cianidina 5=7=3'=4'=OH

Peonidina 5=7=4'=OH 3'=OCH$_3$

Delfinidina 5=7=3'=4'=5'=OH

Petunidina 5=7=4'=5'=OH 3'=OCH$_3$

Malvidina 5=7=4'=OH 3'=5'=OCH$_3$

Figura 4. Estructura química de los flavonoides

Los principales flavonoles son; quercetina, canferol, miricetina e isoramnetina (Hollman y Arts 2000) y algunos de sus glicósidos son la rutina (quercetina-3-O-ramnosilglucósido) y la hiperina (quercetina-3-O-β-D-galactopiranósido) (Robards et al., 1999). Bebidas, frutas y verduras son las principales fuentes de estos flavonoides (Scalbert y Williamson 2000). Los mayores niveles de quercetina se encuentran en las cebollas (280-490 mg/Kg de porción comestible), en las cerezas los niveles están entre 10 y 15 mg/Kg y la grosella negra aporta 37 mg/Kg de quercetina y 1 mg/Kg de canferol. La grosella roja contiene de 8 a 13 mg/Kg de quercetina, las uvas rojas de 15 a 37 mg/Kg y las uvas blancas entre 2 y 12 mg/Kg. El contenido medio de canferol en uvas rojas y blancas es de 4.5 mg/Kg, las fresas contienen entre 6 y 8.6 mg/Kg de quercetina y de 5 a 12 mg/Kg de canferol. Entre los zumos, el de uva contiene 4.4 mg/L de quercetina y 6.2 de canferol y el de naranja 5.7 mg/L de quercetina. El vino tinto 8.3 mg/L de quercetina y 8 mg/L de canferol y el té negro 15-17 mg/L de quercetina, 14-16 mg/L de canferol y 3 mg/L de miricetina (Hollman y Arts 2000). Los flavonoles totales en extractos de uva estuvieron entre 1.4 y 33.5 mg/L (Meyer et al., 1997), en uva Napoleón (21.6 mg/Kg) (Cantos et al., 2000), en zarzamora (83-87 mg/Kg), arándanos (115-139 mg/Kg), frambuesa (19-20 mg/Kg), fresa (6-78 mg/Kg), cereza (10-23 mg/Kg) (Heinonen et al., 1998b) y en grosella negra (157 mg/Kg) (Häkkinen et al., 2000).

Los flavanoles predominantes son la catequina y su isómero epicatequina, la galocatequina y su isómero epigalocatequina, y los ésteres con ácido gálico en posición 3 como el epicatequín galato y el epigalocatequín galato. Los flavanoles glicosilados son poco frecuentes. Algunas fuentes de flavanoles en la dieta son el té, las frutas y las legumbres. Las uvas rojas, se ha determinado que aportan entre 18 y 21 mg de epicatequina por Kg de porción comestible (peso fresco) y menos de 10 mg/Kg de catequina, las cerezas hasta 15 mg/Kg de catequina y entre 4 y 152 mg/Kg de epicatequina. Un zumo de uva comercial, hasta 2 mg/L y 1 mg/L de catequina y epicatequina respectivamente. Los vinos tintos alcanzan contenidos de 208 mg/L de catequina y entre 15 y 88 mg/L de epicatequina (Hollman y Arts 2000). Los flavanoles totales en extractos de uva llegaron hasta los 10 mg/L (Meyer et al., 1997), en zarzamora estuvieron entre 96 y 108 mg/Kg de peso fresco, en arándanos entre 63 y 70 mg/Kg, en frambuesa variaron entre 470 y 480 mg/Kg, en fresas entre 126 y 184 mg/Kg y en cerezas el contenido fue de 26 mg/Kg (Heinonen et al., 1998b).

Las antocianidinas tienen la estructura básica del catión flavilio. Seis antocianidinas son importantes en los alimentos; cianidina, delfinidina, peonidina, pelargonidina, petunidina

y malvidina, contribuyendo a la coloración de numerosas frutas. En los vegetales frescos, generalmente se encuentran unidas a azúcares dando lugar a los antocianos o antocianinas, que son responsables de los colores azules, púrpuras, rojos y matices intermedios de estas coloraciones de frutas rojas como cerezas, ciruelas, fresas, frambuesas, zarzamoras, uvas y grosellas (Clifford 2000; Scalbert y Williamson 2000). Los azúcares más comunes son; glucosa, galactosa, ramnosa y arabinosa, normalmente unidos a la posición 3 o a las posiciones 3 y 5, generando diglicósidos (Clifford 2000). Los antocianos más comunes en las frutas son los 3-monoglucósidos de cianidina, delfinidina, peonidina, pelargonidina y petunidina, la cianidina-3-galactósido y la cianidina-3-arabinósido (Robards et al., 1999). También se encuentran disacáridos de glucosa (soforósidos), de ramnosa y glucosa (rutinósidos), de xilosa y glucosa (sambubiósidos) y trisacáridos (Clifford 2000). Además de las glicosilaciones, se pueden encontrar antocianos acilados (Robards et al., 1999) con ácidos cinámicos (cafeico, *p*-cumárico, ferúlico y sinápico) y ácidos alifáticos (acético, málico, malónico, oxálico o succínico) (Clifford 2000). El contenido en antocianos totales de algunas frutas y vino tinto se muestra en la **Tabla 5** (Clifford 2000).

Tabla 5: Contenido de antocianos totales de algunas frutas y vino tinto.

Fruta	Contenido (mg/kg o mg/L)
Zarzamora	1150
Cereza	20-4500
Grosella roja	1300-4000
Uva roja	3000-7500
Frambuesa roja	100-600
Frambuesa negra	1700-4277
Arándano	600-2000
Fresa	150-350
Vino tinto	240-320

2.2.2 Actividad antioxidante de los compuestos fenólicos

La actividad antioxidante de los compuestos fenólicos se ve determinada por su estructura química, por lo que existen grandes diferencias en la efectividad como antioxidantes entre los distintos grupos de compuestos. Los compuestos fenólicos pueden actuar como antioxidantes mediante dos mecanismos principales (Rice-Evans et al., 1997):

Como captadores de radicales libres. Los compuestos fenólicos pueden actuar como donantes de hidrógeno o electrones en reacciones de terminación que rompen el ciclo de generación de nuevos radicales libres, deteniendo las reacciones en cadena en las que están implicados los radicales libres. El radical fenoxilo generado es menos reactivo ya que se estabiliza por resonancia con los electrones π del anillo aromático. Así, las características estructurales que determinan la capacidad de los compuestos fenólicos para captar radicales son:

- La presencia de dos grupos hidroxilo en posición *orto* (3', 4') en el anillo B (e.j. quercetina, catequina).
- La presencia de dos grupos hidroxilo en posición *meta* (5, 7) en el anillo A (e.j. canferol).
- La presencia en el anillo del doble enlace entre los carbonos 2 y 3 y, junto con el grupo 4-ceto (e.j. quercetina). Estas estructuras son importantes para la deslocalización de electrones y estabilización del radical fenoxilo, siempre que además estén presentes los dos *orto*-hidroxilos en el anillo B.

Como quelantes de metales. Esta acción requiere la presencia de grupos hidroxilos cercanos en el anillo aromático. De este modo, los *o*-dihidroxifenoles son secuestradores efectivos de iones metálicos e inhiben la generación de radicales libres por la reacción de Fenton. Generalmente, los siguientes grupos funcionales se consideran importantes para la actividad quelante de metales (Kokhar y Apenten 2003):

- La presencia de grupos hidroxilo en posición *orto* (e.j. 3'-4' o 7-8).
- La presencia del grupo 4-ceto y grupos hidroxilo en posición 5 y/o 3 (e.j. quercetina).
- La presencia de un gran número de grupos hidroxilo (e.j. ácido tánico).

Además de las características estructurales anteriormente mencionadas, existen otros factores que afectan la actividad antioxidante de los compuestos fenólicos. Así, el número y posición de grupos hidroxilo, el grado de polimerización o la presencia de

azúcares unidos determinarán propiedades de los compuestos fenólicos tales como la solubilidad y la tendencia a ceder electrones o átomos de hidrógeno.

El grado de polimerización de los compuestos fenólicos tiene un marcado efecto sobre la actividad antioxidante. Así, los compuestos poliméricos son más potentes como antioxidantes que los monómeros. Por ejemplo, los taninos son más efectivos frente a los radicales peroxilo que los fenoles simples. La actividad para captar $O_2^{\bullet-}$ aumenta con el grado de polimerización de los flavanoles y los dímeros de ácido ferúlico inhiben la peroxidación lipídica en mayor extensión que los monómeros (Moure et al., 2001). La eficacia antioxidante de las teaflavinas aumenta cuando se unen con ácido gálico o sus polímeros (Rice-Evans et al., 1997). Compuestos fenólicos con un elevado número de grupos hidroxilo en sus estructuras moleculares muestran una mayor actividad antioxidante *in vitro*. Es el caso de las teaflavinas y catequinas del té (Rice-Evans et al., 1997). Además, en los flavonoles, los hidroxilos en las posiciones 2', 3' y 4' incrementan la estabilidad de los radicales permitiendo la deslocalización de electrones en los dobles enlaces del anillo benceno. La presencia de sustituyentes voluminosos en los anillos, que inducen la donación de electrones, aumenta la efectividad como antioxidantes de los compuestos fenólicos al disminuir la fuerza de los enlaces O-H. Por otro lado, el impedimento estérico generado por los sustituyentes en la región del radical, disminuye la velocidad de las reacciones de propagación en la que está implicado el propio radical fenoxilo, contribuyendo a su estabilización (Robards et al., 1999).

La actividad antioxidante de los compuestos fenólicos varía además en función de su solubilidad relativa en fase acuosa o lipofílica. Los flavonoides y los ácidos cinámicos poseen coeficientes de partición intermedios, que dependen en gran medida de su estructura química precisa y de los sustituyentes asociados (grupos hidroxilo, metoxilo, azúcares, etc.). Generalmente, los compuestos hidrofóbicos entran en las células más rápido que los hidrofílicos por procesos de difusión simple. Una vez en el organismo, los compuestos fenólicos más hidrofóbicos tendrán su destino en ambientes lipídicos y los más hidrofílicos quedarán en medios más acuosos (Parr y Bolwell 2000). Así, la unión de azúcares hace a los compuestos fenólicos más hidrosolubles pero disminuye su actividad antioxidante (Rice-evans et al., 1997). Por ello, los compuestos fenólicos con más afinidad por los ambientes lipídicos del organismo podrían tener una mayor relevancia en la prevención de enfermedades. De hecho, los compuestos fenólicos de caracter liposoluble y capaces de unirse a lípidos previenen la oxidación de las LDL *ex vivo* de forma directa y/o

mediante la preservación de otros antioxidantes liposolubles como α-tocoferol (Zhu et al., 2000).

2.2.3 Biodisponibilidad de compuestos fenólicos

Los efectos beneficiosos derivados del consumo de compuestos fenólicos dependen de la cantidad consumida y de su biodisponibilidad. La gran variedad estructural de los compuestos fenólicos, así como la influencia de factores genéticos, agronómicos, del procesado y almacenamiento sobre sus niveles en los alimentos, hace difícil estimar con exactitud la ingesta de compuestos fenólicos en la dieta (Duthie et al., 2003). Se estima que la ingesta aditiva de flavonoles, flavanonas, flavanoles e isoflavonas en las sociedades occidentales es de 100-150 mg/día. A estas cantidades habría que añadir una ingesta variable de ácidos hidroxicinámicos, antocianos y proantocianidinas, aportada principalmente por café, té, bayas y vino. Así, la ingesta total de compuestos fenólicos probablemente alcanza 1 g/día en personas que ingieran varias raciones de fruta y verdura al día (Manach et al., 2004).

Absorción y metabolismo

El metabolismo de los compuestos fenólicos sigue una ruta común que implica reacciones de conjugación y reconjugación (Scalbert y Williamson 2000; Karakaya 2004). La mayoría de compuestos fenólicos se encuentran en los alimentos en forma de ésteres, glicósidos o polímeros que no pueden ser absorbidos directamente en el intestino. Para ello deben ser previamente hidrolizados por enzimas intestinales o por la microflora del colon. La acción de las bacterias del colon implica la rotura de los anillos aromáticos de los compuestos fenólicos y la consiguiente producción de ácidos aromáticos simples, generalmente de menor biodisponibilidad y actividad. Durante el curso de la absorción, los compuestos fenólicos son conjugados en el intestino e hígado mediante reacciones de metilación, glucuronidación, sulfatación, o sus combinaciones. Estos conjugados son transportados en plasma a los distintos tejidos y órganos, principalmente unidos a albúmina. Su eliminación se produce mayoritariamente en orina y bilis. Los compuestos fenólicos que siguen la ruta biliar son secretados en el duodeno, donde son sometidos a la acción de enzimas bacterianas, especialmente la β-glucuronidasa, tras la cual son reabsorbidos. Este reciclaje enterohepático prolonga la presencia de los compuestos fenólicos en el organismo **(Figura 5)**.

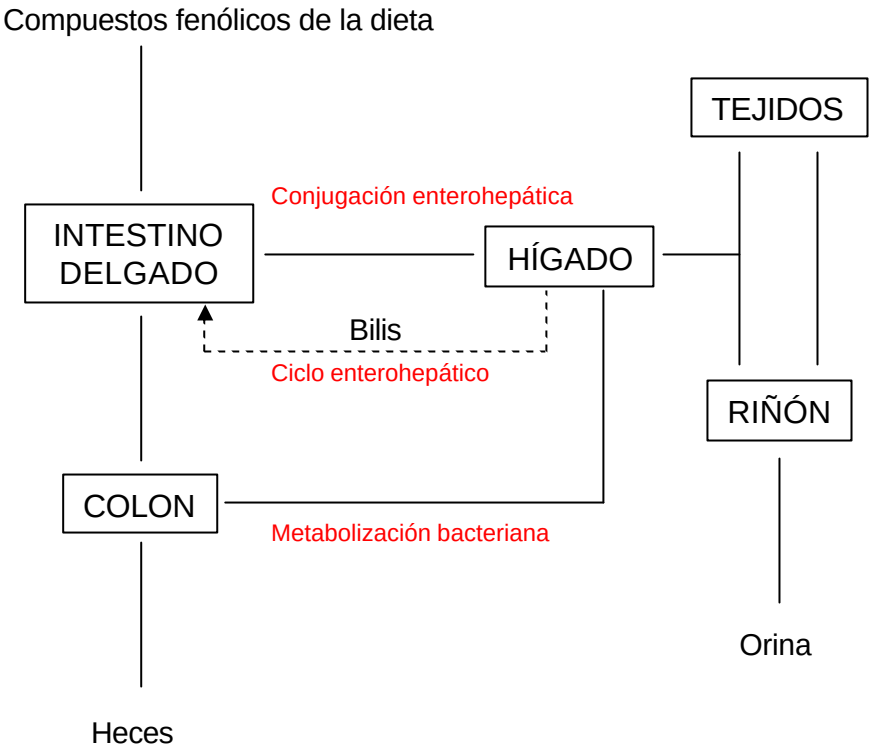


Figura 5. Posibles rutas de los compuestos fenólicos de la dieta (Scalbert y Williamson 2000)

La absorción y metabolización de compuestos fenólicos está influida por factores tales como la liposolubilidad o hidrosolubilidad de la molécula, la unión a azúcares o ácidos orgánicos, o el grado de polimerización. Los coeficientes de partición parecen ejercer un efecto importante en la absorción de agliconas, glicósidos y compuestos fenólicos unidos a ácidos orgánicos. Así, las agliconas hidrofílicas no son capaces de atravesar la pared intestinal por difusión pasiva y por tanto su absorción depende de transportadores de membrana. Por ejemplo, se ha identificado en ratas un mecanismo de transporte saturable dependiente de sodio para los ácidos ferúlico y cinámico (Manach et al., 2004). El coeficiente de partición influirá también en el caso de compuestos fenólicos hidrofóbicos glicosilados, unidos a ácidos orgánicos o con sustituyentes esterificados. Compuestos hidrofílicos de similares características estructurales serán degradados por esterasas de la microflora del colon, como paso previo a su absorción (Karakaya 2004).

La glicosilación influye claramente en la absorción de compuestos fenólicos. La absorción gástrica de flavonoides como quercetina y daidzeína ha sido observada en estudios en ratas, pero no así la de sus glicósidos (Manach et al., 2004). Las agliconas y los polifenoles unidos a glucosa, galactosa o xilosa son absorbidos en el intestino delgado tras

su deglicosilación por los enzimas β-glucosidasa y lactasa floricina hidrolasa (Karakaya 2004), mientras que los compuestos fenólicos unidos a ramnosa deben llegar al colon y ser hidrolizados por las ramnosidasas bacterianas antes de su absorción (Manach et al., 2004). Flavonoides acilados como la epicatequina y epigalocatequina son absorbidos sin deconjugación ni hidrólisis previa. Las agliconas de las isoflavonas son absorbidas en el estómago, mientras que sus glicósidos son absorbidos en el intestino (Karakaya 2004). Los antocianos, por contra, parecen constituir una excepción ya que las formas predominantes en sangre son sus glicósidos intactos. Algunos autores han sugerido la existencia de un mecanismo específico de absorción de los antocianos a nivel gástrico, que podría implicar su transporte vía bilitranslocasa gástrica (Passamonti et al., 2002; Manach et al., 2005).

El grado de polimerización de los compuestos fenólicos determina la facilidad con que son absorbidos en el intestino. Un claro ejemplo es el de las proantocianidinas (e.j. procianidinas B2 y B3), que se caracterizan por su naturaleza polimérica y su elevado peso molecular. Esta característica estructural limita su absorción intestinal de modo que oligómeros de más de tres unidades es poco probable que sean absorbidos en el intestino delgado. No obstante, estudios *in vitro* sugieren que los oligómeros de procianidina podrían ser hidrolizados a mezclas de monómeros y monómeros de flavonoles. Sin embargo, a pesar de su baja biodisponibilidad, las proantocianidinas podrían ejercer efectos fisiológicos en el tracto gastrointestinal a nivel local tras su degradación a ácidos fenólicos por la acción bacteriana (Manach et al., 2004).

Transporte de compuestos fenólicos en sangre

Los metabolitos de los compuestos fenólicos no circulan libres en la sangre. Estudios *in vitro* e *in vivo* han mostrado su afinidad por las proteínas plasmáticas, aunque esta afinidad varía de acuerdo a las características estructurales de los compuestos fenólicos. La albúmina plasmática está considerada la principal responsable de la unión y por tanto del transporte de compuestos fenólicos en el torrente sanguíneo. Así, el grado de unión a la albúmina determinará la distribución de los metabolitos de los compuestos fenólicos a las células y tejidos. De este modo, cambios conformacionales en la albúmina que facilitan la disociación de los polifenoles ligados permitirían la incorporación de los compuestos fenólicos a las células y tejidos (Manach et al., 2004). A este respecto, un estudio *in vitro* ha revelado la posibilidad de un flujo bidireccional de quercetina entre la albúmina y la hemoglobina. Esta observación sugiere que los eritrocitos pueden desempeñar un papel importante en la distribución y biodisponibilidad de los compuestos fenólicos circulantes, al actuar como reservorios naturales de estas sustancias (Fiorani et al., 2003).

En general, los compuestos fenólicos y sus metabolitos muestran una mayor afinidad por los ambientes acuosos del organismo debido a su carácter hidrofílico y a su unión a la albúmina (Manach et al., 2004). No obstante, estudios *in vitro* e *in vivo* revelan la capacidad de los compuestos fenólicos para unirse a estructuras lipídicas del organismo y ejercer efectos fisiológicos. De hecho, uno de los efectos más relevantes de los compuestos fenólicos es su capacidad para inhibir la peroxidación lipídica, particularmente al nivel de las lipoproteínas plasmáticas de baja densidad (LDL), lo que proporciona una evidencia indirecta de su capacidad de unión a lípidos. Así, estudios *in vitro* empleando un modelo de lipoproteínas de baja y muy baja densidad (LDL+VLDL) han mostrado que la capacidad de los compuestos fenólicos para inhibir la peroxidación lipídica depende en gran medida de su capacidad para unirse a las lipoproteínas (Vinson et al., 1999). La capacidad de los compuestos fenólicos para proteger frente al daño selectivo de la apolipoproteina B100 (Apo-B) de las LDL ha sido asociada con su capacidad de union a las LDL *in vitro* (Filipe et al., 2002). Una molécula de LDL se estima que puede unir 10 moléculas de quercetina. De este modo, en el estudio de Filipe et al. (2002), la quercetina se mostró efectiva para reparar el daño inducido en los aminoácidos de la Apo-B, mientras que la rutina, que no es capaz de unirse a la LDL, fue incapaz de reparar el daño. Otros estudios *in vitro* han mostrado la capacidad de la genisteína para unirse a las lipoproteínas de alta densidad (HDL) (Kaamanen et al., 2003). Por otro lado, estudios *in vivo* han puesto de manifiesto la presencia de compuestos fenólicos unidos a las LDL humanas en individuos que consumieron vino tinto (Nigdikar et al., 1998), así como en el caso de individuos no suplementados (Lamuela-Raventós et al., 1999). Estas observaciones sugieren que, aunque en menor medida, las lipoproteínas estarían implicadas el transporte de los compuestos fenólicos.

2.2.4 Diseño de alimentos funcionales con efecto antioxidante

En los últimos años se ha incrementado el interés por parte de las industrias alimentarias y los consumidores por el concepto de alimento funcional. Así, con un consumidor cada vez más interesado en alimentos más saludables y una industria alimentaria que ha comprendido la potencialidad del mercado de los alimentos funcionales, se ha iniciado a nivel mundial una intensa actividad investigadora en el área de estos nuevos alimentos. El término alimento funcional hace referencia a alimentos o ingredientes que mejoran el estado general de salud y/o reducen el riesgo de enfermedad (Rafter 2002). Se trata además de productos alimenticios que deben consumirse dentro de la dieta habitual para conseguir efectos beneficiosos que van más allá de los requerimientos nutricionales tradicionales (Roberfroid 2002). Un alimento puede hacerse funcional siguiendo alguna de las siguientes estrategias o sus combinaciones (Roberfroid 2000; 2002):

1. Eliminando componentes perjudiciales presentes en el alimento (e.j. alergenos).
2. Incrementando la concentración de un componente presente de forma natural en el alimento hasta unos niveles en que pueda inducir los beneficios esperados (e.j. fortificación con micronutrientes) o incrementando la concentración de una sustancia no nutritiva hasta niveles en que se conoce su efecto beneficioso.
3. Añadiendo un componente que no está presente de forma natural en el alimento y que no es necesariamente un macronutriente o un micronutriente, pero cuyos efectos beneficiosos son reconocidos (e.j. prebióticos, antioxidantes no vitaminicos).
4. Sustituyendo un componente, generalmente un macronutriente (e.j. grasas), cuyo consumo excesivo tenga efectos perjudiciales por un componente de reconocido efecto beneficioso (e.j. inulina).
5. Incrementando la biodisponibilidad o estabilidad de un componente que se sepa que es capaz de producir un efecto funcional o reducir un potencial riesgo de enfermedad del propio alimento.

Estos efectos beneficiosos deben demostrarse científicamente con el objetivo de validar sus efectos y para poder aprobar las declaraciones nutricionales en su etiqueta (Roberfroid 2002). Recientemente ha sido publicado un documento en el que se establecen los criterios consensuados para la evaluación del apoyo científico a las declaraciones nutricionales relacionadas con la salud de los alimentos funcionales (PASSCLAIM) (Aggett et al., 2005), las cuales aparecen recogidas en la **Tabla 6**.

Tabla 6. Criterios consensuados para la comprobación científica de las declaraciones nutricionales en el ámbito de la Unión Europea (PASSCLAIM).

1. El alimento o componente del alimento al cual se atribuye en efecto beneficioso debe ser caracterizado.
2. La comprobación de una declaración nutricional debe estar basada principalmente en datos obtenidos en estudios de intervención en humanos. El diseño de los estudios de intervención debe incluir las siguientes consideraciones: - Los grupos de estudio deben ser representativos de la población a la cual va destinado en alimento funcional. - Deben establecerse unos grupos control adecuados. - La duración de la intervención y el seguimiento deben ser adecuados para demostrar el efecto propuesto. - Debe caracterizarse la dieta habitual de los participantes así como los aspectos más relevantes de sus hábitos de vida. - La cantidad de alimento o componente activo debe ser consecuente con el modo propuesto de consumo. - Ha de tenerse en cuenta el efecto de la matriz del alimento así como el contexto dietético del efecto funcional del componente activo. - Comprobación de la conformidad con la ingesta del alimento o componente activo bajo examen. - Ha de considerarse el poder estadístico para el contraste de la hipótesis.
3. Cuando en efecto beneficioso no puede evaluarse de forma directa, los estudios de intervención deben emplear (bio) marcadores. Estos deben ser: - Biológicamente válidos. Para ello, su relación con el efecto final y la su variabilidad dentro de la población deben ser bien conocidas. - Debe ser metodológicamente válido en cuanto a sus características analíticas.
4. En un estudio de intervención, la variable estudiada debe cambiar de un modo estadísticamente significativo y el cambio observado debe ser biológicamente relevante de acuerdo a la declaración nutricional que se pretende apoyar.
5. Una declaración nutricional debe ser comprobada científicamente considerando la totalidad de los datos obtenidos.

Una de las áreas más prometedoras para el desarrollo de alimentos funcionales se fundamenta en la posibilidad de modular los sistemas redox y antioxidante del organismo (Roberfroid 2000). Por esta razón, en la actualidad muchos alimentos funcionales tienen como finalidad incrementar el aporte de antioxidantes naturales en la dieta. En este contexto, la adición de extractos vegetales ricos en compuestos fenólicos ha sido propuesta como una estrategia factible para el desarrollo de alimentos funcionales con una actividad antioxidante incrementada (Larrosa et al., 2002). De hecho, en el campo del desarrollo de nuevos ingredientes se está produciendo un aumento en la producción de este tipo de extractos vegetales en los cuales los compuestos bioactivos son aislados y concentrados para su uso como suplementos, alimentos nutracéuticos o como ingredientes en la elaboración de alimentos funcionales (Psczcola 2003).

No obstante, una alternativa natural a la adición de estos extractos o del propio compuesto activo previamente aislado podría ser la selección de materias primas ricas en el principio activo cuyos niveles se desean incrementar. Como se ha mencionado anteriormente, las frutas rojas y bayas se caracterizan por su elevado contenido en compuestos fenólicos antioxidantes tales como los antocianos, que a su vez les confieren colores atractivos. Además, existe abundante información en la literatura científica relativa a los niveles de compuestos fenólicos de este tipo de frutas, así como de su actividad antioxidante y de su estabilidad tras el procesado (Wang et al., 1996; Heinonen et al., 1998a; Häkkinen et al., 2000; Zafrilla et al., 2001).

Así, el empleo de estas frutas como ingredientes funcionales en las proporciones adecuadas, podría favorecer el desarrollo de alimentos funcionales ricos en compuestos fenólicos y con una elevada actividad antioxidante. Además, el hecho de ser elaborados de forma natural a partir de frutas de colores atractivos podría favorecer la aceptación del producto final por parte del consumidor.

2.3. EVALUACION DE LA EFECTIVIDAD DE ALIMENTOS RICOS EN ANTIOXIDANTES

La evaluación de la efectividad de un alimento rico en antioxidantes puede abordarse de distintas maneras. Por lo general pueden adoptarse las siguientes estrategias complementarias (Collins 2005):

- Evaluar la actividad antioxidante total *in vitro* mediante pruebas químicas.
- Evaluar su efectividad *in vitro* para proteger frente al daño oxidativo inducido en cultivos celulares.
- Comprobar su actividad *in vivo* tras la suplementación en humanos, generalmente evaluando cambios en biomarcadores del daño oxidativo, y/o en el estado antioxidante total de los individuos.

2.3.1 Pruebas químicas *in vitro* de medida de la actividad antioxidante

En investigación, la medida de la actividad antioxidante total de un alimento o una muestra biológica aporta información valiosa sobre procesos de adición y sinergia que se producen como consecuencia de interacciones entre distintas moléculas bioactivas en la matriz de la muestra analizada. Estos procesos dan lugar, por lo general, a una actividad antioxidante total mayor que la correspondiente a la suma de las actividades de los antioxidantes individuales que la componen, lo cual nos dará una aproximación más real al efecto potencial que podría ejercer *in vivo* un alimento rico en sustancias antioxidantes (Liu 2003).

Así, existen numerosos métodos de medida de la actividad antioxidante total, los cuales se han aplicado a diferentes muestras biológicas y alimentos. Estos métodos se basan generalmente en la captación o secuestro de radicales libres generados en la mezcla de reacción ($O_2^{\bullet-}$,$^{\bullet}OH$, $ROO^{\bullet}$, $ONOO^{-}$, etc.), mientras que otros están basados en la reducción de iones metálicos tales como el Fe^{3+} o el Cu^{2+} (Sánchez-Moreno 2002; Schlesier et al., 2002; Prior et al., 2005). A continuación se describen brevemente algunos de los métodos más comúnmente utilizados para la medida de la actividad antioxidante total de compuestos químicos, alimentos y muestras biológicas (plasma/suero, orina, etc.).

Actividad reductora del hierro férrico/poder antioxidante (Ensayo FRAP):

Esta técnica fue desarrollada por Benzie y Strain (1996) como método de medida de la capacidad antioxidante plasmática, aunque posteriormente ha sido aplicado a muestras de alimentos. El método determina la capacidad de la muestra para reducir un complejo por hierro férrico con la molécula tripiridil-*s*-triazina (TPTZ) a su forma ferrosa. De este modo se genera una coloración de intensidad proporcional a la actividad reductora de la muestra, que

puede cuantificarse por colorimetría en base a un patrón de hierro ferroso. La capacidad para reducir el hierro se considera un índice del poder antioxidante de la muestra.

El ensayo FRAP es sencillo y fácilmente automatizable. Es rápido, generalmente la reacción se completa entre 4 y 8 minutos. Sin embargo, en el caso de algunos polifenoles se han descrito reacciones más lentas, llegando incluso a requerir 30 minutos hasta completar la reducción del complejo. El poder reductor de los compuestos fenólicos se asocia con el número de grupos –OH y en grado de conjugación de la molécula. Debido al potencial redox del complejo Fe^{3+}-TPTZ (0.7 V), el ensayo FRAP es capaz de detectar compuestos con un menor potencial redox y por lo tanto se considera un método adecuado para evaluar la capacidad de los antioxidantes para modular el tono redox de células y tejidos. Sin embargo, cuando se aplica en plasma o suero no es capaz de detectar la actividad de antioxidantes con grupos sulfhidrilo como el glutatión o algunas proteínas, por lo que dará valores de capacidad antioxidante del suero o plasma ligeramente más bajos de los reales. Además, cuando se analizan alimentos vegetales, se han descrito interferencias de azúcares y ácido cítrico (Prior et al., 2005).

Capacidad antioxidante expresada en equivalentes Trolox (Ensayo TEAC)

El ensayo TEAC o ensayo del ácido 2,2-azinobis-(3-etilbenzotioazolín-6-sulfónico) (ABTS) está basado en la captación por los antioxidantes del radical catión $ABTS^{\bullet+}$ generado en el medio de reacción. Como patrón se emplea el ácido 6-hidroxi-2,5,7,8-tetrametil-cromán-2-carboxílico (Trolox), un análogo sintético hidrosoluble de la vitamina E. El radical catión del ABTS posee una coloración verde-azulada con un máximo de absorción a 415 nm y una serie máximos secundarios de absorción a 645, 660, 734, 815 y 820 nm (Sánchez-Moreno 2002). Dependiendo de la variante del método TEAC utilizada se emplean distintas longitudes de onda, aunque las más frecuentes son 415 y 734 nm (Prior et al., 2005). Para el desarrollo del método se suelen emplear dos estrategias; inhibición y decoloración. En la primera los antioxidantes se añaden previamente a la generación del radical $ABTS^{\bullet+}$ y lo que se determina es la inhibición de la formación del radical, que se traduce en un retraso en la aparición de la coloración verde-azulada. En la segunda estrategia, los antioxidantes se añaden una vez el $ABTS^{\bullet+}$ se ha formado y se determina entonces la disminución de la absorbancia debida a la reducción del radical, es decir la decoloración de este (Sánchez-Moreno 2002). El ensayo TEAC presenta además variaciones en el modo mediante el cual se genera el radical catión ABTS. Principalmente se consideran dos modos (Schlesier et al., 2002: Prior et al., 2005).

- La generación por reacciones enzimáticas, en las que el ABTS se incuba con H_2O_2 y con metamioglobina, hemoglobina o peroxidasa de rábano.
- La generación por reacciones químicas, el las que el ABTS se hace reaccionar con dióxido de manganeso, persulfato potásico o cloruro de 2,2'-azo-bis-(2-amidinopropano) (ABAP). Estas reacciones químicas por lo general requieren tiempos largos de incubación (16 horas en el caso del persulfato potásico) o altas temperaturas (60ºC en el caso del ABAP).

Así por ejemplo, el método original descrito por Miller et al. (1993) emplea metamioglobina y H_2O_2 para generar ferrilmioglobina, la cual reacciona con el ABTS para generar el $ABTS^{\bullet+}$. La muestra a analizar se añade antes de la formación del $ABTS^{\bullet+}$ por lo que se trata de un ensayo de inhibición. Este orden de adición de los reactivos ha hecho que el método sea criticado, ya que posibles interferencias de los antioxidantes con el sistema de generación de radicales puede llevar a una estimación de los valores de actividad antioxidante por debajo de los reales (Prior et al., 2005). Sin embargo, se trata de un método ampliamente utilizado en ensayos clínicos, al ser un método rápido, sencillo y automatizable. Incluso existen kits comercializados por Randox Laboratories Ltd. (Reino Unido) para su uso en investigación. Además, el $ABTS^{\bullet+}$ es soluble en solventes acuosos y orgánicos, lo cual lo hace un método apto para determinar la capacidad antioxidante hidrofílica y lipofílica de extractos y fluidos biológicos (Schlesier et al., 2002; Prior et al., 2005).

Captura del radical 2,2-difenil-1-picrilhidracilo (Ensayo DPPH)

Este método se basa en la reducción del radical $DPPH^{\bullet}$ por los antioxidantes de la muestra (Brand-Williams et al., 1995). El radical es estable y tiene una coloración púrpura que se pierde progresivamente cuando se añade la muestra conteniendo sustancias antioxidantes. La decoloración del radical se determina a 515 nm y la cuantificación se realiza por lo general empleando soluciones patrón de Trolox. Los tiempos de reacción son variables dependiendo de la naturaleza de los antioxidantes. En particular, las moléculas pequeñas con mejor accesibilidad al centro activo del radical poseen aparentemente una mayor actividad antioxidante por este método (Prior et al., 2005). En general la reacción puede medirse a los 2, 3, 4, 5 y 10 minutos del inicio, ya que en este intervalo se espera que la mayoría de sustancias completen la reacción con el DPPH, y posteriormente en intervalos de 5 minutos hasta que las variaciones de absorbancia esté en torno a 0.003/minuto, lo cual indicaría que la reacción se ha completado (Schlesier et al., 2002).

El ensayo DPPH es un método rápido y sencillo que no requiere un equipamiento sofisticado, sencillamente un espectrofotómetro. A diferencia del ensayo TEAC, en este ensayo no es necesario preparar radical previamente puesto que el DPPH se comercializa ya en la forma de radical y sencillamente requiere su disolución en metanol para el desarrollo del método. Por esta razón se trata de un método ampliamente utilizado para evaluación de la actividad antioxidante total. Es un método adecuado para medir la actividad antioxidante en alimentos y extractos vegetales, mientras que no es adecuado para la determinación de la capacidad antioxidante del plasma o suero, ya que las proteínas precipitan con el metanol del medio de reacción (Sánchez-Moreno 2002). No obstante algunos antioxidantes pueden causar interferencias si poseen un espectro de absorción similar al del DPPH, como es el caso de los carotenoides (Prior et al., 2005).

Ensayo de los fenoles totales con el reactivo de Folin-Ciocalteu.

El ensayo Folin-Ciocalteu ha sido utilizado durante muchos años como una medida del contenido en compuestos fenólicos totales en productos naturales. Sin embargo, el mecanismo básico del método es una reacción redox por lo que puede considerarse como otro método de medida de la actividad antioxidante total (Prior et al., 2005). El método que se utiliza actualmente es una modificación efectuada por Singleton y Rossi (1965) de un método empleado para la determinación de tirosina, el cual se basaba en la oxidación de los fenoles por un reactivo de molibdeno y wolframio (tungsteno). La mejora introducida por Singleton y Rossi fue el uso de un heteropolianión fosfórico de molibdeno y wolframio que oxida los fenoles con mayor especificidad ($3H_2O$- P_2O_5-$13WO_3$-$5MoO_3$-$10H_2O$ y $3H_2O$-P_2O_5-$14WO_3$-$4MoO_3$-$10H_2O$). La oxidación de los fenoles presentes en la muestra causa la aparición de una coloración azul que presenta un máximo de absorción a 765 nm y que se cuantifica por espectrofotometría en base a una recta patrón de ácido gálico.

Se trata de un método simple, preciso y sensible pero que sin embargo sufre de numerosas variaciones cuando es aplicado por diferentes grupos de investigación, fundamentalmente en lo relativo a los volúmenes empleados de muestra, concentraciones de reactivos, y tiempos y temperaturas de incubación. Además, se producen variaciones en el modo de expresar los resultados de modo que el patrón recomendado de ácido gálico se ha sustituido en ocasiones por los ácidos ferúlico, tánico, cafeico, clorogénico, protocatécuico, vanílico o por catequina. Esto da lugar a variaciones de varios órdenes de magnitud en las medidas de compuestos fenólicos obtenidas por diferentes grupos, lo que dificulta la comparación de resultados. Existen además diversas sustancias de naturaleza no fenólica que interfieren en las determinaciones y que pueden dar lugar a concentraciones de

compuestos fenólicos aparentemente elevadas, por lo que deben hacerse correcciones para estas sustancias. Entre ellas destacan las proteínas, el ácido ascórbico, el ácido úrico, algunos aminoácidos y nucleótidos, azúcares y algunas sales inorgánicas (Prior et al., 2005).

No obstante, a pesar de estos inconvenientes, el ensayo de los fenoles totales se emplea con frecuencia en el estudio de las propiedades antioxidantes de alimentos vegetales (ej. frutas y zumos de frutas), al tratarse de un parámetro que generalmente muestra una estrecha correlación con diferentes métodos de medida de la actividad antioxidante (Schlesier et al., 2002; Sun et al., 2002). Así, cuando se evalúan las propiedades antioxidantes de estos alimentos, el análisis de fenoles totales constituye un método complementario al análisis cromatográfico de los principales grupos de compuestos fenólicos que caracterizan a cada variedad de fruta o verdura, a la vez que proporciona información valiosa a la hora de seleccionar variedades con mayor potencial antioxidante. El ensayo de Folin-Ciocalteu también se ha empleado en estudios *in vivo* para la determinación de los niveles de compuestos fenólicos totales en plasma/suero tras la ingesta de vino (Duthie et al., 1998; Serafini et al., 1998) y zumos de frutas ricos en compuestos fenólicos (Pedersen et al., 2000). Para su aplicación a plasma o suero se requiere una serie de pasos de extracción e hidrólisis que comprenden el tratamiento con HCl y NaOH en metanol. Para evitar interferencias con las proteínas, estas se precipitan con ácido metafosfórico y posteriormente se realiza una extracción de los compuestos fenólicos ligados a las proteínas plasmáticas con una mezcla de acetona y agua (Serafini et al., 1998).

3.3.2 Estudios *in vitro* empleando modelos de células en cultivo

Los modelos animales y estudios en humanos son costosos y por tanto no son apropiados en principio para la caracterización inicial de alimentos ricos en antioxidantes. Por esta razón, es interesante realizar una evaluación previa de estos alimentos mediante ensayos *in vitro* para posteriormente proceder a su evaluación *in vivo*. A este respecto, los modelos celulares son considerados una herramienta útil en investigación nutricional así como en la investigación encaminada a la validación de alimentos funcionales ricos en antioxidantes naturales, ya que proporcionan información valiosa sobre los mecanismos de acción y la eficacia protectora de sustancias bioactivas puras y extractos de alimentos ricos en antioxidantes (O'Brien et al., 2000; Glei et al., 2003; Liu y Finley 2005). En la estrategia general a seguir cuando se evalúa la eficacia de un antioxidante en concreto o un alimento rico en antioxidantes, se pueden distinguir 4 etapas fundamentales:

I. Caracterización de las dosis citotóxicas de la muestra (compuesto activo o extracto de un alimento rico en antioxidantes).
II. Suplementación de las células con dosis no citotóxicas de la muestra.
III. Inducción del estrés oxidativo.
IV. Determinación de la protección ejercida por la muestra.

La primera etapa tiene como objetivo definir las dosis de muestra que sean tóxicas para las células en cultivo, para posteriormente emplear aquellas dosis no tóxicas en la caracterización del efecto protector de la muestra. Una vez conocidas las concentraciones no tóxicas de la muestra, se procede a evaluar el efecto protector frente al estrés oxidativo inducido. Para ello, las células son incubadas con el compuesto puro o el extracto y posteriormente son expuestas a la acción de una sustancia oxidante. Las sustancias utilizadas para inducir del estrés oxidativo en estos ensayos son muy variadas; peróxidos orgánicos e inorgánicos, sistemas oxidantes basados en reacciones de Fenton, glutamato, menadiona, peroxinitrito, etc. Sin embargo, de entre estas sustancias, las más frecuentemente usadas y mejor caracterizadas son el peróxido de hidrógeno (H_2O_2) y el *tert*-butilhidroperóxido (tB-OOH), dos compuestos capaces de inducir eficazmente la muerte celular y causar daño oxidativo al ADN, lípidos de membrana y otras biomoléculas (Alía et al., 2005).

La valoración del efecto protector proporcionado por la muestra se realiza mediante la evaluación de diferentes biomarcadores o parámetros indicadores de la extensión del daño causado por el oxidante. Así, se evaluarán cambios en la:

- Tasa de muerte celular.
- Formación de productos de peroxidación lipídica (malondialdehido, isoprostanos).
- Generación intracelular de especies reactivas del oxígeno (ROS).
- Oxidación del ADN (formación de 8-oxo-7,8-dihidroguanosina, rotura de las hebras).
- Oxidación proteica (formación de grupos carbonilo).
- Actividad de los sistemas enzimáticos antioxidantes y/o niveles de GSH y GSSG.

Mediante el empleo de estos modelos experimentales de células en cultivo se han descrito efectos protectores ejercidos por compuestos fenólicos comunes en la dieta y extractos de alimentos ricos en dichas sustancias frente al estrés oxidativo inducido por diferentes agentes oxidantes, en diferentes líneas celulares **(Tabla 7)**.

Tabla 7. Ejemplos de algunos estudios de evaluación del efecto protector de los compuestos fenólicos en células tumorales humanas.

LINEA CELULAR	SUSTANCIA	OXIDANTE	EFECTO	REFERENCIA
U937	Ácido cafeico	tB-OOH	↓Oxidación lipídica ↓Muerte celular	Nardini et al. (1998)
		H_2O_2	↑GSH	Sestili et al. (2002)
	Flavonoides Cinamatos	tB-OOH	↓Daño al ADN ↓Muerte celular	
HepG2	Teaflavinas	H_2O_2 tB-OOH	↓Generación de ROS ↓Daño al ADN	Feng et al. (2002)
	Flavonoides	tB-OOH Menadiona	↓Muerte celular ↓Daño al ADN	Aherne y O'Brien (2000)
Caco-2	Flavonoides	H_2O_2	↓Muerte celular ↓Daño al ADN	O'Brien et al. (2000)
Jurkat-T	Extracto de té	Fe^{2+}	↓Muerte celular ↓Oxidación lipídica ↓Muerte celular ↓Daño al ADN	Erba et al. (1999)
HUVECs	Zumo de fruta	H_2O_2	↓Generación de ROS	Miranda-Rottmann et al. (2002)

U937, leucemia mieloide; HepG2, carcinoma hepático; Caco-2, carcinoma de colon; Jurkat-T, leucemia; HUVECs, células endoteliales de la vena umbilical

Es evidente la idoneidad de los sistemas *in vitro* en modelos celulares para la identificación de compuestos bioactivos de alimentos vegetales y el estudio de sus mecanismos de acción a nivel de células y tejidos. Los modelos celulares presentan una serie de ventajas frente a otros modelos experimentales entre las que se incluyen; su menor coste económico, la posibilidad de criopreservarción de las líneas celulares durante largos periodos de tiempo, la capacidad para desarrollar estudios a nivel molecular o la facilidad de control de las condiciones experimentales. Sin embargo, los modelos celulares no pueden reproducir con total exactitud las condiciones fisiológicas del organismo en su conjunto, ya que estos ensayos no consideran la absorción y metabolismo de los nutrientes (O'Brien et al., 2000; Liu y Finley 2005). Además, no están presentes tampoco funciones sistémicas del organismo tales como los sistemas nervioso y endocrino. Por este motivo, el control del metabolismo *in vitro* debe ser menos constante que en la situación *in vivo*, lo que impide a las células cultivadas representar fielmente al tejido del que provienen (O'Brien et al., 2000).

Por esta razón, la extrapolación de los efectos observados *in vitro* a la situación *in vivo* debe hacerse con precaución y teniendo siempre presentes las limitaciones de estos modelos experimentales. No obstante, la información obtenida en estudios con células en cultivo en conjunto con datos obtenidos mediante pruebas químicas y estudios de intervención en humanos, hacen posible una caracterización adecuada de la seguridad y eficacia de un alimento funcional, así como de los mecanismos de acción de sus compuestos bioactivos. Todos estos requisitos son necesarios para la validación de las declaraciones nutricionales y sus potenciales efectos beneficiosos.

3.3.3 Estudios de suplementación en humanos

Cuando se evalúa la eficacia de un alimento rico en antioxidantes naturales, la prueba más concluyente de su funcionalidad es sin duda su capacidad para producir mejoras en el estado antioxidante total de los individuos y cambios en determinados biomarcadores del daño oxidativo (Collins 2005). En las últimas décadas, diferentes estudios epidemiológicos han asociado el consumo de una dieta rica en alimentos vegetales con un menor riesgo de enfermedades relacionadas con el estrés oxidativo (Lampe 1999). Esta observación ha dado lugar a la realización de numerosos estudios de intervención en humanos encaminados a la identificación de los compuestos bioactivos responsables de dichos efectos preventivos **(Tabla 8)**. Entre estos estudios, un gran número se han centrado el potencial efecto beneficioso atribuido a los compuestos fenólicos (Prior 2003).

Tabla 8. Ejemplos de algunos estudios de intervención en humanos consistentes en una ingesta elevada de antioxidantes.

SUJETOS	SUPLEMENTACION	DURACION	EFECTOS	REFERENCIA
6 individuos sanos (24-31 años)	Zumo de bayas (400 mL)	0-8 horas	↓Oxidación del plasma ↑Transitorio de la AAT ↑Vitamina C y antocianos en orina	Netzel et al. (2002)
10 mujeres (22-40 años)	280 g de cerezas	0-5 horas	↓Ácido úrico plasmático ↓índices de inflamación (PCR, NO) ↑AAT	Jacob et al. (2003)
22 hombres fumadores (18-50 años)	Concentrado de verduras (500 g) Concentrado de frutas (330 mL)	3 semanas	↑AAT, vitamina C y carotenos Sin efecto oxidación de proteínas, lípidos y ADN Sin efecto en GST y relación GSH/GSSG	Van den Berg et al. (2001)
10 diabéticos	Flavonoles (76-110 mg/día) (400 g cebolla+6 tazas de té)	2 semanas	↑Flavonoles en plasma ↑Excreción urinaria de flavonoles ↓Oxidación del ADN *ex vivo* (linfocitos)	Lean et al. (1999)
10 individuos sanos (25-50 años)	Vino desalcoholizado	0-2 horas	↑Polifenoles en plasma ↑AAT plasmática	Serafini et al. (1998)
28 ancianos	Extractos de frutas y verduras	80 días (2 tomas diarias)	↓Daño al ADN (linfocitos)	Smith et al. (1999)

AAT, actividad o capacidad antioxidante total del plasma o suero sanguíneo; PCR, Proteína C reactiva; NO, óxido nítrico

En este tipo de estudios lo ideal es poder detectar presencia de determinados compuestos fenólicos o sus metabolitos en plasma/suero y orina como indicadores de su biodisponibilidad. Aunque los datos existentes sobre biodisponibilidad de compuestos fenólicos en humanos se considera aún escasa y controvertida, diversos estudios evidencian la absorción y excreción urinaria de estos compuestos (Prior 2003; Manach et al., 2005) e incluso su incorporación a las LDL (Nigdikar et al., 1998; Lamuela-Raventós et al., 1999). No obstante, el análisis de compuestos fenólicos en los fluidos biológicos es complicado debido a las amplias diferencias estructurales de los compuestos fenólicos y a la gran variedad de metabolitos generados en los procesos digestivos. Además, estos estudios requieren técnicas analíticas específicas como el HPLC-MS para obtener datos consistentes (Cerdá et al., 2004; Ichiyanagi et al., 2005; Manach et al., 2005).

Por esta razón, en los estudios de intervención con alimentos ricos en antioxidantes es muy útil la evaluación de disminuciones en biomarcadores del daño oxidativo a las principales moléculas diana del organismo (ADN, lípidos y proteínas). Además, en estos estudios es habitual determinar la actividad o capacidad antioxidante total del plasma o suero, que nos da información de la capacidad general de respuesta ante la oxidación y sobre posibles efectos cooperativos entre los distintos componentes de los sistemas de defensa antioxidante. De este modo, obtenemos información sobre el efecto de la exposición del organismo a las sustancias antioxidantes de la dieta (Mayne 2003; Collins 2005). A continuación se describen brevemente algunos de los biomarcadores del daño oxidativo más frecuentemente empleados en estudios de intervención y en modelos de células en cultivo.

3.3.4 Biomarcadores del daño oxidativo

Biomarcadores del daño oxidativo al ADN: La oxidación del ADN da lugar a un gran número de diferentes productos de la oxidación de azúcares y bases nitrogenadas, así como a la escisión de las hebras que componen la doble hélice. Así, una determinación habitual en células sanguíneas y células en cultivo es el grado de rotura de las hebras de ADN que se detecta habitualmente mediante electroforesis en gel (ensayo Comet). La rotura de las hebras permite que los bucles de ADN superenrollado se desplacen en el gel durante el desarrollo de la electroforesis, generando una imagen al microscopio que se asemeja a la de un cometa **(Figura 6)**. La proporción de ADN en la cola indica la extensión de la fragmentación del ADN y por tanto de la extensión del daño. (Mayne 2003; Collins 2005). Un

método de sensibilidad similar es el ensayo Halo alcalino (Sestili y Cantoni 1999), en el cual no se realiza electroforesis. Las células fijadas en gel se incuban en un tampón alcalino (pH 13) y los fragmentos de ADN se desplazan de modo radial generando una imagen al microscopio que se asemeja a un halo concéntrico a los restos del núcleo **(Figura 6)**. El área del halo aumenta a medida que aumenta la fragmentación del ADN, en un proceso asociado a una reducción progresiva del área correspondiente a los restos del núcleo.

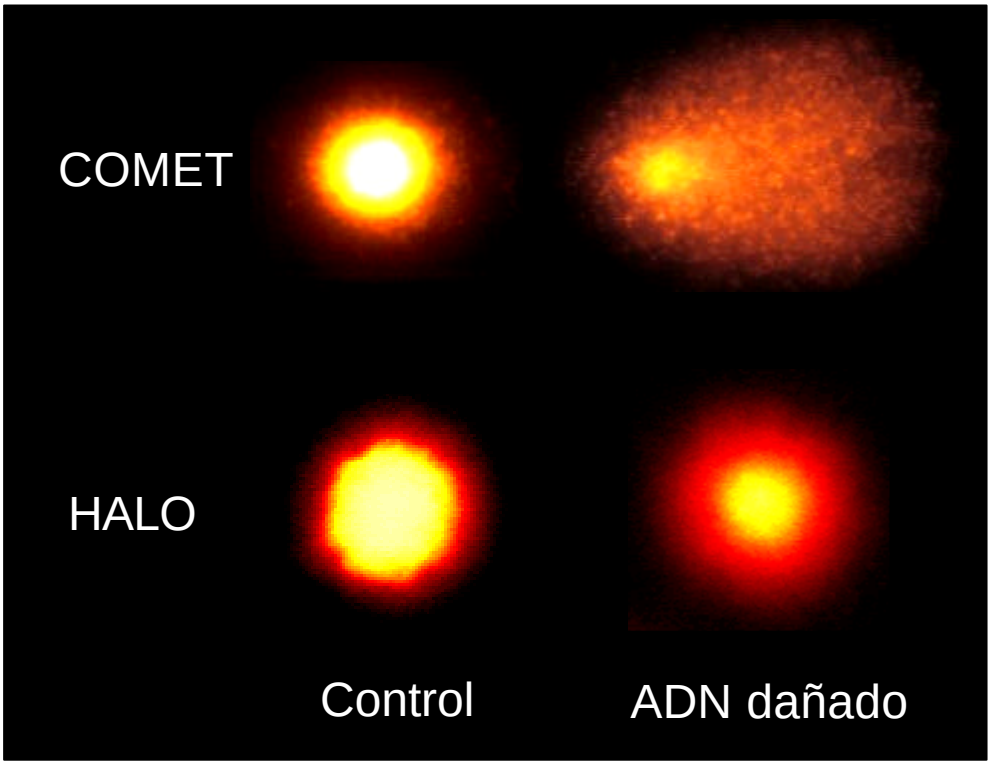


Figura 6. Imágenes características del daño al ADN detectado mediante los ensayos Comet y Halo.

A menudo se miden los niveles productos de la oxidación de las bases nitrogenadas del ADN como la 8-oxo-7,8-dihidroguanina (8-OHdG). Las determinaciones se pueden realizar mediante pruebas cromatográficas (CG-MS, HPLC-ED, HPLC-tandem MS), pruebas inmunoquímicas (ELISA), o con el uso del enzima formamidopirimidina ADN glicosilasa que convierte la 8-OHdG en hebras de ADN rotas, las cuales posteriormente son detectadas mediante el ensayo Comet (Collins 2005).

Biomarcadores del daño oxidativo a lípidos: La peroxidación lipídica es un proceso complejo que genera un gran número de productos finales de oxidación (e.j.

aldehídos, peróxidos lipídicos) en cantidades variables (Halliwell y Whiteman 2004). Por ello la peroxidación lipídica puede medirse de diversas maneras. El método más rápido y sencillo es la determinación colorimétrica de las sustancias reactivas con el ácido tiobarbitúrico (TBARS). Esta técnica mide un cromógeno formado por el ácido tiobarbitúrico (TBA) con el malondialdehído (MDA), que es un producto final de la peroxidación lipídica. Actualmente es frecuente el empleo de una modificación de este método en la cual el cromóforo TBA-MDA se separa por HPLC. De este modo se reducen las interferencias de otras sustancias reactivas con el TBA, aumentando la sensibilidad y especificidad de la determinación (Mayne 2003). El mejor indicador de la peroxidación lipídica *in vivo* parece ser la determinación de isoprostanos, que son productos específicos de la oxidación mediada por radicales libres de ácidos grasos poliinsaturados de cadena larga. Entre ellos se distinguen los isoprostanos F_2, derivados de la oxidación del ácido araquidónico, y los isoprostanos F_4, procedentes de la oxidación del ácido docohexanoico. La determinación de isoprostanos se hace mediante CG-MS o inmunoensayo (Mayne 2003). Otros indicadores de la peroxidación lipídica *in vivo* son los hidrocarburos exhalados, tales como pentano, que deriva de la oxidación de ácidos grasos n-6, y etano, que resulta de la oxidación de ácidos grasos n-3 (Mayne 2003). No obstante, estos hidrocarburos son considerados productos minoritarios de la peroxidación lipídica (Halliwell y Whiteman 2004).

Biomarcadores del daño oxidativo a proteínas: El proceso de oxidación proteica frecuentemente introduce nuevos grupos funcionales, como hidroxilos y carbonilos, que contribuyen a la alteración de la función de la proteína y a su posterior catabolismo (Griffiths et al., 2002). El indicador más frecuentemente usado para la evaluación del daño oxidativo a proteínas es la determinación de la formación de grupos carbonilo mediante el ensayo colorimétrico de la dinitrofenilhidracina o mediante pruebas inmunoquímicas (ELISA). Aunque ambos métodos proporcionan valores absolutos diferentes, estos han mostrado una buena correlación cuando se han empleado en determinaciones plasmáticas (Mayne 2003). Como alternativa esta medida global de la oxidación proteica están las determinaciones mediante ELISA de los semialdehídos aminoadípico (AAS) y glutámico (GGS), principales contribuidores a la medida total de grupos carbonilo (Halliwell y Whiteman 2004).

3

Resultados y Discusión

Results and Discussion

3.1. Estudio 1

Assessment of the antioxidant properties during storage of a dessert made from grape, cherry and berries

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ABSTRACT

A one-year storage trial was designed involving different temperatures (8, 21 and 30 ºC) to assess the antioxidant properties of a dessert formulated with concentrated juices of grape, blackberry, blackcurrant, raspberry and cherry. The total antioxidant activity (TAA) of the dessert was measured as the scavenging capacity of the 2,2'-azino-di-[3-ethylbenzothiazoline-6-sulphonate] radical cation (ABTS) and as its iron-reducing ability (FRAP). Color was spectrophotometrically measured, and vitamin C and phenolics were analysed by HPLC. The color parameters and the concentrations of different antioxidant compounds were affected by storage conditions, especially in samples stored at 30 ºC, although the TAA remained practically invariable during storage.

Key words: antioxidant properties, grape, berries, vitamin C, phenolic compounds.

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INTRODUCTION

Oxidative damage has been implicated in aging processes and in the pathogenesis of several degenerative diseases (Ames et al., 1993). Epidemiological studies have shown that diets rich in plant foods significantly reduce the incidence and mortality rates of degenerative diseases caused by oxidative stress (Tibble 1998). This protective effect has been attributed to the fact that such foods may provide an optimal mix of phytochemicals, such as phenolic compounds and other natural antioxidants (Steinmetz and Potter 1991a, 1991b; Ames et al., 1993). Berries such as strawberry, blueberry and raspberry are traditional desserts throughout the world, either in the form of fresh fruit or as processed food materials in juices, jams, dried fruit, ice cream and so on (Amakura et al., 2000). These fruits are among the most important sources of phenolic compounds in our diets, especially hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, catechins and tannins (Macheix et al., 1990), which continue to be present in their processed products (Heinonen et al., 1998). Grapes and grape juice share the same properties, both containing substantial amounts of important antioxidants, including stilbenoids (resveratrol and piceid) (Cantos et al., 2000). In cherries, the main phenolics are anthocyanins and phenolic acids (Gao and Mazza 1995), which can be detected in cherry jams as anthocyanins (García-Viguera et al., 1997) and in cherry juice as chlorogenic acid (Shahrzad and Bitsch 1996).

The importance of the antioxidant properties of foods in the maintenance of health and in protection from degenerative diseases is of growing interest among scientists, food manufacturers, consumers and health organizations. Since the 1990`s several international organizations have recommended increasing the consumption of fruits and vegetables to five or more daily servings, in order to provide a desirable intake of antioxidants and to improve human health (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997). For this reason, there is a trend in the foodstuff industry toward functional foods, which produce healthy effects based on their antioxidant properties (Velioglu et al., 1998; Kähkönen et al., 1999; Robards et al., 1999). The aim of this study was to evaluate the antioxidant properties of a dessert formulated with concentrated juices of grape, cherry, blackberry, blackcurrant and raspberry. This product was formulated and designed using the data of total antioxidant activity available in the scientific literature for these fruits (Cao et al., 1996; Wang et al., 1996) with the objective of producing a dessert with the average antioxidant capacity equivalent to ten servings of fruits and vegetables, double the five recommended servings mentioned above. To ascertain any relation between these properties and its composition, phenolic compounds and vitamin C

were also evaluated. A one-year storage trial was designed involving different temperatures to assess the ability of the product to maintain its antioxidant properties.

MATERIALS AND METHODS

Material

A dessert was prepared with commercially available concentrated juices of grape (26% v/v), cherry (2% v/v), raspberry (1% v/v), blackberry (0.6% v/v) and blackcurrant (0.6% v/v) and water by the Department of Research and Development of Hero Spain (Alcantarilla, Murcia, S.A.). Pectin was added to jellify the product. The resulting product was highly pasteurized in order to obtain a microbiologically stable product and bottled hot in jars to ensure head space vacuum. Samples were taken immediately after manufacture and analyzed to ascertain the proximate composition, pH, tritratable acidity, soluble solids, color, vitamin C, polyphenol content, antioxidant properties and microbiology. Nine batches were made and stored at 8, 21 and 30 ºC for one year, and samples were taken for analysis every three months. The phenolic profile was also analyzed in the concentrated juices used as ingredients.

Proximate composition, pH, titratable acidity, soluble solids and color

Proximate composition of the dessert (moisture, ash, total protein, total fat and total dietary fiber) was determined following the official methods of the AOAC (AOAC 1999). The pH was measured using a Crison pH-meter, model micro pH 2000 (Crison, Barcelona, Spain), and total titratable acidity (expressed as percentage of citric acid) was analyzed by titrating the product with 0.1 N NaOH to pH 8.2. Soluble solids were quantified using a Leica Abbe Mark II refractometer (Leica, Buffalo, NY, USA) following the procedures described by Shams and Thompson (1987), and color with a tristimulus color spectrophotometer Minolta CM 508i (Osaka, Japan). L* a* b* values were calculated using illuminant D65 and a 10º observer angle according to the CIELAB 76 convention (McLaren 1980). Hue angle (H) was calculated from $H=tg^{-1}\ b^*/a^*$, metric chroma (C) from $C=(a^{*2}+b^{*2})^{1/2}$ and color difference (δE) from $dE=[(L^*)^2+(a^*)^2+(b^*)^2]^{1/2}$. All analyses were made in triplicate.

Determination of phenolic compounds

Phenolic compounds were analyzed by HPLC according to the method described by Cantos et al., (2000). For this, 10 g of sample were homogenized using an Ultraturrax T-25 (Janke and Kunkel, Ika-Labortechnic) at 24000 rpm for 1 min after the addition of 3 mL of methanol of HPLC grade plus 3% formic acid per gram of sample. The extracts were

centrifuged at 5000 x g for 5 min in a Centromix centrifuge (Selecta, Barcelona, Spain), filtered through 0.45 μm Nylon filter (Whatman) and HPLC analyzed on a L-7100 liquid chromatograph equipped with a Merck-Hitachi 7455 UV diode array detector and a Licrochart RP-18 column of 25 x 0.4 cm, 5 μm particle size (Merck, Darmstadt, Germany). Solvents were water plus 5% formic acid (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 mL/min. Elution was performed with a gradient starting with 2% B to reach 32% B at 30 min, 40% B at 40 min and 95% B at 50 min and then isocratic for 5 min. Chromatograms were recorded at 510, 360, 320 and 255 nm. The different phenolic compounds were identified from their UV spectra, which were recorded with a diode array detector and from chromatographic comparisons with the different standards assayed. Anthocyanins were quantified at 510 nm as cyanidin-3-glucoside (previously isolated and identified from pigmented lettuce), flavonols at 360 nm as quercetin-3-rutinoside, stilbenoids at 320 nm as resveratrol, hydroxycinnamic acid derivatives at 320 nm as chlorogenic acid and ellagic acid derivatives at 255 nm as ellagic acid. All analyses were made in triplicate.

Antioxidant properties

To evaluate these properties samples were diluted 1:20 (w/v) in water and then analysed in triplicate to evaluate the scavenging capacity and iron reducing ability. The scavenging capacity of the 2,2'-azino-di-[3-etylbenzothiazoline-6-sulphonate] radical cation (ABTS) was assayed using a kit manufactured by Randox Laboratories Ltd. (Cat. No. NX2332, Ardmore, UK), according to the method described by Miller et al., (1993). This spectrophotometric technique measures the relative abilities of antioxidants to scavenge the ABTS radical cation in comparison with standard amounts of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Results were multiplied by the dilution factor and expressed as mM Trolox equivalents. The iron reducing capacity was evaluated following the metodology of Benzie and Strain (1996). At low pH, when a ferric-tripyridyltriazine complex is reduced to the ferrous form, an intense blue color with an absorption maximum at 593 nm develops. FRAP reagent was freshly prepared by mixing 25 mL acetate buffer 300 mM (pH 3.6), 2.5 mL 2,4,6-tripyridyl-s-triazine 10 mM in 40 mM HCl solution and 2.5 mL $FeCl_3 \cdot 6H_2O$ 20 mM. Aqueous solutions of known Fe^{II} concentration in the range of 0.2-2.0 mM ($FeSO_4 \cdot 7H_2O$) were used for calibration, obtaining the following lineal equation:

$$mM\ Fe^{II}\ equivalents/L = 1.55 \times dAbsorbance + 0.03\ (R^2 = 1)$$

$$dAbsorbance = Absorbance\ (sample\ solution) - Absorbance\ (FRAP\ reagent)$$

Results were multiplied by the dilution factor and expressed as mM Fe^{II} equivalents. One Fe^{II} equivalent per liter equals the amount of Fe^{II} per liter required to give the same absorbance change. The antioxidant properties in our samples were compared with the values obtained in different samples used as reference. The samples consisted of a solution of 1 mM vitamin C, Spanish red wine from Penedés (grape varieties Tempranillo, Garnacha and Monastrell; year 1999) and black tea (1.15 g/250 mL water), while the antioxidant properties were 0.92, 17.88 and 2.96 mM Trolox equivalents for ABTS scavenging capacity and 2.07, 19.20 and 1.96 mM Fe^{II} equivalents for the iron reducing ability, respectively.

Determination of Vitamin C

The ascorbic acid content was measured by reversed phase HPLC, as described by Esteve et al., (1995). 10 g of sample were diluted to 100 mL with 1% (w/v) meta-phosphoric acid solution and shaken for 10 min. The extracts were filtered first through Whatman No 1 paper and then through a 0.45 μm Millipore filter before being HPLC analyzed. The HPLC analyses were performed on a 2690 Waters system (Waters, Milford, MA, USA) equipped with a Waters 996 UV diode array detector. System conditions were: injection volume 20 μL, detector wavelength 245 nm, flow rate 1 mL/min, column Lichospher 100 RP-18 of 12.5 x 0.4 cm, 5 μm particle size (Merck, Darmstadt, Germany). The mobile phase was phosphate buffer 0.1 M (pH 3.5) and vitamin C was quantified as L-(+)-ascorbic acid (Merck, Darmstadt, Germany).

Microbiological analysis

To ascertain the microbiological quality of the dessert immediately after manufacture and during the one-year storage trial, total bacteria and total enterobacteria were analyzed, the former with plate count agar (No. 105463, Merck, Darmstadt, Germany), while the latter were isolated with violet red bile glucose medium (No. 110275, Merck, Darmstadt, Germany) (International Commission on Microbiological Specifications for Foods 1982).

Statistical analysis

Data were analyzed by Statistical Package SPSS 10.0 version for Windows. An analysis of variance was included in the data treatment to study the variation in the analyzed compounds as a function of the time and temperature of storage. Tukey's test for pairwise comparison was used to determine significant differences at a level of 5%. Pearson correlation was carried out to understand any relation between all analyzed parameters.

RESULTS AND DISCUSSION

Compositional analysis of the dessert

Table 1 shows the compositional indices of the dessert made of grapes, cherries, and berries, immediately after manufacture. The product showed a high water content, while the levels of nutrients such as protein, fiber and minerals (expressed as ash content) were very low, representing less than 0.5% of total weight. Fat was not detected in the compositional analysis.

Table 1-Compositional indices of the dessert

Moisture (%)	78
Total protein (%)	0.50
Total fat (%)	nd*
Total dietary fiber (%)	0.3
Ash (%)	0.3
Total soluble solids (ºBrix)	21
pH	3.65
Total titratable acidity (% citric acid)	0.62

*nd: Not detected

Effect of storage on phenolic compounds

Figure 1 shows the content of total anthocyanins (TAn), hydroxycinnamic (THy) acids and stilbenoids (TSt), while **Figure 2** shows the total ellagic acid (TEll) and flavonol (TFl) contents of the dessert stored at 8, 21 and 30 ºC for 3, 6, 9 and 12 months.

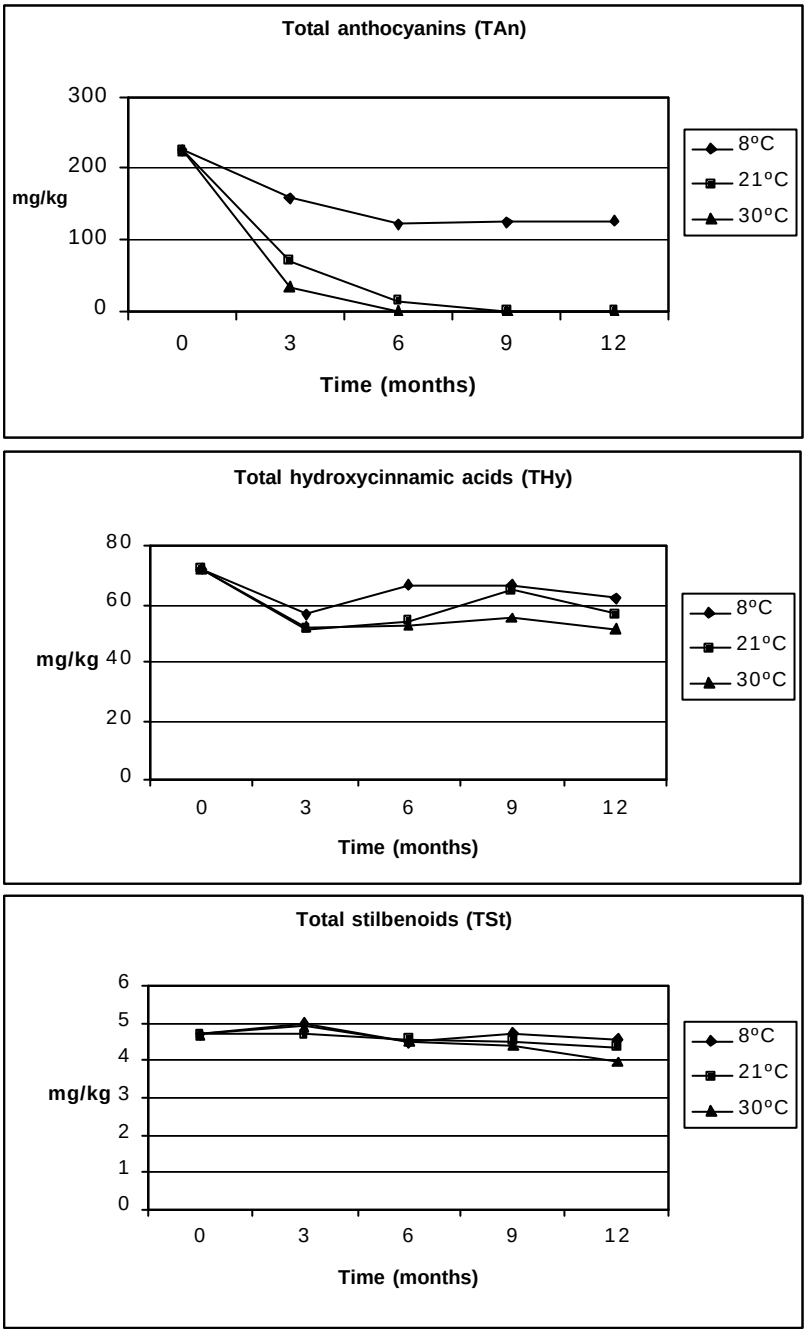

Figure 1-Changes in total anthocyanins, total hydroxycinnamic acids and total stilbenoids during storage at 8, 21 and 30 ºC

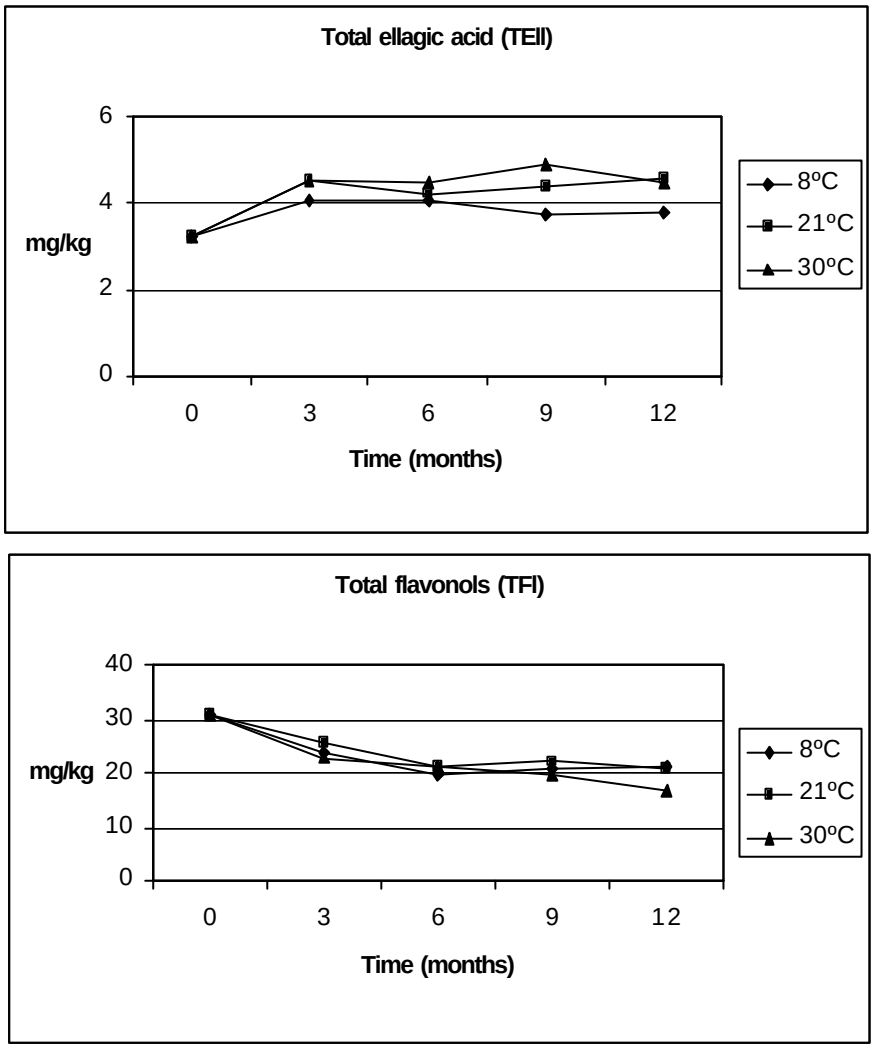

Figure 2-Changes in total ellagic acid and total flavonols during storage at 8, 21 and 30 ºC.

Table 2 shows the phenolic profile of the concentrated juices used as ingredients. The phenol content of fruit, vegetables and derived products depends on different factors, including genetic factors and environmental, processing and storage conditions. The phenolics determined in all the concentrates were total anthocyanins (ranging between 8000 and 51300 mg/kg), followed by total flavonols (with values between 700 and 5600 mg/kg). Total hydroxycinnamic acids were the major phenolic compounds in the cherry concentrate and were not detected in the raspberry and blackberry concentrates. Total stilbenoids were only detected in the grape concentrate and total ellagic acids only in concentrated raspberry and blackberry juices. Obviously, the phenolic composition of the dessert was determined by the phenolic content of the fruit concentrates used as ingredients **(Table 2).**

Table 2. Phenolic profile of the fruit concentrates used as ingredients, expressed as mg/kg of juice[1]

Phenolics	**Fruit concentrate**				
	Grape (26%)[2]	**Cherry** (2%)	**Raspberry** (1%)	**Blackberry** (0.6%)	**Blackcurrant** (0.6%)
Total anthocyanins	13700	8000	12200	10500	51300
Total hydroxycinnamic acids	10500	52600	nd	nd	5100
Total flavonols	2800	5600	700	5300	2500
Total stilbenoids	500	nd	nd	nd	nd
Total ellagic acids	nd*	nd	13200	3400	nd

[1]Mean

[2]Values between brackets indicate the percentage (v/v) of the fruit concentrate in the dessert.

*nd: Not detected

In the dessert, the main phenolic compounds detected immediately after manufacture were anthocyanins followed by hydroxycinnamic acids, flavonols, stilbenoids and ellagic acid. During storage, the phenolic composition significantly ($P < 0.01$) depended on the temperature and time of storage, with the exception of total stilbenoids **(Figures 1** and **2).** The initial total anthocyanins content was 224.5 mg/kg, which decreased significantly during storage. The greatest losses in total anthocyanins were observed during the first 3 months of storage. The samples exhibited a very sharp decrease when stored at high temperature (30 ºC) but less so when stored at 21 and 8 ºC **(Figure 1).** In samples stored at 8 ºC, the final content after 12 months was around 50% of the initial content, whereas in samples stored at

21 and 30 ºC a total loss of total anthocyanins was observed at 9 and 6 months, respectively **(Table 3, Figure 1)**.

The total hydroxycinnamic acids content decreased slowly from 72 mg/kg to values of 62, 57 and 52 mg/kg after storage for 12 moths at 8, 21 and 30 ºC. The total stilbenoids content detected in the dessert was provided by grapes **(Table 2)**, and its levels were more or less constant at around 4-5 mg/kg. However, total ellagic acids increased during storage from 3.2 to 4.6 mg/kg, although this increase was lower in refrigerated samples (8 ºC) **(Figure 2)**. This could be explained by a temperature-dependent release of ellagic acid from ellagitannins, since the greatest increase was observed in samples stored at 30 ºC. The ellagitannins were provided by the raspberry and blackberry concentrates used as ingredients **(Table 2)**. The total flavonols content was also affected by the time of storage, decreasing after 12 months from 31 mg/kg to 21, 20.8 and 16.7 mg/kg for samples stored at 8, 21 and 30 ºC **(Figure 2)**. Different authors have described similar behavior for the phenolic compounds in berry jams. For example, García-Viguera et al. (1998) described anthocyanin degradation in raspberry and strawberry jams, with the greatest losses occurring during the first months of storage at higher temperatures. Zafrilla et al. (2001) also described an increase in free ellagic acid in raspberry jams after processing and during storage. These authors suggested that the increase in free ellagic acid observed during jam cooking can be related to a release of hexahydroxydiphenic acid from ellagitannins, which is transformed to ellagic acid, or to the increased extractability of this compound from processed products due to degradation of the cell structures.

Effect of storage on antioxidant activity

Figure 3 shows the total antioxidant activity of the fruit dessert and its evolution during the storage trial at 8, 21 and 30 ºC, measured as the capacity to scavenge ABTS radical (ABTS method) and as its iron reducing capacity (FRAP assay). The antioxidant activities of the dessert ranged from 19 to 22 mM Trolox equivalents, as assessed by the ABTS scavenging capacity and from 19 to 24 mM Fe^{II} equivalents in the FRAP assay. Overall, the analysis of variance showed that storage time and temperature significantly determined the total antioxidant activity of the dessert. However, the total antioxidant activity of the dessert was only slightly modified by storage conditions, values remaining more or less constant when samples were stored at 8 ºC and decreasing slightly in samples stored at 21 and 30ºC. For this reason, the refrigerated storage of the dessert is recommended in order to maintain its antioxidant activity.

If we compare the antioxidant activity of the dessert with those of other foods and foodstuffs, this product showed higher antioxidant activity than many fresh fruits and vegetables expressed as mM Trolox equivalents: 0.58-3.50 for tomatoes (Martínez-Valverde et al., 2002) 4.68-6.32 for onions, 2.30-3.78 for red peepers (Martínez-Valverde 2001), 0.61 for apricot nectar, 2.67 for black plum, 6.84 for red grape, 1.65 for cherry (Karakaya et al., 2001).

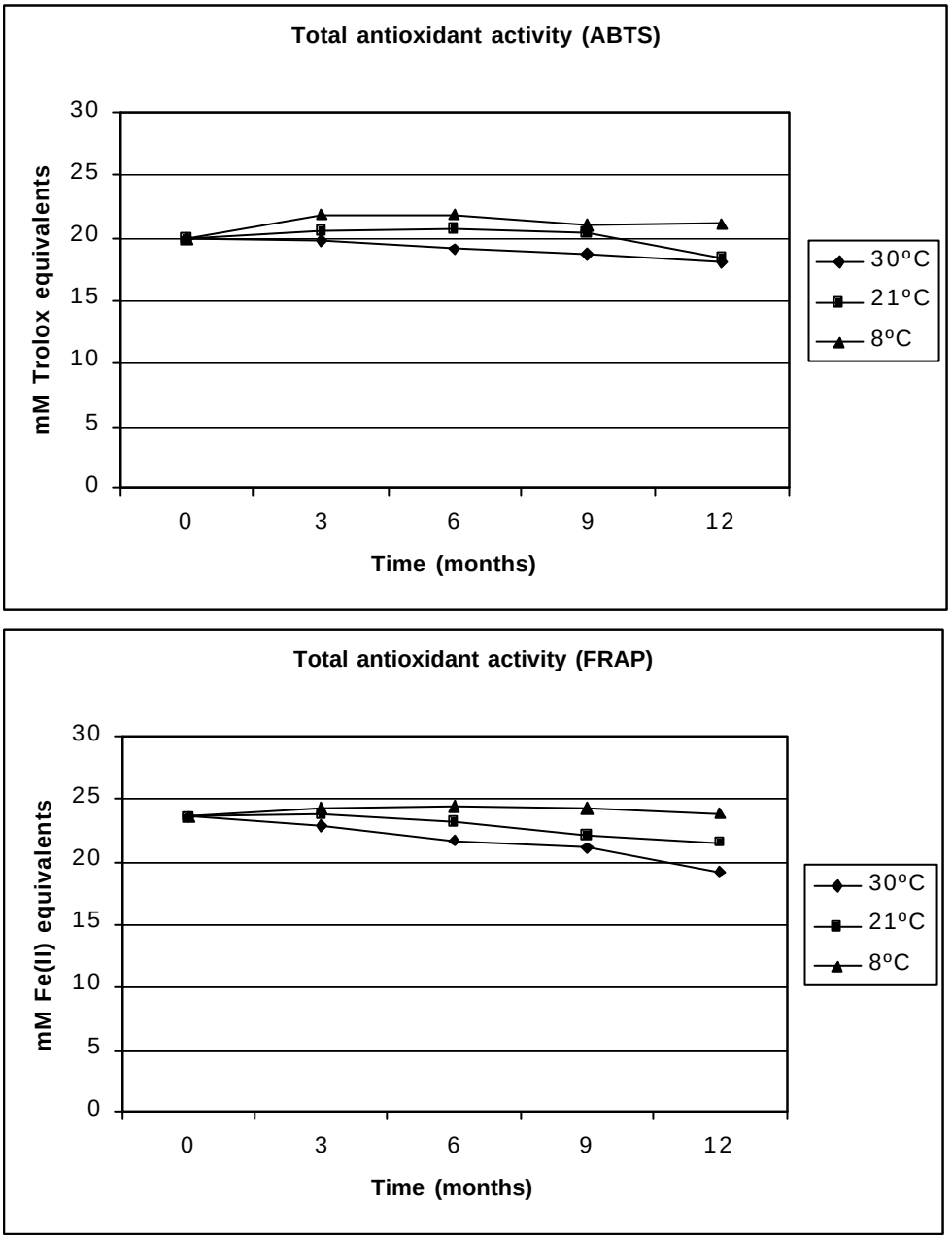

Figure 3-Changes in total antioxidant activity of the dessert during storage at 8, 21 and 30 ºC

As regards the iron reducing ability, Gardner et al., (2000) reported average total antioxidant activity values of 6 mM Fe^{II} equivalents for orange juice, 4 mM Fe^{II} equivalents for grapefruit juice and of 1.5 mM Fe^{II} equivalents for pineapple juice, as assessed by the FRAP assay. In addition, the antioxidant properties of dessert are also higher than those measured in beverages recognized as important antioxidants in the diet (4.88 for Turkish coffee, 4.44 for black tea, 12.61-22.24 in red wines and 1.0-2.0 in beers, expressed as mM Trolox equivalents) (Walters et al., 1997; Karakaya et al., 2001). In general, the data show that the total antioxidant properties of the dessert are similar to those of red wine and higher than those of tea, coffee and beers. These results are also in accordance with the values observed in the reference samples, as described the Material and Methods section.

The total antioxidant properties of this product are related to the phenolic compounds. There was a positive and significant correlation between total anthocyanins and the total antioxidant activity assessed by the ABTS method ($r = 0.37$, $p < 0.05$) and the FRAP assay ($r = 0.72$, $p < 0.001$). The FRAP values were also correlated with total hydroxycinnamic acids ($r = 0.60$, $p < 0.001$), total flavonols, ($r = 0.59$, $p < 0.001$), total stilbenoids ($r = 0.57$, $p < 0.001$) and ascorbic acid content ($r = 0.50$, $p < 0.001$), whereas ABTS values were only related with total anthocyanins and total stilbenoids. Although phenolic compounds decreased, the total antioxidant activity remained more or less constant. These findings suggest that other phenolics, such as the products of anthocyanin degradation, contribute to maintaining the total antioxidant activity of the dessert. This process could be similar to that which occurs in red wines during aging, in which it has been suggested, the larger polyphenolic complexes and condensation products that appear during aging make a sizable contribution to overall antioxidant activity (Burns et al., 2001). The anthocyanins exist in wines in three forms: free, combined and polymerised anthocyanins, the last belonging to the group of condensed tannins. The free anthocyanins rapidly disappear during the first few years of wine maturation, whilst the combined and polymerised anthocyanins generally remain present regardless of wine age (Glories 1978). Such an effect is not surprising in the dessert because of the high anthocyanin content of grape concentrate and because of the high proportion of this fruit in the final formulation.

Quality parameters and total anthocyanins content

As quality parameters, the ascorbic acid content was ascertained; color measurements and microbiological analyses were performed (See in **Table 3** and **Figure 4**). The dessert was microbiologically safe and stable, since no microbial growth was detected in the samples during the storage trial (total bacteria and total enterobacteria < 10^1 ufc/g). Changes in vitamin C have been considered as an indicator of the enzymatic and non-enzymatic degradative reactions which take place during processing or storage (Skrede 1996). **Figure 4** shows the degradation of ascorbic acid during storage at 8, 21 and 30 ºC. Losses in vitamin C were not temperature-dependent and were only affected by storage time. Major losses occurred during the first 3 months of storage, when values decreased from 14 mg/100 g to around 3 mg/100 g. After 12 months of storage losses of more than 90% of the initial ascorbic acid content were observed. Many reports have indicated that ascorbic acid tends to accelerate anthocyanin degradation rates (Skrede et al., 1992; Martí et al., 2002). In this study, anthocyanin losses were significantly affected by time and storage temperature **(Figure 1)**, whereas vitamin C degradation was affected only by storage time **(Figure 4)**. Iversen (1999) reported similar results for blackcurrant nectar in dark and light storage at 20 ºC, when anthocyanin degradation was 3-4 times greater than that observed for ascorbic acid.

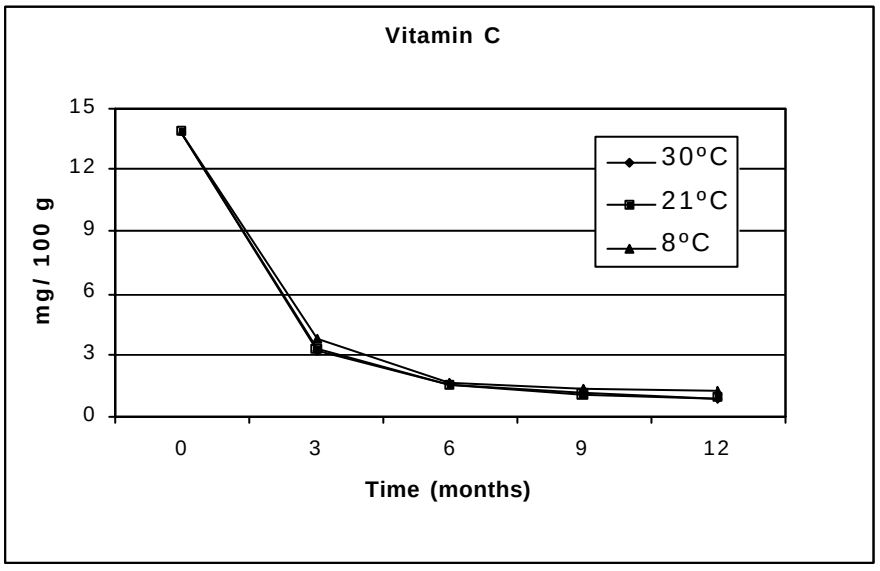

Figure 4-Changes in vitamin C during storage at 8, 21 and 30 ºC

Table 3. Color parameters and losses in parameters a* and total anthocyanins (TAn) in the dessert during storage at 8, 21 and 30 ºC[1]

Time	L*	a*	b*	Chroma	Hue angle	dE	% loss a*	% loss TAn
8 ºC								
0	24.02 ± 0.52^{b}	17.79 ± 0.39^{a}	6.04 ± 0.34^{a}	18.79 ± 0.48^{a}	18.78 ± 0.52^{a}	-	-	-
3	23.97 ± 0.85^{b}	13.67 ± 0.55^{b}	4.80 ± 0.68^{a}	14.49 ± 0.74^{b}	19.27 ± 2.05^{a}	4.38 ± 0.72^{c}	23.2	29.3
6	23.60 ± 0.60^{b}	16.44 ± 0.44^{a}	6.00 ± 0.50^{a}	17.50 ± 0.58^{a}	19.96 ± 1.27^{a}	1.53 ± 0.56^{d}	7.6	46.1
9	22.70 ± 0.53^{b}	12.00 ± 0.63^{c}	4.70 ± 0.58^{a}	12.89 ± 0.80^{b}	21.46 ± 1.74^{a}	6.10 ± 0.84^{b}	32.6	44.5
12	26.57 ± 0.50^{a}	10.28 ± 0.49^{d}	2.52 ± 0.49^{b}	10.59 ± 0.59^{c}	13.85 ± 1.90^{b}	8.68 ± 0.47^{a}	42.2	56.6
21 ºC								
0	24.02 ± 0.52^{b}	17.79 ± 0.39^{a}	6.04 ± 0.34^{a}	18.79 ± 0.48^{a}	18.78 ± 0.52^{a}	-	-	-
3	23.25 ± 0.44^{bc}	13.38 ± 0.49^{b}	4.46 ± 0.46^{b}	14.11 ± 0.61^{b}	18.26 ± 1.04^{a}	4.76 ± 0.68^{b}	24.8	68.6
6	22.98 ± 0.58^{bc}	17.44 ± 0.66^{a}	6.30 ± 0.30^{a}	18.54 ± 0.72^{a}	19.80 ± 0.00^{a}	1.28 ± 0.43^{c}	2	93.9
9	22.49 ± 0.49^{c}	12.33 ± 0.33^{b}	4.41 ± 0.41^{a}	13.10 ± 0.45^{b}	19.62 ± 1.28^{a}	5.91 ± 0.54^{b}	30.7	100
12	26.94 ± 0.73^{a}	9.76 ± 0.46^{c}	2.12 ± 0.35^{b}	9.99 ± 0.52^{c}	12.22 ± 1.38^{b}	9.43 ± 0.31^{a}	45.2	-
30 ºC								
0	24.02 ± 0.52^{b}	17.79 ± 0.39^{a}	6.04 ± 0.34^{a}	18.79 ± 0.48^{a}	18.78 ± 0.52^{a}	-	-	-
3	22.89 ± 0.79^{bc}	12.15 ± 0.45^{c}	4.16 ± 0.44^{b}	12.84 ± 0.57^{c}	18.94 ± 1.29^{a}	6.08 ± 0.70^{c}	31.7	85.5
6	23.56 ± 0.71^{bc}	15.01 ± 0.28^{b}	5.64 ± 0.37^{a}	16.04 ± 0.39^{b}	20.64 ± 0.77^{a}	2.91 ± 0.43^{d}	15.6	100
9	22.17 ± 0.57^{c}	10.21 ± 0.43^{d}	3.75 ± 0.63^{b}	10.88 ± 0.62^{d}	20.11 ± 2.28^{a}	8.15 ± 0.71^{b}	42.6	-
12	26.80 ± 0.47^{a}	6.76 ± 0.57^{e}	1.24 ± 0.27^{c}	6.87 ± 0.61^{e}	10.38 ± 1.39^{b}	12.36 ± 0.51^{a}	62	-

[1]Mean and standard deviation of three determinations. Different letters in the same column for the same temperature mean significant differences for $p<0.05$

The loss of red color and increased browning during the production and/or storage of processed foods are influenced by many factors, which include Maillard and enzymatic browning, ascorbic acid degradation and the polymerization of anthocyanins with other phenolics (Wrolstad et al., 1990). As regards the color parameters **(Table 3)**, a slight non temperature-dependent increase in L* was observed between 9 and 12 months of storage, indicating changes in lightness. The decrease in a* values observed during storage was significantly higher in samples stored at 30 ºC, leading to samples with a less red hue **(Table 3)**. The decreases in b* values and Hue angle were not affected by storage temperature, while decreases in Chroma, which indicate losses in color saturation, were significantly higher in samples stored at 30 ºC. Changes in color difference (ΔE), related to the product immediately after pasteurization, showed a significant increase during storage, particularly at 30 ºC. Anthocyanins are responsible for the characteristic red-purple color of berries and berry products. However, anthocyanins disappear as monomeric compounds and are transformed into polymeric forms, resulting in a color change to a more brownish shade. Consequently, color changes during storage of the dessert are more likely to be due to polymerization phenomena mediated by other phenolics, similar to that which occurs in wines and fruit products (Skrede et al., 1992; Johnston and Morris 1997). Nevertheless, the rate of color loss was much slower than the rate of total anthocyanins decrease **(Table 3)**, which reflects the findings for other fruit products such as strawberry and raspberry jams (García-Viguera et al., 1998, 1999).

CONCLUSIONS

We conclude that the dessert made with grapes, cherries, and berries has high antioxidant activity, and could be considered as a functional food with potential healthy effects for humans. Although, there was a strong decrease in antioxidant compounds, such as anthocyanins and vitamin C, during storage antioxidant activity remained practically invariable for one year at different temperatures. These findings could be useful for researchers and processors wishing to predict the stability of natural antioxidants and total antioxidant activity in novel fruit-based functional foods. Further studies are being carried out involving the intake of this dessert and evaluating biomarkers of oxidative damage (García-Alonso et al., 2002; Linde et al., 2002).

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3.2. Estudio 2

Antiproliferative and cytoprotective activities of a phenolic-rich juice in HepG2 cells

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ABSTRACT

A variety of fruit and vegetable extracts have been found to have antiproliferative activities and to protect against oxidant-induced damage in cell-culture models. In the present study carried out in HepG2 cells (human caucasian hepatocyte carcinoma), we evaluate the ability of a juice rich in phenolics (3 g/L) made from grapes, cherries and berries to inhibit cell proliferation and to protect cells from both tert-butylhydroperoxide (tB-OOH) and hydrogen peroxide (H_2O_2)-induced cell death and lipid peroxidation. Cells exposed to tB-OOH were also analyzed for glutathione peroxidase (GPx) and glutathione-s-transferase (GST) activities. In addition, the radical scavenger N,N'-diphenyl-1,4-phenylene-diamine (DPPD) and the intracellular iron chelator o-phenanthroline (o-phe) were assayed in order to elucidate the action mechanisms of juice phenolics. Cell proliferation at 24, 48, 72 and 96 hours was inhibited in a dose-dependent manner by juice extracts with a median effective dose (EC_{50}), which is the amount of phenolic compounds able to inhibit cell proliferation by 50%, in the range of 45-55 μg of total phenolics per mL of medium. Cells preincubated with sub-toxic concentrations of juice extracts showed increased resistance to oxidative challenge, as revealed by an increase in cell survival and a decrease in lipid peroxidation after exposure to either H_2O_2 or tB-OOH. Both DPPD and o-phe protected cells from the lipid peroxidation caused by either H_2O_2 or tB-OOH. Detoxification of tB-OOH by cells caused an increase in both GPx and GST activity. Both juice and o-phe decreased GPx activity, but not that of GST. The tB-OOH-induced GST activation was only suppressed by DPPD. After a short preincubation period (3 hours), phenolics in the functional juice protected cells from induced oxidative stress, indicating that phenolics were rapidly absorbed by cells and contributed to the cellular antioxidant defences. Data also suggest that the protective activity of the phenolics in the functional juice may partly reside in their iron-chelating ability.

Keywords: phenolics, berries, antiproliferative, iron chelators, radical scavengers, tert-butylhydroperoxide, hydrogen peroxide, HepG2

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INTRODUCTION

Phytochemicals, especially the phenolics found in fruits and vegetables, have been proposed as the major bioactive compounds providing the health benefits associated with diets rich in plant-foods. Since the prevention of chronic diseases is a more effective strategy than their treatment, reducing the risk of diseases such as cardiovascular disease and cancer is a subject of great interest for doctors, scientists in general, consumers and the food industry (Liu 2003). In this context, redox and antioxidant systems are among the most promising targets for functional food science. For this reason, many functional foods are nowadays aimed at boosting intakes of antioxidants in order to reduce the risk of chronic disease linked to oxidative stress. Functional foods refer to foodstuffs or ingredients that improve overall health and/or reduce the risk of disease, and are food products to be taken as part of the usual in order to have beneficial effects that go beyond what are known as traditional nutritional effects (Rafter 2002; Roberfroid 2002).

Among the most common dietary sources of natural antioxidants, grapes and berries are rich in phenolic compounds, particularly flavonoids, which are valued for their role in anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial and antioxidant activities (Robards et al., 1999; Moure et al., 2001). Furthermore, most of these compounds remain present in berry and fruit products (Heinonen et al., 1998). Based on this knowledge, in a previous study we proposed the use of selected concentrated juices of grapes and berries as ingredients for the development of phenolic-rich functional foods providing potential nutritional benefits (García-Alonso et al., 2003). Important to the acceptance of health claims is the necessity to generate enough scientific evidence to support the efficacy of the claim, but, in addition, functional foods need to be assessed for toxic effects. In fact, polyphenolic compounds of plants are well known as dietary antioxidants, but they also exhibit contrasting pharmacological actions, such as prooxidant activity and the induction of cell death (Lapidot et al., 2002; Saleem et al., 2002). In this regard, cell culture is a powerful technique for studying physiological, biochemical and toxicological processes modulated by pure phytochemicals or by whole foods *in vitro* (Glei et al., 2003).

Since our ongoing research focuses on the validation of fruit-based functional foods, for the present study a functional juice rich in phenolic compounds was formulated by using concentrated juices of grapes, cherries and berries, and investigated for its antiproliferative and cytoprotective activities in the human HepG2 cell line. This cell line has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resembles the human hepatocyte in culture (Ramos et al., 2005). In addition, steady-state functioning of the antioxidant defences in HepG2 is relatively higher

than in hepatocytes and other non-transformed cells. Therefore, variations in the responses to different conditions are more easily detected (Alia et al., 2005).

By using this cell-culture model, we aimed to evaluate the modulatory effect of the phenolic-rich juice on cell proliferation and on the protection from oxidative stress caused by *tert*-butylhydroperoxide and hydrogen peroxide, two compounds frequently used as models to study the mechanisms of cellular alteration resulting from free radical action.

MATERIALS AND METHODS

Reagents and chemicals

Minimum essential medium with Earle's salts (MEM), penicillin, streptomycin, non essential aminoacids and L-glutamine were purchased from Gibco (Paisley, Scotland, UK). 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), foetal bovine serum, trypsin, 1,10-phenanthroline monohydrate (*o*-phenantroline), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 1-chloro-2,4-dinitrobenzene (CDNB), Folin-Ciocalteu's phenol reagent 2 N, 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS), 2,4-dinitrophenylhydrazine, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ethilenediaminetetra-acetic acid (EDTA), *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD), reduced glutathione (GSH), *tert*-butyl hydroperoxide (tB-OOH) 70% solution in water and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich (St. Louis, USA). Hydrogen peroxide (H_2O_2) aqueous solution 30% and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). 2,4,6-tripyridil-*s*-triazine (TPTZ) and ferulic acid were from Fluka (Buchs, Switzerland). Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) was from Panreac (Barcelona, Spain). All other reagents were of the highest grade obtainable.

Test product

The test product was an experimental juice prepared by the Research and Development Department of Hero Spain S.A (Alcantarilla, Murcia, Spain). This product was formulated and designed using the data of total antioxidant activity available in the scientific literature for the fruits used (Cao et al. 1996; Wang et al. 1996), with the objective of reaching per serving (a bottle of 200 mL) an average antioxidant capacity equivalent to 10 servings of fruits and vegetables, 2-fold higher than the "Five a day" recommended by several international organizations (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997). The major ingredient was water, which was mixed with commercially available concentrated juices of grape (26%),

cherry (2%), blackberry (0.6%), blackcurrant (0.6%) and raspberry (1%). The resulting product was pasteurized in order to obtain a microbiologically stable product and hot-bottled to ensure headspace vacuum.

Analysis of phenolic compounds

The main groups of phenolic compounds were HPLC analyzed on a L-7100 liquid chromatograph equipped with a Merck-Hitachi 7455 UV diode array detector and a Licrochart RP-18 column of 25 x 0.4 cm, 5-mm particle size (Merck, Darmstadt, Germany), according to the method described by Cantos et al. (2000). Total phenolics in the juice were analyzed by a colorimetric assay using Folin-Ciocalteu's phenol reagent, as described by Singleton and Rossi (1965). Ferulic acid was used as standard and the total phenolic content is expressed as mg/L of ferulic acid equivalents or as μM of ferulic acid equivalents, for the cell culture assays.

Antioxidant properties in the juice

Total antioxidant activity of the juice was evaluated by using four common tests: the Trolox equivalent antioxidant capacity assay (TEAC I-II assay), 2,2-diphenyl-1-picrylhydrazyl assay (DPPH assay), and the ferric reducing/antioxidant power (FRAP assay). The colorimetric TEAC assay measures the ability of antioxidants to scavenge the ABTS radical cation. The TEAC-I assay was carried out using a kit manufactured by Randox Laboratories Ltd. (Cat. No. NX2332, Ardmore, UK), according to the method described by Miller et al. (1993). This method is based on the inhibition by antioxidants of the absorbance of the radical cations of ABTS at 600 nm. ABTS radical cations are formed by incubation of ABTS with metmyoglobin and H_2O_2. The TEAC-II assay (Miller et al., 1996) is based on the reduction of the radical cation of ABTS which is generated by filtering an ABTS solution through manganese dioxide powder. The antioxidant activity of the samples is calculated by determining the decrease in absorbance at 734 nm. In the DPPH assay (Brand-Williams et al., 1995) antioxidants reduce the free radical DPPH, and the decrease in absorbance is measured at 515 nm at 0, 2, 3, 4, 5 and 10 minutes, and then in intervals of 15 minutes until the reaction reaches a *plateau* (ΔAbsorbance = 0.003 minutes^{-1}). In all these tests, Trolox was used as standard, and results are expressed as mM of Trolox equivalents. The FRAP assay (Benzie and Strain 1996) measures the iron reducing capacity of the sample as its ability to reduce a ferric (Fe^{3+})-TPTZ complex to the ferrous (Fe^{2+}) form. Then, an intense blue colour develops, which is measured at 593 nm. Aqueous solutions of known Fe^{2+} ($FeSO_4 \cdot 7H_2O$) concentration were used for calibration. The final results were expressed as

milimoles of Fe^{2+} equivalents per litre. One Fe^{2+} equivalent per litre equals the amount of Fe^{2+} per litre required to give the same absorbance change.

Cell culture and treatments

HepG2 human caucasian hepatocyte carcinoma cells (European Collection of Cell Cultures; ECACC No. 85011430, CAMR, Salisbury, Wiltshire, UK) were routinely grown in MEM supplemented with 10% (v/v) foetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% non essential aminoacids and 2 mM L-glutamine, in a humidified atmosphere of 95% air-5% CO_2 at 37°C. Stock solutions of tB-OOH and H_2O_2 were freshly prepared in serum-free MEM. The radical scavenger *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD) and the intracellular iron chelator *o*-phenanthroline (*o*-phe), were also assayed with the aim of elucidating the mechanisms of action of phenolics in the juice. DPPD and *o*-phenanthroline were dissolved in DMSO. At the treatment stage, the final DMSO concentration was never higher than 0.1%. Juice extracts were prepared by directly diluting the juice in cell growth medium, and then filtered through 0.2 μm sterile cellulose acetate syringe filters (Filalbet S.L., Barcelona, Spain).

Antiproliferative assays

The MTT-assay (Mosmann 1983) was used to evaluate the antiproliferative activities of the juice extracts. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells, which can be photometrically quantified. An increase in the number of living cells results in an increase in total metabolic activity, which leads to a stronger colour formation. For the assays, cells (5×10^3 cells/well in 180 μL of complete MEM) were placed in each well of a 96 well flat bottom plate. Cells were allowed to adhere for 24 hours, and then 20 μL of complete cell growth medium containing increasing concentrations of juice extracts were added to the cells. The concentrations of total phenolics in the tests ranged from 0 to 500 μM of ferulic acid equivalents per well. Blank wells contained the above concentrations of juice extracts in 200 μL of growth medium but with no cells. After 24, 48, 72 and 96 hours of incubation, medium was replaced for 200 μL of serum-free MEM and 50 μL of MTT solution (5 mg/mL) was added to each well. After 4 hours of postincubation, the medium was removed and 100 μL of DMSO was added to each well in order to solubilize the formazan. The plate was read using a Labsystem multiskan MCC/340 microplate reader (Thermo Electron Corporation, Barcelona, Spain) at a wavelength of 570 nm with a reference wavelength of 690 nm. The results are expressed as the percentage of viable cells with respect to the control.

$$\text{Cell proliferation (\%)} = \frac{\text{Mean absorbance of the control - Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

The median effective dose (EC_{50}), which is the amount of phenolic compounds able to inhibit cell proliferation by 50%, was calculated graphically for each cell proliferation curve. In order to exclude any possible effect on cell proliferation due to changes in osmolality, the latter was measured in the cell growth medium before and after the addition of juice extracts, using a Vapro vapour pressure osmometer model 5520 (Wescor, Claremont, Ontario, Canada).

Cytotoxicity assays

The protective effect of juice extracts against oxidant-induced cytotoxicity was also evaluated by the MTT-assay. For the experiments, cells (10^4 cells/well) were seeded onto 96-well microtiter plates and allowed to adhere for 24 hours. Then, the cells were preincubated with juice extracts at different concentrations (0-150 μM) for 3 hours in complete growth medium (200 μL). After that, the medium was replaced by 200 μL of serum-free MEM containing different concentrations of tB-OOH or H_2O_2, and the cells were incubated for 24 hours. At the end of incubation, 50 μL of MTT solution in serum-free MEM (5 mg/mL) was added to each well and incubation was allowed to continue for a further 4 hours. Finally, the medium was removed and 100 μL of DMSO was added to each well in order to solubilize formazan. The plate was read using a Labsystem multiskan MCC/340 microplate reader (Thermo Electron Corporation, Barcelona, Spain) at a wavelength of 570 nm with a reference wavelength of 690 nm. The results are expressed as the percentage of viable cells with respect to the control.

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance of the control - Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

Lipid peroxidation assays

HepG2 cells (10^6 cells) were seeded onto 60 mm Petri dishes and were grown to confluence (1 week). The medium was removed and replaced by 3 mL serum-free MEM containing different concentrations of juice extracts (0-150 μM) and incubated for 0.5, 1.5, 3 or 14 hours. DPPD and *o*-phenantroline were added to the cultures 5 minutes prior to the addition of tB-OOH. After incubation, cells were challenged with 10 mM tB-OOH for 30 minutes or 20 mM H_2O_2 for 2 hours at 37ºC in serum-free MEM. After the treatments, cells were washed twice in prewarmed PBS (pH 7.2) and scraped into 2 mL of PBS. This cell

suspension was used for protein determination (Lowry et al., 1951) and the thiobarbituric acid reacting substances (TBARS) assay (Buege and Aust 1978). For the assay, 0.9 mL of cell suspension was mixed with 2 mL of thiobarbituric acid (TBA) reagent (15% trichloroacetic acid, 0.25 N HCl and 0.5% TBA) and heated at 100ºC in a water bath for 25 minutes. After centrifugation at 5000 rpm for 10 minutes, the absorbance of the supernatants was measured at 535 nm. The total content of malondialdehyde (MDA) and related aldehydes capable of reacting with TBA to form chromophores absorbing at 535 nm was estimated using a molar absorption coefficient for the malondialdehyde–TBA complex of 1.56×10^5 $M^{-1}\cdot cm^{-1}$. The results are expressed as nanomoles of MDA per mg of protein.

Enzyme activities

The activity of the glutathione-related enzymes, glutathione peroxidase (GPx) and glutathione-*s*-transferase (GST), which are involved in the reduction and detoxification of *tert*-butylhydroperoxide, were also evaluated. For the experiments, $2\cdot10^6$ cells were seeded into 75 cm^2-cell culture flasks and grown to confluence (10 days) in complete MEM. After that, the medium was removed and the cells were exposed to the 150 µM-juice extract in 10 mL of serum-free MEM for 3 hours. After the preincubation period, the cells were washed twice in serum-free MEM, and later challenged with 10 mM tB-OOH for 30 minutes in serum-free MEM (10 mL). DPPD and *o*-phe were added to the cultures 5 minutes prior to the addition of tB-OOH. After the treatments, cells were detached by trypsinization (0.25% trypsine and 0.25% EDTA in PBS), washed twice in prewarmed PBS (pH 7.2) and resuspended into 2 mL of PBS. Cells were then disrupted by sonication for 1 minute, and centrifuged at 15000 x g for 15 minutes at 4ºC. The supernatant was used for protein determination (Lowry et al., 1951) and the enzyme activity assays. Protein was immediately analyzed, whereas for enzyme measurements the supernatants were frozen at -80ºC until analyzed.

Assay of glutathione peroxidase activity: GPx activity was determined by an UV method using a commercially available kit (RANSEL, Cat. No. RS 504, Randox Laboratories Ltd., Ardmore, UK), following the manufacturer's instructions. In this method, which is based on that of Paglia and Valentine (1967), GPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured at 340 nm and 37ºC. For the assay, 1000 µL of working reagent (glutathione 4 mM, glutathione reductase =0.5 U/L, and NADPH 0.34 mM in 50 mM phosphate buffer pH 7.2 containing 4.3 mM EDTA) and 40 µL of cumene hydroperoxide (0.18 mM) was added to 20 µL of sample or water (blank) The

indicated concentrations are those yielded in the test for a final volume of 1060 µL in a semi-micro quartz glass cuvette. After mixing, absorbance readings at 340 nm were taken after 1 minute, and again after 2 minutes. For calculations, the following formula was used and the results were expressed as mU/mg protein.

$$\text{GPx activity (U/L)} = \frac{\Delta \text{ absorbance of the sample - } \Delta \text{ absorbance of the blank}}{2} \times 8412$$

Assay of glutathione-s-transferase activity: GST activity was determined according to the method of Habig et al. (1974). In this assay, GST catalyzes the conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB), producing a dinitrophenyl thioeter chromophore which is accompanied by the appearance of an absorption band at 340 nm. Prior to the assay, the CDNB working reagent was prepared by mixing 44 volumes of 0.1 M potassium phosphate pH 6.5 with 1 volume of 50 mM CDNB in absolute ethanol. The 0.1 M potassium phosphate buffer was prepared by diluting a 1 M phosphate buffer (St. Louis, USA) 10-fold into water, and adjusting pH to 6.5 with 5 N NaOH. For the assay, the following solutions were added sequentially to a semi-micro quartz glass cuvette: 900 µL of CDNB working reagent, 50 µL of 20 mM reduced glutathione (GSH) aqueous solution, and 50 µL of sample or water (blank). The increase in absorbance was monitored at 340 nm at 25ºC over a 5-minute time period. For calculations, the following formula was used:

$$\text{GST activity (mU/mL)} = \frac{\text{Net rate}}{\varepsilon_{340\,nm}} \times \text{Dilution factor}$$

where $\varepsilon_{340\,nm}$ is the extinction coefficient of the CNDB-GSH adduct (0.0096 $M^{-1} \cdot Cm^{-1}$). The dilution factor was 20 since 50 µL of sample was used in a final volume of 1000 µL. The sample and blank slopes were calculated as the variation in absorbance per minute (ΔAbsorbance/5), and the net rate by subtracting the blank slope from the sample slope. The results were expressed as mU/mg protein.

STATISTICAL ANALYSIS

Data were analyzed by Statistical Package SPSS 11.0 version for Windows (SPSS Inc., Chicago, IL, USA). A Student's *t*-test was used to evaluate differences between the test samples and positive controls. The results are reported as mean±SEM of at least three separate experiments.

RESULTS

Antioxidant properties of the juice

Table 1 shows the phenolic compounds and antioxidant properties of the experimental juice. The main phenolics were anthocyanins, followed by catechins, hydroxycinnamic acids (caffeic acid derivatives), and stilbenoids. As regards the antioxidant properties of the juice, this product showed higher antioxidant activity than many fresh fruits and vegetables, even higher than those of beverages recognized as important antioxidants in the diet (tea, coffee and beer), and similar to those of red wine. These results are in accordance with those observed in a previous study, in which we evaluated the antioxidant properties of a jellified dessert designed as a functional food and prepared by using the same formulation (García-Alonso et al., 2003).

Table 1. Phenolic compounds and total antioxidant activity of the experimental juice.

Parameter	Mean values	Units
Total phenols[1]	3000	mg/L
Anthocyanins	670	mg/L
Catechins	425	mg/L
Hydroxycinnamic acids	200	mg/L
Stilbenoids	9.5	mg/L
FRAP	32	mmol of Fe^{2+} equivalents/L
TEAC-I (ABTS/MetMb)	25	mmol of Trolox equivalents/L
TEAC-II (ABTS/MnO_2)	27	mmol of Trolox equivalents/L
DPPH	15	mmol of Trolox equivalents/L

[1]The total amount of phenolic compounds is 15000 μM when expressed as ferulic acid equivalents.

Antiproliferative activity

As illustrated in **Figure 1**, incubation with extracts of the phenolic-rich juice inhibited HepG2 cell proliferation in a dose-dependent manner, but this antiproliferative activity did not depend on the incubation time, as can be observed from the four cell proliferation curves (24, 48, 72 and 96 hours). Thus, for all incubation times, the median effective dose (EC_{50}), which is the amount of phenolic compounds able to inhibit cell proliferation by 50%, was similar and within the range of 250 to 300 μM ferulic acid equivalents in the medium (45-55 μg of total phenolics per mL of medium). Importantly, osmolality did not significantly change from the value of the medium alone (288 mmol/kg) at any of the concentrations tested. Thus, under these experimental conditions, it seems that cell growth inhibition is not osmotically mediated. For subsequent experiments to examine the cytoprotective effect of the juice, subcytotoxic doses of juice extracts (0-150 μM) were used.

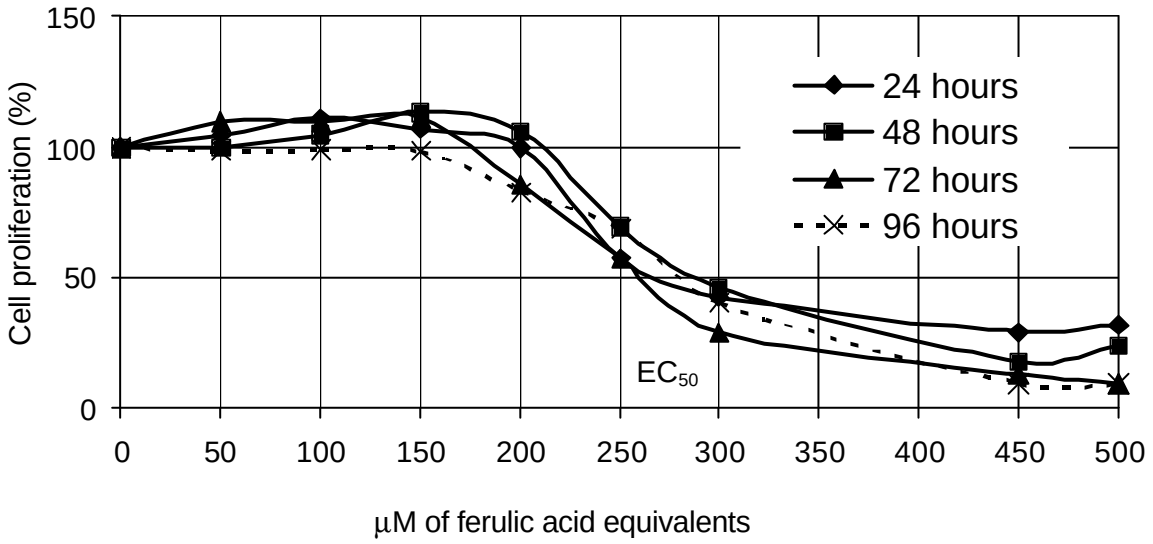

Figure 1. The effect of the phenolic-rich juice on HepG2 cell proliferation. Cells ($5 \cdot 10^3$ cells/well) were incubated with extracts of the juice at different concentrations of phenolics (0-500 μM) for 24, 48, 72 or 96 hours in complete MEM. Cell proliferation was evaluated as the mitochondrial ability to metabolize MTT. Results represent the mean values of at least three separate experiments. EC_{50} values were comprised into the range of 250 to 300 μM (45-55 μg of total phenolics per mL of medium).

Prevention of tB-OOH and H_2O_2 induced cytotoxicity

Table 2 shows the effect of preincubation with extracts of the phenolic-rich juice on HepG2 cell death caused by different concentrations of tB-OOH or H_2O_2. Exposure of cells to either tB-OOH or H_2O_2 increased cell death in a dose-dependent manner. Preincubation of cells with juice extracts at different concentrations for 3 hours partially abolished the cell death induced by both tB-OOH and H_2O_2, even at the low concentration of 10 µM ferulic acid equivalents. A dose-dependent effect was only observed in H_2O_2-treated cells. In the case of tB-OOH-challenged cells, exposure to the lowest concentrations of juice extracts was more effective in affording protection.

Table 2. The effect of the phenolic-rich juice on oxidant-induced cytotoxicity in HepG2 cells[1].

Juice (µM)	+tB-OOH 10 µM	+tB-OOH 25 µM	+H_2O_2 25 µM	+H_2O_2 50 µM
0	92.4±2.2	54.7±2.5	65.0±1.5	35.6±2.0
10	97.1±5.2	80.0±1.0**	71.4±2.4	53.4±2.0**
50	111.2±4.8**	77.0±2.0**	75.0±1.5*	53.5±1.5**
100	107.8±4.1*	70.0±3.0**	85.6±5.0**	49.8±2.3**
150	94.0±5.7	64.8±4.7	86.0±5.0**	59.8±5.0**

[1]Results represent the % of cell viability with respect to untreated control cells. Cells (10^4 cells/well) were preincubated with extracts of the juice at different concentrations of phenolics (0-150 µM of ferulic acid equivalents) for 3 hours and then challenged for 24 hours in serum-free MEM with different concentrations of tert-butylhydroperoxide (tB-OOH) or hydrogen peroxide (H_2O_2). Cell viability was evaluated as the mitochondrial ability to metabolize MTT. Results represent the mean ± SEM of at least three separate experiments. *p <0.05, **p<0.01, compared with control cells exposed to tB-OOH or H_2O_2.

Prevention of oxidant-induced lipid peroxidation

Figure 2 shows the effect of preincubation with extracts of the phenolic-rich juice on tB-OOH- or H_2O_2– induced lipid peroxidation. Exposure of cells to 10 mM tB-OOH for 30 minutes or 20 mM H_2O_2 for 2 hours significantly increased lipid peroxidation. As illustrated in **Figure 2 A**, preincubation of cells with juice extracts at different concentrations for 14 hours prevented tB-OOH-induced lipid peroxidation, but only achieved statistical significance ($p<0.05$) when cells were preincubated with the extract at 150 μM ferulic acid equivalents. Importantly, as can also be seen in **Figure 2 A**, juice extracts alone were not prooxidant and did not increase lipid peroxidation at any of the concentrations tested. The concentration of 150 μM was seen to be the most effective in preventing lipid peroxidation and for this reason was chosen for subsequent experiments.

As shown in **Figure 2 B**, preincubation of cells with the juice extract at 150 μM ferulic acid equivalents for 0.5, 1.5, 3, and 14 hours prevented tB-OOH-induced lipid peroxidation in a time-dependent manner. Interestingly, a rapid but not statistically significant decrease in lipid peroxides was already observed after 30 minutes of preincubation with the juice extract, suggesting that phenolics from the juice were rapidly taken up by cells, and were able to afford protection against tB-OOH-induced lipid peroxidation. Statistical significance ($p<0.05$) was achieved after longer preincubation periods (1.5, 3 and 14 hours). The addition of both the intracellular iron chelator *o*-phenanthroline (25 μM) and the radical scavenger DPPD (10 μM) abolished tB-OOH-induced lipid peroxidation.

As shown in **Figure 2 C**, preincubation with the 150 μM-juice extract for 3 hours significantly ($p<0.05$) prevented the lipid peroxidation caused by H_2O_2. Also *o*-phe (25 μM) and DPPD (10 μM) prevented H_2O_2-induced lipid peroxidation, but did not reach statistical significance. Importantly, DMSO, DPPD nor o-phenanthroline alone induced lipid peroxidation (data not shown).

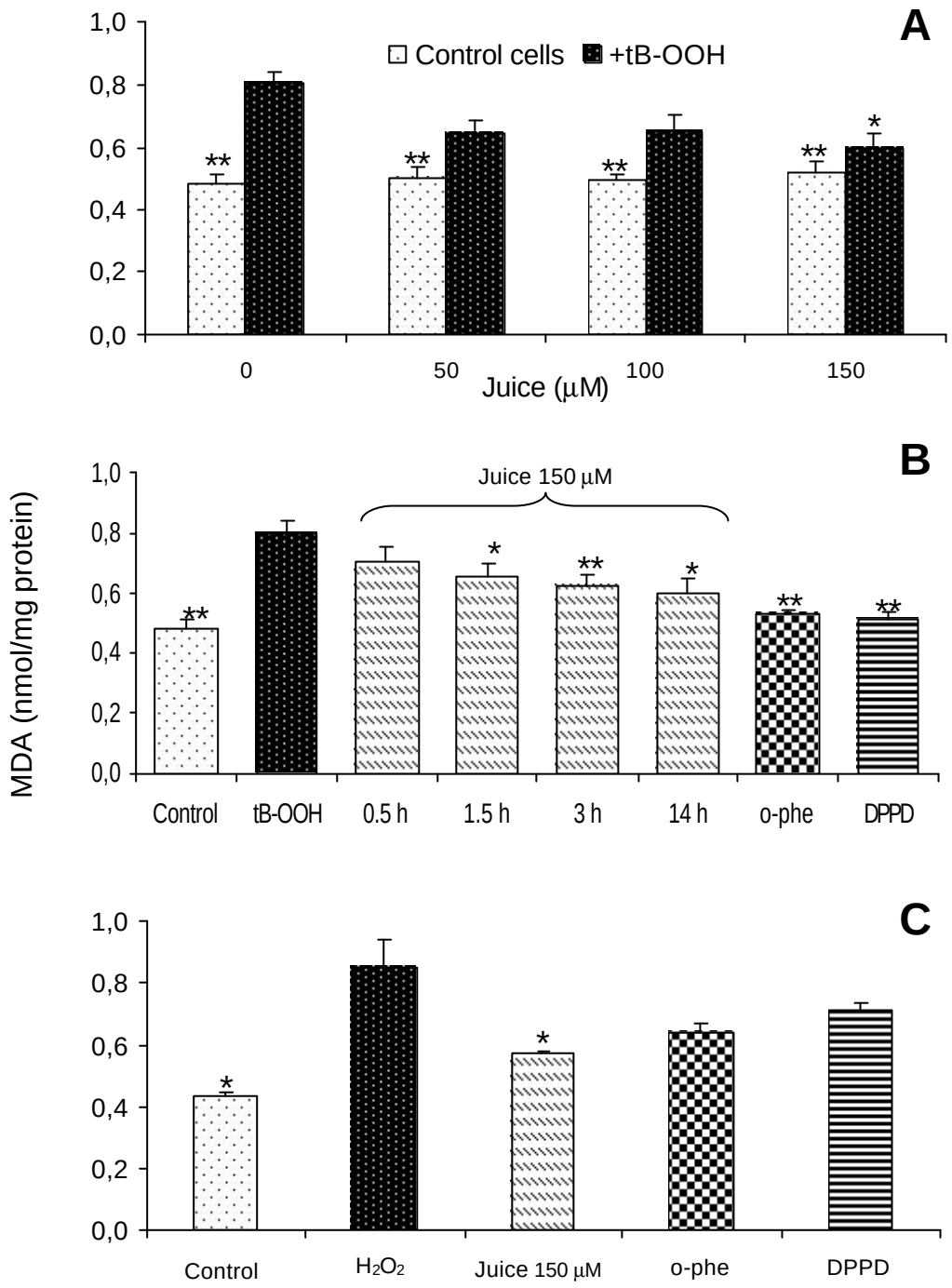

Figure 3. The effect of the phenolic-rich juice on oxidant-induced lipid peroxidation in HepG2 cells. (A) Cells preincubated with juice extracts ranging from 0 to 150 μM for 14 hours and challenged with 10 mM tB-OOH for 30 minutes. (B) Cells preincubated with the 150 μM juice extract for 0.5, 1.5, 3 or 14 hours, and challenged with 10 mM tB-OOH for 30 minutes. (C) Cells preincubated with the 150 μM juice extract for 3 hours and challenged with 20 mM H_2O_2 for 2 hours. DPPD (10 μM) and *o*-phe (25 μM) were added to the cultures 5 minutes prior to the addition of tB-OOH or H_2O_2. Results represent the mean±SEM of at least three separate experiments. *p <0.05, **p<0.01, compared with control cells exposed to tB-OOH or H_2O_2.

GSH-related enzyme activities

Figure 3 shows the effect of the phenolic-rich juice, *o*-phenanthroline and DPPD on tB-OOH-induced GPx and GST activation. Exposure to 10 mM tB-OOH for 30 minutes significantly ($p<0.01$) increased the activity of both enzymes, compared with control cells **(Figure 3 A-B)**. As illustrated in **Figure 3 A**, preincubation of cells with the 150 µM-juice extract for 3 hours significantly ($p<0.01$) reduced GPx activity. The intracellular iron chelator *o*-phenathroline partially reduced GPx activity ($p=0.057$), whereas DPPD did not suppress GPx activation. As shown in **Figure 3 B**, neither the juice nor *o*-phenantroline decreased the GST activity. Only addition of the radical scavenger DPPD (10 µM) to the cultures effectively suppressed the tB-OOH-induced increase in GST activity. Importantly, the juice extracts, DMSO, DPPD and o-phenanthroline alone did not affect the GPx or GST activities (data not shown).

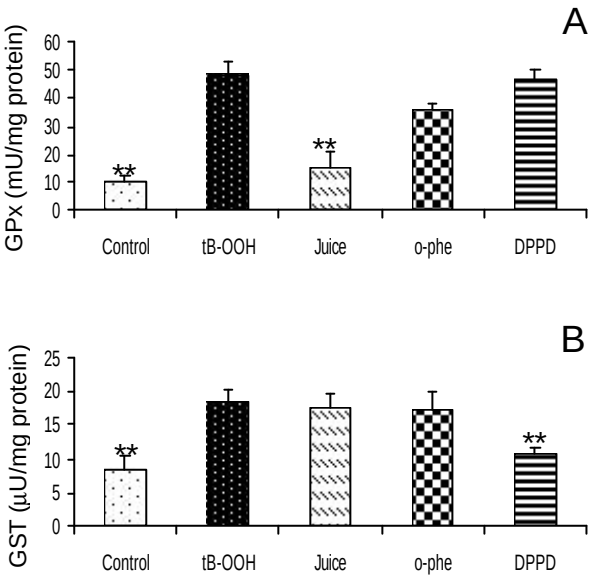

Figure 3. The effect of the phenolic-rich juice on glutathione-related enzyme activities in HepG2 cells. Cells ($2 \cdot 10^6$ cells/75 cm^2-cell culture flask) were grown to confluence in complete MEM and preincubated with the 150 µM-juice extract for 3 hours in serum-free MEM. Later, cells were challenged with 10 mM tB-OOH for 30 minutes in serum-free MEM. DPPD (10 µM) and *o*-phe (25 µM) were added to the culture 5 minutes prior to the addition of tB-OOH. After the treatments, cells were collected in PBS, disrupted by sonication and finally assayed for (A) glutathione peroxidase activity (GPx) and (B) glutathione-*s*-transferase activity (GST). Results represent the mean±SEM of at least three separate experiments. *$p<0.05$, **$p<0.01$, compared with control cells exposed to tB-OOH.

DISCUSSION

Polyphenols are used to represent anticarcinogenic food components in a human diet rich in fruits and vegetables. Previous studies carried out in cell culture systems have looked at the antiproliferative activities of commonly consumed fruits and vegetables, and have reported potent inhibitory effects on HepG2 cell proliferation after incubation with some food extracts, especially those of pigmented fruits such as grapes, raspberries, cranberries or strawberries (Chu et al., 2002; Liu et al., 2002; Sun et al., 2002; Meyers et al., 2003). In agreement with this observation, in the present study we observed a dose-dependent inhibitory effect on HepG2 cell growth after incubation with different concentrations of the juice, with an average EC_{50} value of 50 μg of phenolics per mL of cell growth medium. Interestingly, the arrest of HepG2 cell growth was not time-dependent and was already evident after a treatment lasting 24 hours. This indicates that the polyphenols in the juice promptly initiated a series of cellular events leading to the inhibition of cell proliferation and/or the induction of cell death. However, it should be noted that by using the MTT-assay it is not possible to differentiate between cell growth inhibition and an increase in cell death. In this regard, data from studies focusing on the elucidation of the molecular basis of the putative anticancer activity of polyphenols indicate that both the inhibition of cell growth and the induction of cell death play a role in the antitumour activity of polyphenols (Wenzel et al., 2000; Lazzè et al., 2004). Furthermore, data from other cell culture studies strongly suggest that the mechanism whereby phenolic compounds modulate cell proliferation is remarkably dose-dependent (Ferguson et al., 2004; Ramos et al., 2005). At low concentrations, cell growth arrest has been attributed to both the inhibition of cell replication and the induction of apoptotic cell death, whereas at high concentration has been attributed to a direct toxic effect, leading to necrotic cell death (Saleem et al., 2002).

Other research has examined the protective effects of phenolics against different oxidative insults in cell-culture systems. In our study, juice extracts at phenolic concentration equal to or below 150 μM had no effect on HepG2 cell growth. For this reason, this concentration range was chosen for subsequent experiments aiming to evaluate the cytoprotective effects of juice extracts against oxidative challengers. For that, HepG2 cells were preincubated with juice extracts at different sub-toxic concentrations of phenolic compounds and challenged with *tert*-butyl hydroperoxide (*t*-BOOH) or hydrogen peroxide (H_2O_2), two widely used models of *in vitro* oxidative stress. It is well known that H_2O_2 can directly damage DNA, lipids, and other macromolecules, causing oxidative injury to the cell. Similarly, the organic hydroperoxide tB-OOH induces an array of cellular dysfunctions,

including peroxidation of membrane lipids, glutathione and protein thiol depletion, alteration of calcium homeostasis, and DNA damage, eventually leading to cell death (Alia et al., 2005).

In agreement with other previously reported data (Nardini et al., 1998; Feng et al., 2002; Yau et al., 2002; Lazzè et al., 2003; Hong and Liu 2004; Sohn et al., 2005), the results of the present study demonstrated that cells preincubated with extracts of the phenolic-rich juice showed an increased resistance to oxidative challenge. This was revealed by an increase in cell survival and a decrease in the formation of lipid peroxidation products after exposure to both H_2O_2 and tB-OOH. Lipid peroxidation has been suggested to play an important role in the development of toxicity. It is thought that peroxidation of the cell membrane phospholipids and an accumulation of lipid peroxides alter membrane fluidity and permeability, leading to disruption of membrane structure and function (Sohn et al., 2005). In the present study, the protective effect exerted by the juice was observed after a short preincubation period (3 hours). Interestingly, the juice rapidly afforded protection from tB-OOH-induced lipid peroxidation, as revealed by the decrease in malondialdehyde production even after a preincubation period of 30 minutes, indicating that phenolics from the juice were rapidly taken up by cells, whose antioxidant defences were thus enhanced. This observation suggests that molecular features might aid this rapid absorption of phenolics from the juice. In particular, the molecule lipophilicity is expected to increase the cellular uptake of phenolics, as well as their localization in lipid compartments (Sestili et al., 2002).

Based on these observations, it is reasonable to think that the protective effect exerted by the juice might be strongly related to the ability of its polyphenols to inhibit lipid peroxidation. In this context, it is widely believed that the antioxidant capacity of polyphenols mainly resides in their ability to scavenge the free radicals generated during lipid peroxidation (Arora et al., 1998). In accordance with this premise, the juice showed strong antioxidant activity in terms of reducing capacity and the scavenging of free radicals in different *in vitro* tests **(Table 1).** However, in addition to its radical scavenging activity, polyphenols possess an ideal structural chemistry for metal chelation, supporting the role of polyphenols as preventative antioxidants in terms of inhibiting transition-metal catalyzed free radical formation (Rice-Evans et al. 1997). In this regard, it has been shown that cell death induced by both H_2O_2 and tB-OOH depends on a cellular source if iron. Both peroxides react with ferrous iron to produce more potent oxidants, the hydroxyl and *t*-butyl alkoxyl radicals, respectively, by Fenton chemistry (Farber et al., 1990). Thus, iron chelation will decrease the toxicity of oxygen to cells and may contribute to the cytoprotective activity of phenolic compounds. Accordingly, dietary plant phenolics such as quercetin or various caffeic acid

derivatives have been reported to protect from H_2O_2- and tB-OOH-induced toxicity by an iron-chelating mechanism (Sestili et al., 2002). Furthermore, anthocyanins (Lazzè et al., 2003) and caffeic acid (Nardini et al., 1998) protect cell systems from tB-OOH-induced toxicity and lipid peroxidation.

It is generally thought that the following functional groups are important for iron chelation: (A) *o*-dihydroxyl groups, e.g. 3'-4', 7–8 dihydroxy groups; (B) the presence of 5-OH and/or 3-OH in conjunction with a C4 keto group (e.g. quercetin), and (C) a large number of OH groups (e.g. tannic acid) (Khokhar and Apenten 2003). Of these, the presence of two hydroxyl groups in the *o*-position has been reported to be critical for the chelation of iron and the protection afforded by phenolic compounds (Sestili et al., 2002). This essential structural feature is shared by flavonoids (e.g. quercetin, catechins and anthocyanins) and caffeic acid. For this reason, it is reasonable to think that the protective effects exerted by these polyphenols might partly be explained by an iron-chelating activity. Importantly, as can be seen in **Table 1**, anthocyanins, caffeic acid derivatives and catechins were well represented in the juice. Since these polyphenols possess ideal structural features for iron chelation, the possible role of iron-chelating activity in the protection afforded by the juice should not be ruled out. For comparison, in the present study, we also evaluated the ability of the radical scavenger DPPD and the intracellular iron chelator o-phenathroline to prevent lipid peroxidation. As illustrated in **Figure 2 B-C**, under our experimental conditions, both DPPD and o-phenanthroline prevented lipid peroxidation caused by either H_2O_2 or tB-OOH. These data indicate that both radical scavenging and iron chelation play a role in the prevention of the lipid peroxidation caused by H_2O_2 and tB-OOH. However, our data do not allow us to conclude whether these effects are mediated by a radical scavenging or an iron chelating mechanism, or by a combination of these two activities.

To further characterize the protective effects exerted by the phenolic-rich juice, we also evaluated changes in endogenous enzyme activities, which are considered a fairly sensitive biomarker of the cellular response to oxidative stress. For the experiments, tB-OOH was chosen as oxidative stressor because it has been shown to efficiently increase the activity of endogenous enzymes in HepG2 cells (Alía et al., 2005). The cellular defence system (including glutathione, glutathione-related enzymes, and antioxidant and redox enzymes) plays a crucial role in cell survival and growth in aerobic organisms. Reduced glutathione (GSH), the major intracellular thiol, is believed to be an important protector against free radical damages by providing reducing equivalents for antioxidant enzymes and also by scavenging hydroxyl radicals and singlet oxygen (Lee et al., 2002). Among the GSH-related enzymes, in the present study we measured the activity of glutathione peroxidase

(GPx), and glutathione-*s*-transferase (GST). The enzyme GPx participates in the detoxification of tB-OOH by converting tB-OOH to *t*-butanol and oxidized glutathione (GSSG) (Sohn et al., 2005). When formed, GSSG is quickly recycled back to GSH by the enzyme glutathione reductase (Alia et al., 2005). The enzyme glutathione-*s*-trasferase (GST) catalyzes the conjugation of reduced glutathione (GSH) to a wide variety of hydrophobic molecules, thereby reducing lipid hydroperoxides and providing protection from membrane lipid peroxidation (Marí and Cederbaum 2001). Increases in the activities of these GSH-related enzymes have been reported in cell systems after exposure to tB-OOH and concomitant with a decrease in reduced GSH levels (Aniya and Daido 1994; Alía et al., 2005).

In agreement with these findings, we observed a significant increase in both GPx and GST activity after exposure to tB-OOH. As shown in **Figure 3 A-B**, preincubation with the 150 µM-juice extract effectively suppressed the increase in GPx activity, but not that of GST. Consistent with our results, Murakami et al. (2002) reported the ability of tea catechins to suppress the tB-OOH-induced increase in GPx activity in HepG2 cells. Thus, as regards GPx activity, phenolics in the juice might exert their protective effect by delaying the consumption of GSH and/or other cellular antioxidants, during the process of tB-OOH detoxification. Accordingly, the plant polyphenolic compounds, panduratin A and sylibin, as well as plant extracts showing high antioxidant activity have been shown to reverse the GSH-depleting effect of tB-OOH in hepatocyte systems (Yau et al., 2002; Sohn et al., 2005). Furthermore, as illustrated in **Figure 3 A**, the radical scavenger DPPD did not suppress the tB-OOH-mediated increase in GPx activity, whereas the intracellular iron chelator *o*-phenanthroline slightly, but not significantly, reduced the activity of GPx. This suggests that phenolics in the juice might have acted more like iron-chelators than radical scavengers. In agreement with this supposition, only DPPD was able to suppress GST activation after exposure to tB-OOH **(Figure 3 B)**. Neither the 150 µM-juice extract nor *o*-phenanthroline demonstrated a suppressive effect on GST activation. This indicates that, under our experimental conditions, iron chelation seemed to have no effect on the tB-OOH-induced activation of GST.

Consequently, since the juice behaved more like the iron chelator *o*-phenanthroline, we could reasonably hypothesize that the antioxidant properties of the phenolics relied more on their iron-chelating abilities than on radical scavenging activities. In this context, previous data reported by Sestili et al. (2002) regarding cell death and DNA cleavage might support our hypothesis. These authors observed that U937 cell death caused by tB-OOH is prevented by both radical scavengers (DPPD, trolox and butylated hydroxytoluene) and iron

chelators (*o*-phenanthroline), whereas cell death caused by H_2O_2 is only prevented by iron chelators. Similar results have been reported in renal epithelial cells (Park et al., 2003). In agreement with this notion, Sestili et al. (2002) also observed that among the polyphenolic compounds tested only those showing iron-chelating activity were effective in protecting from H_2O_2-induced cell death. As noted above, in our study juice extracts protected cells from both oxidative insults. Moreover, in a previous work carried out in U937 cells, we evaluated the protective effects of this phenolic-rich juice against tB-OOH-induced oxidative stress (García-Alonso et al. unpublished data). Our main finding was that juice extracts, DPPD and *o*-phenanthroline offered protection against tB-OOH-induced cell death, whereas only juice extracts and *o*-phenanthroline prevented the DNA cleavage caused by tB-OOH. This observation is consistent with the notion that DNA cleavage provoked by tB-OOH is abolished by iron chelators but is insensitive to radical scavengers, whereas tB-OOH-induced cell death is prevented by both radical scavengers and iron chelators (Sestili et al., 2002). Taken together, these data could indicate a more prominent iron-chelating activity in the protection afforded by the phenolic-rich juice. Although iron chelation has generally been regarded as playing a minor role in the antioxidant activity of polyphenols, the presence of iron chelating groups in food and their efficiency in iron chelation may partly explain the health protective role of specific phenolics in the human diet (Duthie et al. 2000; Prior and Cao 2000).

CONCLUSIONS

The data we report above, in conjunction with data reported by other investigators, clearly indicate that dietary constituents can exert significant modulatory effects on cell proliferation, cytotoxicity and oxidative reactions in cellular systems. Under our experimental conditions, phenolics in the functional juice afforded protection against oxidant-induced cytotoxicity, lipid peroxidation, and glutathione peroxidase activation, even after a short preincubation period. This indicates that phenolics from the juice were rapidly absorbed by cells and contributed to the cellular antioxidant defences. The results obtained support the efficacy of natural phenolics from grapes and berries in offering protection against oxidative stress and highlight the fact that phenolic-rich processed foods may provide health benefits. The possible role of an iron chelating mechanism, potentially of biological relevance, is proposed as being partially responsible for these health benefits.

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3.3. Estudio 3

Phenolic-rich juice prevents DNA single-strand breakage and cytotoxicity caused by *tert*-butyl hydroperoxide in U937 cells via an iron-chelating mechanism

This work was carried out at the Institute of Pharmacology of the University of Urbino (Italy) and was supervised by Dr. Orazio Cantoni.

ABSTRACT

Flavonoids, especially anthocyanins, are the main phenolics in pigmented fruits and are recognized as potent antioxidants. The aim of the present study was to evaluate the ability of a phenolic-rich juice made from grape, cherries and berries to protect U937 cells from oxidative stress caused by *tert*-butyl hydroperoxide (tB-OOH). Preincubation of cells with extracts of the phenolic-rich juice at different concentrations (0-200 μM of ferulic acid equivalents) for 3 hours prevented cell death and abolished the DNA cleavage induced by tB-OOH. Moreover, when preincubating cells with the 100 μM-juice extract (the dose which diminished cell death by around 50%), a partial prevention of tB-OOH-induced mitochondrial permeability transition (MPT) pore opening and mitochondrial formation of H_2O_2 was observed. The radical sacavenger *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD) and the intracellular iron chelator *o*-phenanthroline (*o*-Phe) were also tested with the aim of elucidating the mechanisms of action of phenolics in the juice. *o*-Phe (25 μM) prevented cell death, DNA cleavage, and H_2O_2 generation, whereas DPPD (10 μM) only prevented cell death. Therefore, under the experimental conditions used, phenolics in the juice afforded protection against induced cytotoxicity, DNA cleavage, MPT pore opening and intracellular H_2O_2 generation, most probably by means of an iron chelating mechanism. The results obtained underline the efficacy of natural phenolics from grapes and berries to protect against oxidative stress and highlight the fact that phenolic-rich processed foods may provide considerable benefits to health.

Key words: Phenolics, iron chelators, radical sacavengers, DNA single-strand breakage, cell death, *tert*-butyl hydroperoxide

INTRODUCTION

Several human chronic disease states have been associated with oxidative stress, which occurs in a cell or tissue when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability of that cell (Sies, 1991). A role for reactive oxygen radicals in the aetiology of cancer is supported by epidemiologic studies, since the oxidative damage to DNA produce mutations which lead to a ROS-induced carcinogenesis (Breimer, 1990). Different world organizations (World Health Organization and World Cancer Research Foundation) have recommended an increase in the intake of dietary antioxidants, with the aim of preventing cancer and many chronic diseases. The human diet contains an array of different compounds that possess antioxidant activities or that have been suggested as ROS scavengers based on their structural properties. The most prominent representatives of dietary antioxidants are ascorbate (vitamin C), tocopherol (vitamin E), carotenoids and phenolic compounds (Diplox et al., 1998). Fruits and vegetables provide a wide range of these antioxidants, whose synergistic effects provide several benefits for human health.

Several plant phenolics have been found to show anticancer activity and to protect against oxidant-induced damage in cell-culture models. Studies performed in U937 cells have shown that flavonoids and several hydroxycinnamic acids (caffeic acid esters) afford protection against *tert*-butylhydroperoxide (tB-OOH)-induced cell death and DNA damage (Sestili et al., 1998; 2000). In addition, anthocyanins have been shown to protect against tB-OOH-induced cell death and DNA damage in the rat smooth muscle (SMC) and rat hepatoma (MH1C1) cell lines (Lazzè et al., 2003). Other studies developed with the Caco-2 and HepG2 cell lines have reported the ability of flavonoids, such as quercetin, myricetin and rutin, to protect against DNA damage caused by tB-OOH, H_2O_2 and menadione (Aherne and O'Brien 1999; 2000). Phenolics from black and green tea have been investigated in many cell culture models, where they have demonstrated a protective effect against oxidant-induced DNA damage (Johnson and Loo 2000; Feng et al., 2002; Jimenez-López and Cederbaum, 2004). However, such biological effects have not only been observed with chemical phenolic compounds but also in phenolic-rich foodstuffs. In this context, phenolics from berries have shown protective effects against H_2O_2–induced intracellular ROS generation in human endothelial cells (Miranda-Rottmann et al., 2002), and this type of fruit is considered a rich dietary source of phenolic antioxidants.

Nowadays, there is growing interest among scientists, food manufacturers and consumers in the study of food properties for maintaining human health (Karakaya et al., 2001). Any effects have to be scientifically demonstrated with human intervention studies

(Roberfroid, 2002), and functional foods need to be evaluated from a toxicological point of view. For this, *in vitro* cell-culture systems can be used to assess cytotoxicity and cellular responses and to perform toxic kinetic modeling (Glei et al., 2003), since they are a valuable tool for elucidating the mechanisms of action of the natural antioxidants present in fruit-derived functional foods. The experimental *in vitro* analysis of biological activities of whole foods will therefore serve several purposes. On the one hand, the determination of toxicity can be used as a tool to define the concentrations at which chemoprotective effects can be further characterized, and on the other hand, cells can be treated with sub-toxic concentrations of the compounds to identify new cellular responses, among them mechanisms of potential chemoprevention (Glei et al., 2003).

In previous studies we have ascertained the *in vitro* and *in vivo* antioxidant properties of a phenolic-rich dessert made with grapes, cherries and berries (García-Alonso et al., 2003; Ramirez-Tortosa et al., 2004). The aim of the present study was to evaluate the ability of a phenolic-rich juice, prepared by using the above formulation, to protect U937 cells against oxidative DNA damage caused by *tert*-butylhydroperoxide, an organic hydroperoxide widely used as a model compound to induce oxidative stress, and to throw light on the antioxidant mechanism of phenolic compounds to prevent the DNA damage.

MATERIALS AND METHODS

Description of the juice

The experimental juice was prepared by the Department of Research and Development of Hero Spain S.A (Alcantarilla, Murcia, Spain). The major ingredient was water, which was mixed with commercially available concentrated juices of grape, cherry, blackberry, blackcurrant and raspberry. The resulting product was pasteurized in order to obtain a microbiologically stable product and hot-bottled to ensure headspace vacuum. Phenolic compounds and total antioxidant activity were evaluated in the juice prior to the cell culture assays. The mean content of phenolic compounds was 3000 mg/kg or 15000 μM of ferulic acid equivalents, and total antioxidant activity value was 25 mM of 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxilic acid (Trolox) equivalent antioxidant capacity (TEAC). All the characteristics of this product have been previously published by the authors (García-Alonso et al., 2003).

Cell culture and treatments

Reagent-grade chemicals and *tert*-butylhydroperoxide (tB-OOH) were obtained from Sigma-Aldrich (Milan, Italy). Stock solutions of tB-OOH were freshly prepared in water. *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD) and *o*-phenanthroline (*o*-Phe) were dissolved in dimethyl sulfoxide (DMSO), and cyclosporyn A (CsA) in ethanol. U937 (human myeloid leukaemia) cells were cultured in suspension in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, penicillin (50 UI/mL) and streptomycin (50 µg/mL), at 37°C in a humidified atmosphere of 95% air-5% CO_2. For the experiments, two batches of cells ($2.5x10^5$ cells/mL) were used. One batch was used to assess the effect of preincubation with extracts of the phenolic-rich fruit juice (juice-preloaded cells). For that, cells were exposed for 3 hours to juice extracts at different concentrations of total phenolics in 2 mL of RPMI 1640 medium. The extracts were obtained by directly diluting the juice in RPMI 1640 medium. The other batch was used as control as well as to assess the effect of the exposure to DPPD, *o*-Phe and CsA. After being washed, cells were treated for 15 or 30 minutes at 37°C in 2 mL of saline A buffer (8.812 g/L NaCl, 0.372 g/L KCl, 0.336 $NaHCO_3$, and 0.9 g/L glucose) containing tB-OOH. After each treatment, the cells were washed and analyzed immediately for DNA damage and for dihydrorhodamine 123 (DHR) oxidation and calcein staining, or post-incubated for the cytotoxicity assays. DPPD, *o*-Phe and CsA were added to the cultures 5 minutes prior to the addition of tB-OOH. At the treatment stage, the final DMSO and ethanol concentration was never higher than 0.1%. Under these conditions, DMSO and ethanol were neither cytotoxic nor DNA damaging, nor did they affect the cyto-genotoxic properties of tB-OOH.

Cytotoxicity assay

After the treatment with tB-OOH for 30 minutes, the cells were washed with saline A and resuspended in prewarmed RPMI 1640 medium (2 mL) before being plated into 60-mm tissue culture dishes and incubated at 37°C for 4 hours. Cytotoxicity was determined with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue (Sigma) and the cells were counted with a haemocytometer. The results are expressed as the percentage of dead cells (ratio of stained cells versus the total number of cells).

Measurement of DNA single-strand breakage

After treatment with tB-OOH for 30 minutes, cells were analyzed immediately for DNA single-strand breakage by using the Alkaline-halo assay (Sestili and Cantoni 1999), with minor modifications. The alkaline-halo assay allows the measurement of DNA lesions at the

single cell level and its sensitivity is remarkably similar to that of the widely used alkaline elution and comet assays. After the treatments, the cells were resuspended at 2 $x10^4$ cells/100 µL in low-melting-point agarose in phosphate buffered saline containing 5 mM EDTA, and immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling, the coverslips were removed and the slides were immersed in an alkaline buffer (0.1 M NaOH, pH 13) for 10 minutes. Afterwards, the slides were washed and stained for 5 min with 10 µg/mL ethidium bromide.

Fluorescence images were captured with a BX-51 microscope (Olympus) equipped with an SPOT-RT camera unit (Diagnostic Instruments). The excitation and emission wavelength were 488 and 515, respectively, with a 5-nm slit width for both emission and excitation. The images were collected with exposure times of 100-400 milliseconds, digitally acquired and processed for fluorescence determination at the single cell level on a personal computer using Scion Image software (Scion Corp., Frederick, MA, USA). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment/experiment. The level of DNA single-strand breakage was quantified by calculating the nuclear spreading factor values, which represent the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area, nucleus plus halo) and that of the nucleus. Single-stranded DNA fragments spread radially from the nuclear cage and generate a fluorescent image that resembles a halo concentric to the nucleus remnants. The area of the halos increases with increasing DNA fragmentation, a process that is associated with a progressive reduction in the areas of the nuclear remnants. Data are expressed as relative nuclear spreading factor (RNSF) values calculated by subtracting the nuclear spreading factor values of control cells from those of treated cells.

Dihydrorhodamine 123 (DHR) oxidation and calcein staining and imaging.

After treatment with tB-OOH for 15 minutes, cells were analyzed immediately for the formation of H_2O_2 using the fluorescent probe dihydrorhodamine 123 (Emmendörffer et al., 1990, Tommasini et al., 2004) and for mitochondrial permeability transition (MPT) pore opening (Petronilli et al., 1999). Cells ($2.5x10^5$/mL) were pre-treated for 15 minutes with 250 µM tB-OOH and then post-incubated for 10 minutes in the presence of 10 µM DHR or 1 µM calcein-AM and 1 mM $CoCl_2$. After washing three times, the cells were resuspended in 20 µL of saline A and stratified on a slide. Fluorescence images were captured with a BX-51 microscope (Olympus) equipped with an SPOT-RT camera unit (Diagnostic Instruments). The excitation and emission wavelength were 488 and 515, respectively, with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100-

400 milliseconds, digitally acquired and processed for fluorescence determination at the single cell level on a personal computer using Scion Image software (Scion Corp., Frederick, MA, USA). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment/experiment.

Statistical Analysis

Data were analyzed by Statistical Package SPSS 11.0 version for Windows (SPSS Inc., Chicago, IL, USA). A Student's *t*-test was used to evaluate differences between the test samples and positive controls.

RESULTS

Prevention of tB-OOH induced cytotoxicity

Figure 1 shows the effect of preincubation with extracts of a phenolic-rich juice designed as a functional food on U937 cell death caused by tB-OOH. Preincubation of cells with juice extracts at different concentrations for 3 hours partially abolished the cell death induced by tB-OOH. As illustrated in **Figure 1A**, treatment with increasing concentrations of tB-OOH for 30 minutes increased cell death in a dose-dependent manner. Conditions were chosen so that 30 minutes' incubation at a tB-OOH concentration of 250 μM decreases cell viability by about 60%. The cytotoxic effect of tB-OOH was reduced by around 50% in cells preloaded with juice extracts at a phenolic concentration of 100 μM of ferulic acid equivalents. As shown in **Figure 1B**, treatment with 250 μM tB-OOH for 30 minutes was highly toxic in U937 cells. This cytotoxic effect of tB-OOH was partially mitigated in cells preloaded with juice extracts at different concentrations, reaching a plateau when cells were preincubated with juice extracts at a phenolic concentration of 50 μM or above. Importantly, juice extracts alone did not promote cytotoxicity at any of the concentrations tested (data not shown). As illustrated in **Figure 1C**, under similar experimental conditions, the antioxidant DPPD (10 μM), the intracellular iron chelator o-Phe (25 μM) and the immunosuppressive drug CsA (0.5 μM) also promoted cytoprotection.

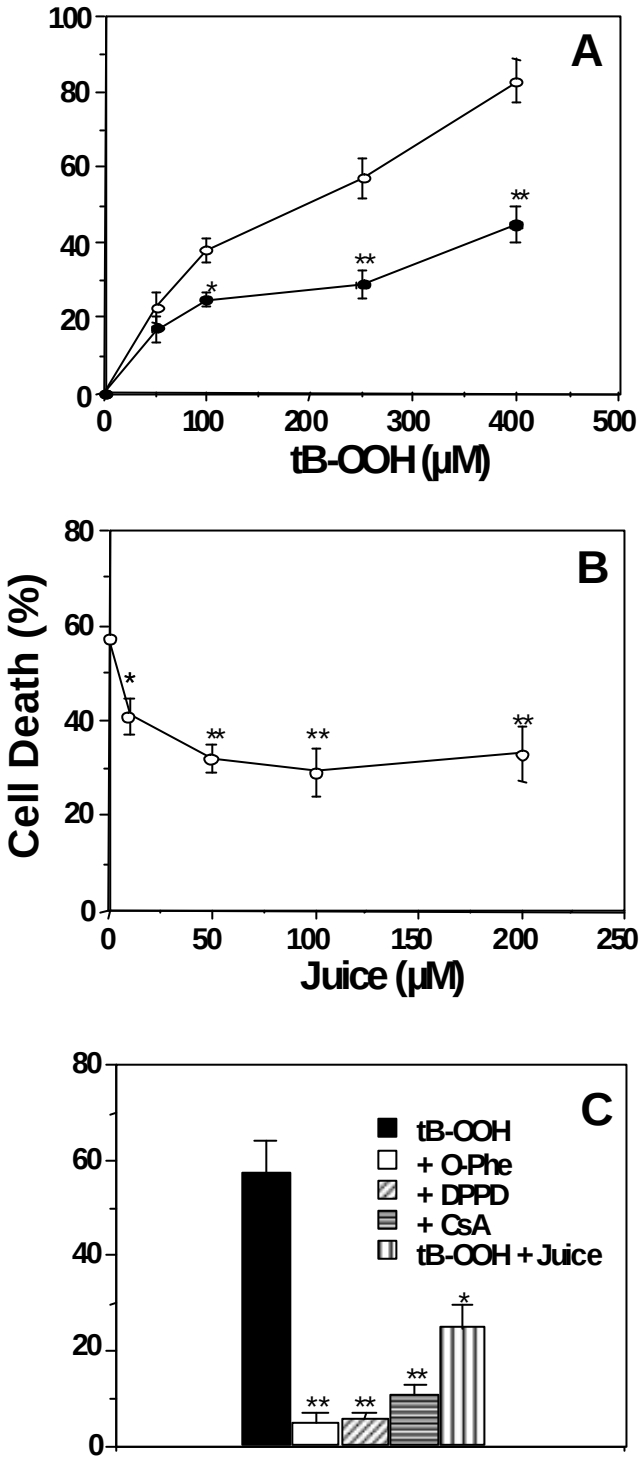

Figure 1. The effect of the phenolic-rich juice, *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD), *o*-phenantroline (*o*-Phe) and cyclosporyn A (CsA) on tB-OOH-induced cytotoxicity. (A) U937 control cells and juice-preloaded cells (at a concentration of phenolics of 100 µM of ferulic acid equivalents) were plated at a density of $2.5x10^5$ cells/mL in saline A and exposed for 30 minutes to different concentrations of tB-OOH. (B) Control and juice-preloaded cells (at different concentrations of phenolics) were exposed to 250 µM tB-OOH for 30 minutes in saline A. (C) Control cells, 100 µM-juice-preloaded cells, and control cells exposed for 5 minutes to 10 µM DPPD, 25 µM *o*-Phe and 0.5 µM CsA, respectively, were exposed to 250 µM tB-OOH for 30 minutes in saline A. The relative number of dead cells was measured after 4 hours of post-challenge growth in fresh culture medium using the trypan blue assay. Results represent the mean ± SEM of at least three separate experiments. *p <0.05, **p<0.01, compared with control cells exposed to tB-OOH.

Prevention of DNA single-strand breakage

Figure 2 shows the effect of preincubation with extracts of the phenolic-rich juice on DNA cleavage produced by tB-OOH. Preincubation of cells with juice extracts at different concentrations for 3 hours prevented the DNA strand scission caused by tB-OOH. **Figures 2A** to **2F** shows nuclear spreading factors and representative photomicrographs of ethidium bromide-stained nuclei. As shown in **Figure 2B**, treatment with 250 μM tB-OOH for 30 minutes was highly DNA damaging and, consequently, led to an increase in the nuclear spreading factor compared to with that observed in untreated control cells **(Figure 2A)**. As shown in **Figure 2D**, preincubation with the 10 μM-juice extract virtually abolished the extensive DNA cleavage caused by tB-OOH. As illustrated in **Figure 2C**, the 10 μM-juice extract alone (juice control) was not DNA damaging and showed a nuclear spreading factor similar to that of the untreated control cells. **Figure 2E** demonstrates that the intracellular iron chelator *o*-Phe (25 μM) abolished tB-OOH-induced DNA cleavage, whilst in **Figure 2F** it can be seen that an antioxidant such as DPPD (10 μM) was not able to protect against such cleavage. As illustrated in **Figure 2G**, the preincubation of cells with extracts of the phenolic-rich juice at different concentrations for 3 hours effectively reduced tB-OOH-induced DNA strand scission in a dose dependent manner. A *plateau* was reached when cells were preincubated with juice extracts at a phenolic concentration of 10 μM or above. Moreover, juice extracts alone were not DNA damaging at any of the concentrations tested (data not shown).

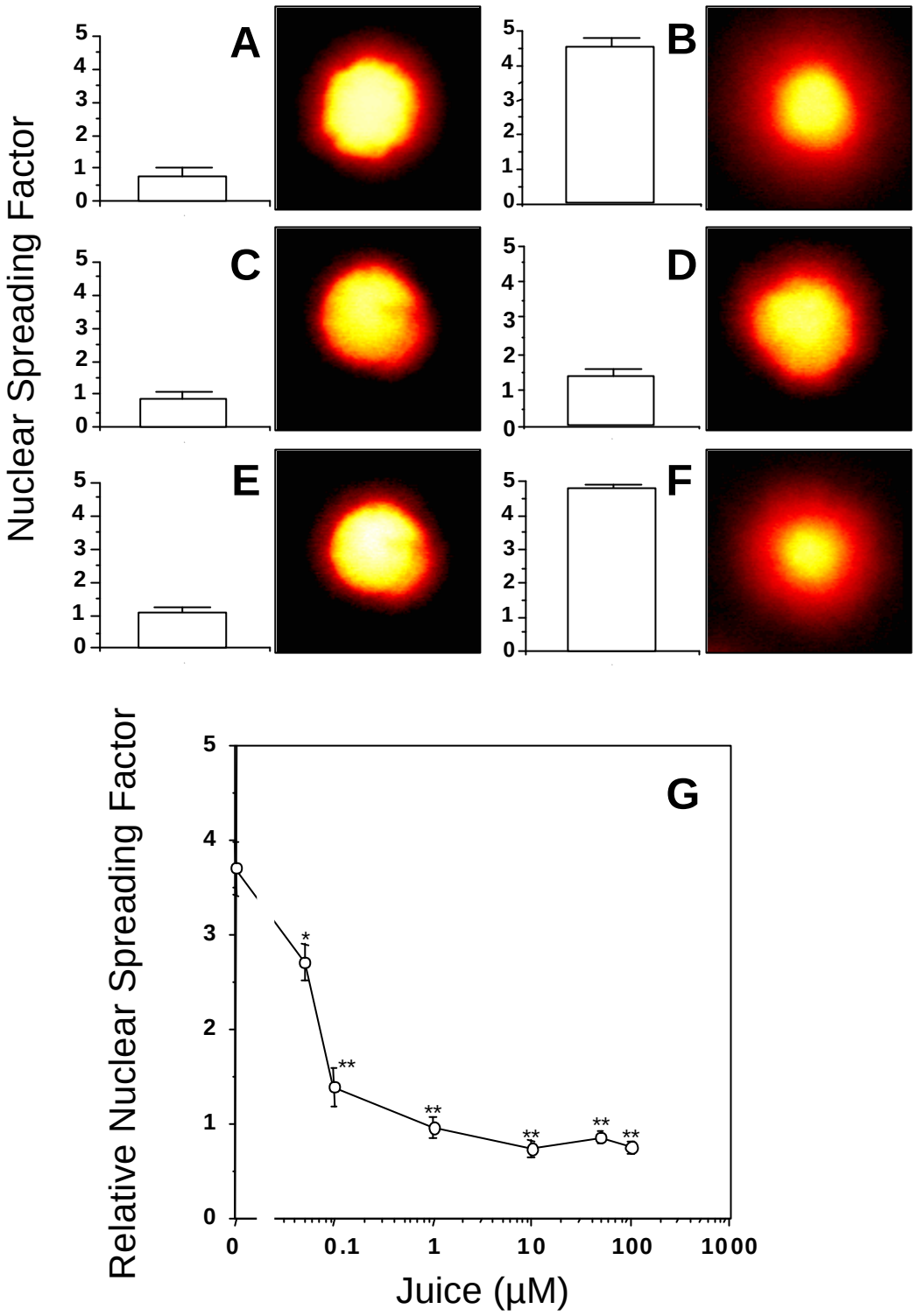

Figure 2. The effect of the phenolic-rich juice, *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD) and *o*-phenantroline (*o*-Phe) on tB-OOH-induced DNA single-strand breakage. (A-F) Representative photomicrographs and nuclear spreading factors of U937 cells under the experimental conditions detailed as follows: (A) Control cells not exposed to tB-OOH, (B) control cells exposed to 250 µM tB-OOH for 30 minutes, (C) 10 µM-juice preloaded cells unexposed to tB-OOH (juice control), (D) 10 µM-juice preloaded cells exposed to 250 µM tB-OOH for 30 minutes, (E and F) control cells treated for 5 minutes with 25 µM *o*-Phe and 10 µM DPPD, respectively, and then exposed to 250 µM tB-OOH for 30 minutes. (G) Control and juice-preloaded cells (at different concentrations of phenolics) were exposed to 250 µM tB-OOH for 30 minutes in saline A. Results are expressed as the relative nuclear spreading factor and represent the mean ± SEM of at least three separate experiments. *p <0.05, **p<0.01, compared with control cells exposed to tB-OOH.

Prevention of intracellular (mitochondrial) reactive oxygen species (ROS) generation

Figure 3 shows the effect of the phenolic-rich juice, *o*-Phe and DPPD on tB-OOH-induced intracellular H_2O_2 generation. The formation of H_2O_2 was assessed as a measure of intracellular oxidative stress by using the fluorescent probe dihydrorhodamine 123, which accumulates in the mitochondria and fluoresces when oxidized by various species, including H_2O_2 (Emmendörffer et al 1990). The results shown in the bar graph in **Figure 3G** are illustrated in panels A-F. **Figure 3B** shows a representative photomicrograph of control cells exposed to 250 μM tB-OOH in which the fluorescence is much stronger than in the untreated control cells of **Figure 3A**. Exposure to tB-OOH significantly increased the intracellular oxidative stress and led to an increase in DHR-derived fluorescence, due to DHR oxidation. As shown in **Figure 3C**, the addition of the iron chelator *o*-Phe (25 μM) to the cultures inhibited the formation of H_2O_2, giving a fluorescence intensity similar to that of untreated control cells. However, as shown in **Figure 3D**, the radical scavenger DPPD (10 μM) did not inhibit intracellular oxidative stress and the observed DHR-derived fluorescence was similar to that of control cells exposed to tB-OOH. As illustrated in **Figure 3F**, preincubation of cells with the 100 μM-juice extract for 3 hours partially inhibited the formation of H_2O_2, leading to a lower fluorescence intensity after exposure to tB-OOH. Importantly, as shown in **Figure 3E**, the juice extract alone did not increase the formation of H_2O_2 and gave a fluorescence intensity similar to that of untreated control cells.

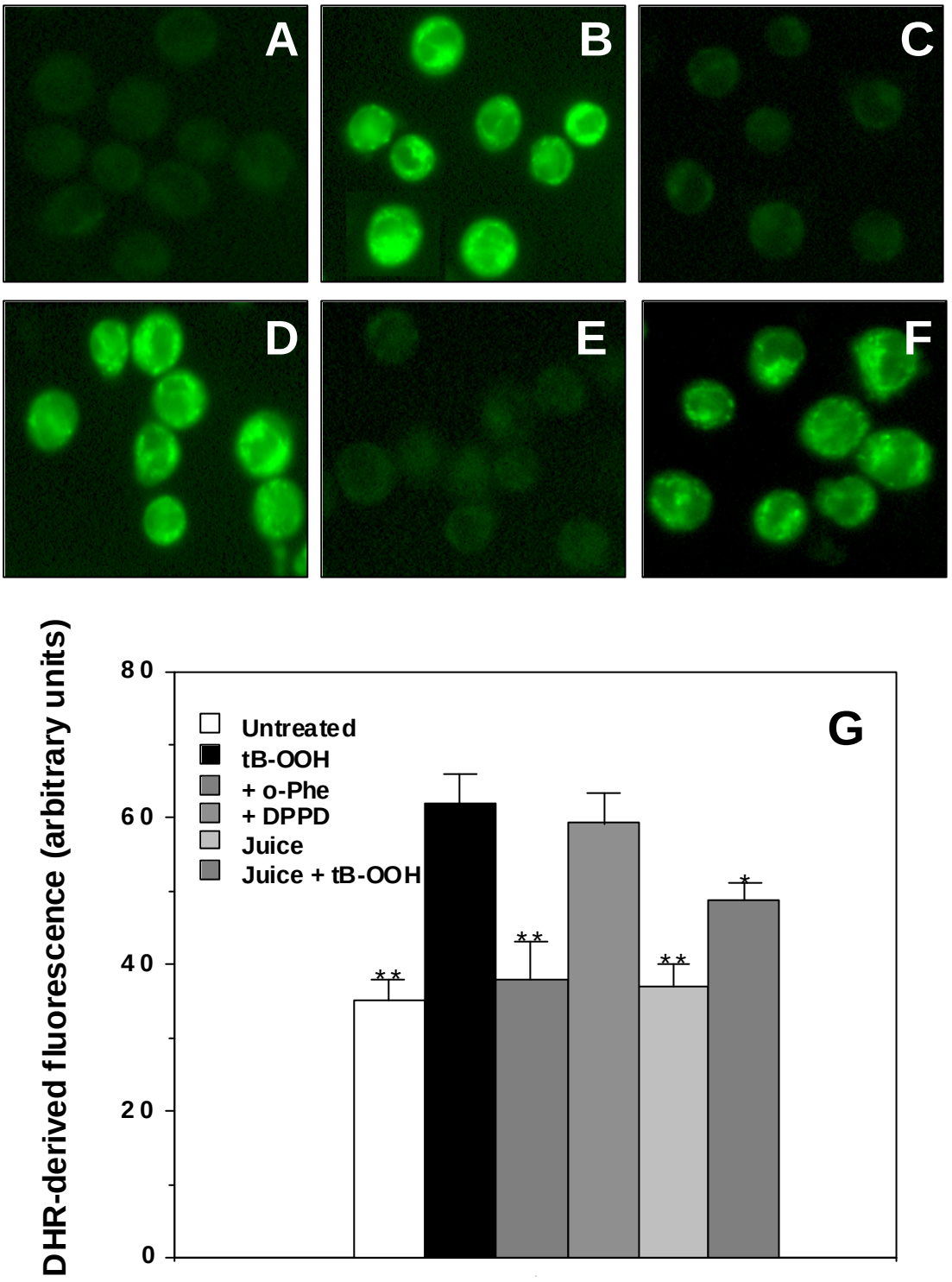

Figure 3. The effect of the phenolic-rich juice, *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD) and *o*-phenantroline (*o*-Phe) on tB-OOH-induced DHR-derived fluorescence in U937 cells. (A-F) Representative photomicrographs of U937 cells pre-treated or not for 15 minutes with 250 μM tB-OOH and then post-incubated for 10 minutes in the presence of 10 μM DHR. (A) Control cells unexposed to tB-OOH, (B) control cells pre-treated for 15 minutes with 250 μM tB-OOH, (C and D) control cells pre-exposed for 5 minutes to 25 μM *o*-Phe and 10 μM DPPD, respectively, and then exposed to 250 μM tB-OOH for 15 minutes (E) 100 μM juice-preloaded cells not exposed to tB-OOH (juice control), (F) 100 μM juice-preloaded cells which exposed to 250 μM tB-OOH for 15 minutes. (G) Quantification of DHR-derived fluorescence under the experimental conditions described above. Results represent the mean ± SEM of at least three separate experiments. *p <0.05, **p<0.01, compared with control cells exposed to tB-OOH.

Prevention of mitochondrial permeability transition (MPT) pore opening

Figure 4 shows the effect of the phenolic-rich juice and cyclosporin A on tB-OOH-induced MTP pore opening in U937 cells. MPT pore opening was assessed by monitoring the changes in mitochondrial calcein fluorescence after quenching the cytosolic and nuclear signal with Co^{2+} (Petronilli et al., 1999). As illustrated in **Figure 4A**, the resulting punctuate fluorescence was caused by the calcein localized in the mitochondrial compartment. As can be seen from **Figure 4B**, the loss of mitochondrial calcein therefore acts as a strong indication of MPT pore opening. This event was clearly detected in cells exposed to 250 µM tB-OOH for 15 minutes, but was not observed in unexposed control cells **(Figure 4A)**. The image shown in **Figure 4B** should be compared to that shown in **Figure 4C**, in which the presence of the cells is demonstrated by darkening the digital image. As illustrated in **Figure 4D**, MPT pore opening was fully prevented by the mitochondrial permeability transition inhibitor CsA (0.5 µM). As shown in **Figure 4E**, preincubation of cells with the 100-µM juice extract alone (juice control) did not cause MPT pore opening, while MPT pore opening was partially prevented in 100-µM juice preloaded cells exposed to tB-OOH **(Figure 4F)**. The image shown in **Figure 4F** should be compared to that shown in Figure 4G, in which the presence of the cells is demonstrated by darkening of the digital image.

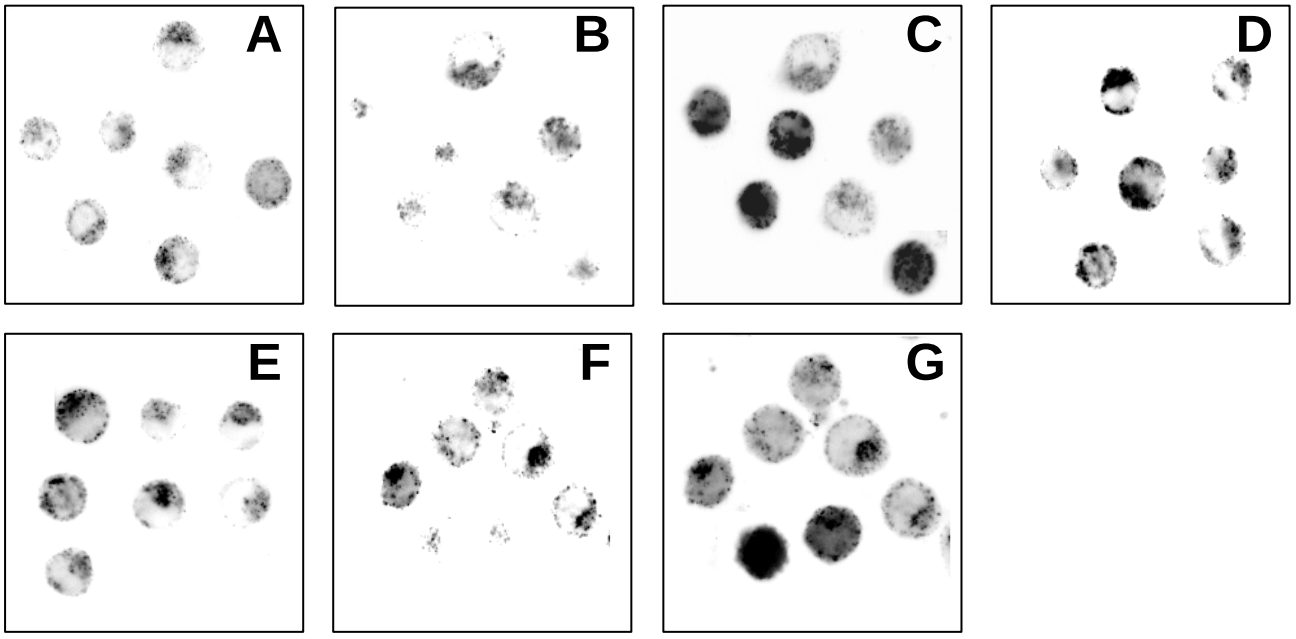

Figure 4. The effect of the phenolic-rich juice and cyclosporin A on tB-OOH-induced mitochondrial permeability transition (MPT) pore opening in U937 cells. Representative photomicrographs of U937 cells pre-treated or not for 15 minutes with 250 µM tB-OOH and then post-incubated for 10 minutes in the presence of 1 µM calcein-AM and 1 mM $CoCl_2$. (A) Control cells not exposed to tB-OOH, (B) control cells exposed to 250 µM tB-OOH for 15 minutes, (D) control cells pre-exposed for 5 minutes to 0.5 µM CsA and then pre-treated for 15 minutes with 250 µM tB-OOH, (E) 100 µM juice-preloaded cells not exposed to tB-OOH (juice control), (F) 100 µM juice-preloaded cells exposed to 250 µM tB-OOH for 15 minutes. (C) and (G) shows the same images as (B) and (F) respectively, in which the presence of the cells is demonstrated by darkening the digital image. The micrographs are representative of at least three separate experiments.

DISCUSSION

The protective effects of phenolics against different oxidative insults in several cell-culture systems have been described by a number of researchers. In the present study, U937 cells were challenged with tB-OOH, which is a well-characterized model compound for the study of mechanisms of oxidative cell injury. The organic hydroperoxide tB-OOH induces an array of cellular dysfunctions, including peroxidation of membrane lipids, depletion of GSH, oxidation of NAD(P)H, perturbation of calcium ion sequestration, DNA single strand breakage and mitochondrial damage (Brambilla et al., 1998). tB-OOH may decompose to other alkoxyl and peroxyl radicals that accelerate lipid peroxidation chain reactions, a decomposition that is aided by metal ions and their complexes. Proposed mechanisms of tB-OOH-induced toxicity include alterations in intracellular calcium homeostasis following glutathione and protein thiol depletion, the production of DNA strand breaks, the onset of lipid peroxidation, and the production of *tert*-butoxyl radicals. The genotoxicity of tB-OOH arises as a result of a transition metal-driven reaction leading to the generation of different reactive oxygen species (ROS), including H_2O_2 (Aherne and O'Brien 2000). The above results show that the preincubation of cells with extracts of the phenolic-rich juice designed as a functional food at different concentrations for 3 hours prevented cell death and abolished the DNA cleavage induced by tB-OOH. Moreover, after preincubating cells with the 100 μM-juice extract (the dose which diminished cell death by around 50%), a partial prevention of tB-OOH-induced MPT opening and mitochondrial formation of H_2O_2 was observed.

Flavonoids, especially anthocyanins, are the main phenolics in pigmented fruits such as grapes, cherries and berries, and are recognized as potent antioxidants (Scalbert and Williamson 1996; Robards et al., 1999). Since fruit phenolics continue to be present in their processed products (Heinonen et al., 1998), the antioxidant properties of the juice may be related to its phenolic compounds. As might be expected from the product design, the juice showed a high total amount of phenolics (3000 mg/kg or 15000 μM of ferulic acid equivalents) and a high total antioxidant activity of 25 mM Trolox equivalents and 32 mM Fe^{2+} equivalents according to the TEAC (Miller et al., 1993) and FRAP (Benzie and Strain 1996) methods, respectively. As expected from the concentrated juices employed as ingredients, the main phenolics identified in the juice were anthocyanins (670 mg/L), followed by catechins (425 mg/L), hydroxycinnamic acids (caffeic acid derivatives) (200 mg/L) and stilbenoids (9.5 mg/L). As regards the antioxidant properties of the juice, this product showed higher antioxidant activity than many fresh fruits and vegetables, even higher than those of beverages recognized as important antioxidants in the diet (tea, coffee and beer), and similar to those of red wine. These results are in accordance with those observed in a previous

investigation, in which we evaluated the antioxidant properties of a jellified dessert designed as a functional food and prepared by using the same formulation (García-Alonso et al., 2003).

Although the antioxidant properties of polyphenols are well documented, and although the product in question showed high antioxidant activity, it is still unclear whether the protective effects exerted by the phenolic-rich juice are dependent on radical scavenging or iron chelating activities. Polyphenols possess an ideal structural chemistry for free radical scavenging activities and metal chelation, supporting the role of polyphenols as preventative antioxidants in terms of inhibiting transition-metal catalyzed free radical formation (Rice-Evans et al., 1997). The chelation of transition metals, such as iron and copper, will decrease oxygen toxicity to cells, since these metals can participate in the generation of reactive oxygen species which are associated with many pathological conditions (cancers and heart disease). It is generally thought that the following functional groups are important for iron chelation: (1) *ortho*-dihydroxyl groups, e.g. 3'-4', 7–8 dihydroxy groups; (2) the presence of 5-OH and/or 3-OH in conjunction with a C4 keto group (e.g. quercetin), and (3) a large number of OH groups (e.g. tannic acid) (Khokhar and Apenten 2003).

To elucidate the mechanisms of protection afforded by the phenolic-rich juice, the radical scavenger DPPD and the intracellular iron chelator *o*-Phe were also tested. By using this experimental approach, which is based on the notion that iron chelators suppress DNA strand scission and cytotoxicity induced by tB-OOH, whereas radical scavenging antioxidants prevent only the latter response, we provide experimental evidence indicating that the most prominent activity of phenolics in the functional juice resides in their ability to chelate iron. Our results are in agreement with those obtained by Sestili et al., (1998; 2000) and Aherne and O'Brien (2000) who demonstrated that radical scavengers such as DPPD, Trolox or butylated hydroxytoluene (BHT) did not afford protection against tB-OOH-induced DNA damage, whilst *o*-phenanthroline was able to abolish DNA cleavage. This indicates that the DNA cleavage induced by tB-OOH requires a source of iron and is insensitive to radical scavenging antioxidants. As regards the protection against cytotoxicity caused by tB-OOH, in this study both DPPD and *o*-Phe prevented cell death, results which are consistent with those obtained by Sestili et al. (1998; 2000). By using an identical toxicity paradigm in U937 cells, these authors observed that various *bona fide* antioxidants (BHT, Trolox, DPPD, a-tocopherol), as well as the iron chelator *o*-Phe, were able to protect cells from tB-OOH-induced cytotoxicity. Consequently, under our experimental conditions the cytoprotective effect of the phenolic-rich juice may be the consequence of the radical scavenging and/or

iron chelating capacity of its phenolics, whereas the protection afforded against DNA damage could be best explained by an iron chelating mechanism.

To further characterize the protection afforded by the phenolic-rich juice, the effect on intracellular reactive oxygen species (ROS) generation and MPT pore opening was also assessed. It is well accepted that the collapse of the mitochondrial potential and opening of permeability transition pores in the inner mitochondrial membrane is the final event leading to cell death (Castilho et al., 1995). MPT is a Ca^{2+}-induced, non-selective inner mitochondrial membrane permeabilization process that may precede necrotic and apoptotic cell death. The occurrence of MPT has been linked to an oxidized shift in the mitochondrial redox state and/or increase in mitochondrial generation of ROS (Vercesi et al., 1997). It is generally thought that tB-OOH-induced MPT pore opening depends on the generation of ROS. tB-OOH initiates a series of mitochondrial alterations that culminate in cell death: the oxidation of pyrimidine nucleotides (NADH and NADPH), an increase in mitochondrial Ca^{2+}, and the stimulation of mitochondrial formation of ROS to promote the onset of MPT pore opening, mitochondrial depolarization, ATP depletion, and eventually cell death (Kim et al., 2003). Cell death under conditions involving MPT can be prevented by antioxidants (Kowaltowsky et al., 2002).

In this study, we observed the partial prevention of MPT pore opening after preincubation with the 100 µM-juice extract, whilst the immunosuppressive drug CsA fully prevented MPT pore opening. After exposure to tB-OOH, increased mitochondrial Ca^{2+} stimulates intramitochondrial ROS generation, which, in turn, initiates MPT pore opening. Thus, an antioxidant such as DPPD is able to prevent events prior to opening of MPT pores, particularly at the ROS generation stage (Kim et al., 2003). Conversely, cyclosporin A protects cell death after oxidative stress by blocking MPT pore opening. CsA shows cytoprotective properties in many cellular models, which may depend on interference in the interaction of cyclophilin A with calcineurin or of cyclophilin D with the MPT pore (Waldmeier et al., 2002). The oxidative stress induced by tB-OOH treatment increases cyclophilin D binding to the inner mitochondrial membrane, at the same time increasing the sensitivity of pore opening to Ca^{2+}. CsA is able to bind cyclophilin D, thus almost totally preventing its binding to the inner mitochondrial membrane and preventing MPT pore opening (Halestrap et al., 2002).

The question therefore arises as to how the phenolic-rich juice exerts its cytoprotective effect. As regards cell death and MPT pore opening, the data obtained in this study, as well as data available in the scientific literature, suggest that the mechanism involves free radical scavenging and/or iron chelating activities. Nevertheless, based on the evidence that MPT pore opening can be induced directly by ROS and depends on the presence of H_2O_2, the data obtained regarding the inhibition of ROS generation and prevention of DNA cleavage suggest that the most prominent activity of the phenolics in the juice involves iron chelation. Mitochondrial H_2O_2 derives from superoxide radicals and is converted to hydroxyl radicals, via the Fe^{2+}-dependent Fenton reaction, before promoting MPT pore opening (Castilho et al., 1995). Thus, the observation that MPT is prevented by the iron chelator *o*-Phe (Castilho et al., 1995) provides an additional clue, supporting the hypothesis that the protection exerted by the phenolic-rich juice against tB-OOH-induced oxidative injuries might arise mainly through an iron chelating mechanism. Mitochondria also play a central role in the process of tB-OOH-induced DNA damage. DNA strand scission generated by tB-OOH involves the calcium-dependent mitochondrial formation of tB-OOH-derived DNA-damaging species, mainly represented by H_2O_2 (Guidarelli et al., 1997). In addition to this calcium-dependent mechanism, the DNA damage caused by tB-OOH may be triggered by metal-catalyzed reactions leading to free radical formation and further radical-mediated processes (Latour et al., 1995). Our results are in agreement with this premise, since we observed that intramitochondrial ROS generation and DNA cleavage were prevented by *o*-Phe, but not by DPPD, whereas both DPPD and *o*-Phe abolished cell death. This fact suggests that the mechanism whereby the juice prevents ROS generation and MPT pore opening might be iron chelation, since MPT pore opening is linked to an increased oxidative status. The presence of iron chelating groups in food and their efficiency in iron chelation may partly explain the health protective role of specific phenolics in human diet (Duthie et al., 2000; Prior and Cao 2000).

CONCLUSIONS

Under the described experimental conditions, phenolics in the functional juice under study afforded protection against induced cytotoxicity, DNA cleavage, MPT pore opening and intracellular ROS generation, most probably via an iron chelating mechanism. The results obtained lend weight to the efficacy of natural phenolics from grapes and berries in protecting against oxidative stress and highlight the fact that phenolic-rich processed foods may indeed provide health benefits to consumers.

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3.4. Estudio 4

Estado nutricional y antioxidante de un grupo de ancianos institucionalizados de Murcia (España).

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SUMMARY.

Nutritional and antioxidant status of an institutionalized elderly group in Murcia (Spain).

The aging of the population is giving rise to a large number of studies aimed at improving the quality of life, considering diet as a major contributing factor in this life stage. The objectives of the present study were to evaluate the nutritional status of a group of institutionalized elderly in Murcia, Spain, and to relate the diet to anthropometric and biochemical indices, paying special attention to total antioxidant status. The dietary evaluation showed excessive intakes of proteins and lipids, and deficiencies in those of carbohydrates, dietary fiber, zinc, iodine and vitamins A, E and D. The high proportion of overweight observed was not significantly correlated to the energy intakes. Although total plasma antioxidant status was low (0.62 mM eq Trolox and 0.98 mM eq Fe^{II}) compared with reference values, estimated oxidative risk in this group of elderly was low according to plasma levels of vitamins C, A and E.

KEY WORDS: Total antioxidant status, elderly, macronutrients, micronutrients, nutritional evaluation, plasma antioxidants, dietary antioxidant.

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INTRODUCCIÓN

La mejora de las condiciones socioeconómicas en la mayor parte de los países europeos ha contribuido a una mejor calidad de vida y como consecuencia a una mayor esperanza de vida, por ello uno de los aspectos más destacados, desde el punto de vista demográfico, es el progresivo aumento de la vida media de las personas. En la actualidad en España se cuenta con una esperanza de vida al nacer de 75.3 años para los hombres y de 82.3 años para las mujeres (BERM 2003). Además, si tenemos en cuenta que las tasas de natalidad han decrecido en todos los países europeos en las tres últimas décadas, de manera particularmente acusada en Italia, Portugal y sobretodo en España, se está produciendo un envejecimiento de la población europea (Aranceta y Pérez-Rodrigo 1998). Si se considera la edad cronológica de 65 años como comienzo de la ancianidad, que coincide generalmente con el cese de la actividad laboral, se estima que para el año 2030 las personas mayores de 65 años constituirán el 21% de la población (Schlenker 1994).

El proceso de envejecimiento lleva asociadas una serie de modificaciones del metabolismo y por tanto de los requerimientos nutricionales de las personas de edad avanzada. La inactividad física y la disminución de la tasa metabólica basal hacen que las necesidades calóricas se vean disminuidas, pero no así las de proteínas, vitaminas y minerales, que incluso pueden verse aumentadas en determinadas patologías (Koehler y Garry 1993; Rusell y Sutter 1993). La disminución del apetito, las dificultades de masticación, el consumo de fármacos y la menor eficacia en la absorción de nutrientes, pueden conducir a estados carenciales de vitaminas, minerales y a una menor ingesta de antioxidantes (Rusell y Sutter 1993; Monget et al. 1996; Sastre-Gallego 1999; Rusell 2001). El envejecimiento implica además un peor funcionamiento de los sistemas de defensa antioxidante del organismo, haciendo necesario un aporte adecuado de sustancias antioxidantes para hacer frente a la agresión de los radicales libres. El estrés oxidativo causado por el aumento y acumulación de radicales libres en el organismo está implicado en el origen y desarrollo de enfermedades comunes en la edad adulta, muchas de las cuales están además íntimamente relacionadas con la alimentación (enfermedades cardiovasculares, cáncer, aterosclerosis, hipertensión, diabetes, hiperlipemias, etc.) (Halliwell 1996; Sastre-Gallego 1999). La situación social de esta población es heterogénea y los estudios realizados en nuestro país establecen que el 77% de nuestras personas ancianas viven acompañadas (53% de cónyuge y 24% de la familia), el 19% viven solos mientras que el 4% restante viven en régimen de residencia (Sastre-Gallego 1999). Sin embargo, en un futuro próximo el incremento de la población anciana así como los cambios sufridos en los núcleos familiares, va a determinar un incremento en el número de residencias y por tanto un incremento en las personas que adopten este régimen de vida. El

objetivo del presente estudio ha sido evaluar en un grupo de adultos mayores el estado nutricional y el potencial de respuesta ante la agresión oxidativa de acuerdo a su estado antioxidante total y a su nivel plasmático de vitaminas antioxidantes. El fin último es obtener información que permita planificar actuaciones futuras encaminadas a incrementar el bienestar y la calidad de vida de este colectivo.

SUJETOS Y MÉTODOS

Sujetos

El grupo de estudio estuvo constituido por 26 ancianos (8 hombres y 18 mujeres) de una Residencia para Mayores de Murcia (España), con una media de edad de 81 años (65-92). La residencia pertenece al Instituto de los Servicios Sociales de Región de Murcia (ISSORM), por lo que es estatal, aunque es necesario realizar una aportación económica de acuerdo a los ingresos que reciben como subsidio de jubilación las personas que en ella residen. Los individuos que participaron en el estudios fueron seleccionados entre todos los residentes que no presentaban patologías agudas o crónicas, según la información proporcionada por el servicio médico de la residencia, los cuales firmaron el consentimiento informado mostrando su acuerdo de participación en el estudio. Antes de realizar dicho estudio se solicitó la aprobación del Comité de Ética y de la directora del ISSORM. Para valorar el estado nutricional y la capacidad de defensa frente a la oxidación, se utilizaron indicadores dietéticos, bioquímicos y antropométricos.

Indicadores dietéticos

Los datos de la dieta se recopilaron durante 15 días mediante recuentos dietéticos de 24 horas. Debido a que en el centro la dieta está estandarizada y se conocen las cantidades de los alimentos distribuidos durante el desayuno, almuerzo y cena, en el recordatorio de 24 horas se solicitaba a cada individuo que recordara los alimentos que había ingerido el día anterior, para conocer si realmente había seguido el menú preestablecido y si había realizado una ingesta de alimentos fuera de las horas de comida establecidas en la residencia. Ninguno de los individuos ingería complementos dietéticos por lo que el aporte de los nutrientes era realizado exclusivamente a través de la dieta. Para la valoración de los datos y el cálculo de los porcentajes de adecuación se empleó el programa informático ALIMENTACIÓN Y SALUD Versión 0698.046 (BitASDE General Médica Farmaceútica, Valencia, España). Como referencia se emplearon tablas españolas de ingestas diarias recomendadas (IDRs) para adultos mayores de 50 años, que lleva incorporadas el programa.

Indicadores antropométricos

Se determinaron el peso y la talla de los individuos, para posteriormente calcular el índice de masa corporal (IMC), mediante la fórmula $IMC=P/T^2$ (kg/m^2). Los individuos se clasificaron según el grado de obesidad siguiendo los criterios aceptados por la Organización Mundial de la Salud (Seidell y Fleigal 1997). Los aparatos utilizados fueron una balanza digital Seca modelo 812 (Vogel y Halke GMBH, Alemania) con un peso máximo de 150 kg y una sensibilidad de 100 g, y un tallímetro con una escala graduada desde 45 hasta 200 cm con divisiones de 1 mm.

Indicadores bioquímicos

Los distintos indicadores bioquímicos fueron analizados en las muestras de sangre de cada uno de los individuos sometido a estudio. La extracción sanguínea fue realizada por el equipo sanitario del centro, a primera hora de la mañana con los individuos en ayunas. Se determinaron en plasma los niveles de albúmina, bilirrubina, hierro, ácido úrico, vitaminas A (retinol), E (tocoferol), C (ácido ascórbico), β-caroteno y el estado antioxidante total. Estos indicadores bioquímicos fueron seleccionados por ser los componentes plasmáticos que tienen mayor relación con el estado antioxidante total de un individuo. El hierro sérico, la albúmina, la bilirrubina y el ácido úrico se determinaron mediante sistemas automáticos de análisis clínico, empleando kits comerciales y un analizador automático COBAS-MIRA Plus (ABX Diagnostics, Montpellier, Francia). Los niveles plasmáticos de retinol, tocoferol y β-caroteno se determinaron por HPLC (Thurnham et al. 1988) y la concentración de vitamina C fue cuantificada mediante un test enzimático-colorimétrico (Böehringer-Manheim Cat. No. 409677, Manheim, Alemania). Para la evaluación de la actividad antioxidante total del plasma se aplicaron dos métodos diferentes; el ensayo FRAP (Benzie y Strain, 1996), basado en la reducción del Fe^{3+} a Fe^{2+} y el ensayo del ABTS (Miller et al., 1993), basado en la captación del radical $ABTS^{\bullet+}$, para el que se empleó un test colorimétrico (Randox Laboratories Ltd. Cat. No. NX2332, Ardmore, Reino Unido).

Tratamiento estadístico

Para el tratamiento de los datos se empleó el paquete estadístico SPSS 10.0 para Windows (SPSS Inc., Chicago, USA). Los resultados se expresaron como media y desviación típica y para detectar diferencias entre medias se aplicó la prueba *t*-Student, con un nivel de significación de 0.05. Para el estudio de las relaciones existentes entre las distintas variables se aplicó un análisis de correlación de Pearson, con niveles de significación de 0.05 y 0.01, así como un análisis de regresión lineal simple.

RESULTADOS

Indicadores dietéticos

La **Tabla 1** muestra la ingesta media diaria estimada de energía, macronutrientes, vitaminas y minerales del grupo de ancianos, durante los 15 días en los que se realizó el estudio, estableciendo las diferencias de acuerdo al sexo y comparándolos con los valores de IDRs. En general, los valores estimados en el grupo de ancianos fueron similares a los datos publicados para otros grupos de población española de 65-74 años, residentes en las Islas Canarias. Las diferencias más destacables entre las poblaciones de Canarias y Murcia fueron relativas al consumo de lípidos, vitamina A y ácido fólico, que resultaron superiores el grupo de ancianos de Murcia (ENCA 1997-1998), hecho que está ligado a las diferencias gastronómicas entre las dos áreas geográficas.

Tabla 1. Ingesta media diaria de energía y nutrientes del grupo de ancianos[1].

	IDR[2]		Total del grupo (n=26)	Mujeres (n=18)	Hombres (n=8)
	M[3]	H[4]			
Energía (Kcal)	2075	2700	1877±239	1920±217	1781±261
Proteínas (g)	41	54	74.5±13.8	74.2±15.2	75.2±10.3
Hidratos de carbono (g)	--		235±49	246±50.4	208±35.8
Lípidos (g)	--		74.2±15.1	74.1±16.5	74.5±11.8
Ácidos grasos saturados (g)	--		18.1±4.5	17.7±4.9	19.3±2.8
Ácidos grasos monoinsaturados (g)	--		26.0±9.2	25.56±10.4	27.0±5.9
Ácidos grasos poliinsaturados (g)	--		9.4±4.9	9.0±5.1	10.5±4.0
Colesterol (mg)	<310		252±61.9	252±66.1	251±52.6
Fibra dietética(g)	25		17.6±4.7	16.6±3.9	20.0±5.4
Vitamina A (µg)	800	1000	1467±1501	1188±1096	2096±2046
Vitamina B_1 (mg)	0.8	0.96	14.2±29.9	20.0±34.4	1.2±0.2
Vitamina B_2 (mg)	1.2	1.6	1.5±0.5	1.5±0.6	1.4±0.1
Vitamina B_6 (mg)	1.6		1.7±0.4	1.6±0.4	1.9±0.2
Vitamina B_{12} (µg)	2		4.7±2.7	4.7±3.2	4.5±1.3
Vitamina C (mg)	60		143±81.6	144±89.8	142±60.7
Vitamina D (µg)	5		9.3±54.1	12.1±64.9	2.8±1.5
Vitamina E (mg)	12		5.2±2.0	4.8±2.1	5.9±1.7
Niacina (mg)	14		18.7±5.1	18.3±5.6	19.6±3.5
Ácido Fólico (µg)	200		231±84.8	221±90.5	253±66.9
Sodio (mg)	--		1465±336	1505±304	1375±392
Potasio (mg)	--		2801±679	2687±697	3059±569
Calcio (mg)	800		836±140	804±151	909±76.0
Fósforo (mg)	800		1133±190	1136±200	1126±169
Ratio Ca/P	1		0.8±0.1	0.7±0.1	0.8±0.1
Magnesio (mg)	300	350	260±57.5	250±57.9	282±51.4
Hierro (mg)	10		12.0±2.7	11.8±2.9	12.7±2.3
Cinc (mg)	15		6.0±1.8	5.7±1.9	6.6±1.3
Yodo (µg)	110	140	55.3±20.1	52.0±19.6	62.6±19.8

[1]Media±desviación estándar; [2]Ingesta Diaria Recomendada según las tablas españolas para mayores de 50 años recogidas en el programa ALIMENTACIÓN Y SALUD Versión 0698.046; [3]Mujer; [4]Hombre

La **Tabla 2** muestra los porcentajes de adecuación para la ingesta de energía y macronutrientes a las IDR, estableciendo tres grupos en función de la adecuación de la ingesta. Las ingestas estimadas de energía estuvieron por debajo de las cantidades recomendadas en ambos grupos (hombres y mujeres), destacando que un 19% de los individuos del grupo (hombres) se encontraba por debajo del 70% de los requerimientos energéticos, mientras que por el contrario las mujeres presentaron una mejor adecuación de la ingesta energética.

Tabla 2. Porcentajes de adecuación de la ingesta de energía y macronutrientes.

	<70% de la IDR[1]			**90-110% de la IDR[1]**			**>120% de la IDR[1]**		
	Total	***Mujer***	***Hombre***	***Total***	***Mujer***	***Hombre***	***Total***	***Mujer***	***Hombre***
Energía	19	0	28	46	67	0	0	0	0
Proteínas	0	0	0	4	6	0	86	94	75
Carbohidratos	46	4	50	8	11	0	0	0	0
Lípidos	19	22	13	42	39	50	4	6	0
Fibra	58	67	38	12	17	0	4	0	13

[1]Ingesta Diaria Recomendada según las tablas españolas para mayores de 50 años recogidas en el programa ALIMENTACIÓN Y SALUD Version 0698.046.

La ingesta media de proteínas del grupo de ancianos (74.5 g/día, **Tabla 1**) constituyó el 16.1% del valor calórico total (VCT), algo por encima del 12% considerado como porcentaje adecuado en una dieta equilibrada. Por ello el 86% de los individuos presentaron un consumo excesivo de proteínas, por encima del 120% de la IDR. La ingesta media estimada de hidratos de carbono (235 g/día, **Tabla 1**) constituyó el 47.6% del VCT, quedando por debajo del 55% establecido como adecuado. En un 46% de los individuos se estimó una ingesta de hidratos de carbono inferior al 70% de la IDR, estando este grupo principalmente constituido por hombres. En cuanto al consumo medio de grasas (74.2 g/día) supuso aproximadamente el 36% del VCT de la dieta, superando ligeramente el 33% establecido como referencia, observando una ingesta inferior al 70% de la IDR en un 19% de los individuos y sólo un 4% mostró una ingesta superior al 120% de la IDR, siendo en este caso mujeres. El excesivo aporte proteico y graso en detrimento del aporte de hidratos de carbono, es la pauta dietética general que se registra en la alimentación española (MAPA 2003) y también ha sido descrito por otros autores en diferentes poblaciones (Payette y Gray-Donald 1991; Falque-Madrid et al. 1997; Suriah et al. 1998).

Este comportamiento alimentario constituye un gran problema en los países desarrollados al existir una estrecha relación entre el aporte de estos macronutrientes en la dieta y distintas patologías. Incluso se agrava más desde un punto de vista nutricional, si observamos que el 58% de los ancianos ingirieron cantidades inferiores al 70% de la IDR para la fibra dietética.

La **Tabla 3** muestra los porcentajes de adecuación para la ingesta de vitaminas. Las deficiencias más destacadas se apreciaron en las vitaminas liposolubles (A, D y E), observando un alto porcentaje de individuos con ingestas inferiores a las IDRs. Así, por debajo del 70% de las IDRs se encontraron un 54% (de los individuos) en el caso de la vitamina A, un 58% para la vitamina D y un 96% para la vitamina E. Únicamente las mujeres mostraron ingestas inferiores al 70% de las IDRs para las vitaminas B_6, B_{12}, niacina y ácido fólico. Entre las ingestas vitamínicas del grupo B superiores al 120% de las IDRs, destacan las de vitamina B_1 (81% de los individuos), vitamina B_{12} (85% de los individuos), niacina (61% de los individuos) y ácido fólico (54% de los ancianos del grupo). Para la vitamina C hay que destacar igualmente una ingesta muy alta a partir de la dieta al estimar que el 77% de los ancianos superaron el 120% de la IDR, alcanzando en algunos individuos ingestas de vitamina C superiores al 300%, dada la gran variabilidad existente en la ingesta de esta vitamina según individuos **(Tabla 1)**.

Tabla 3. ***Porcentajes de adecuación de la ingesta de vitaminas.***

	<70% de la IDR[1]			**90-110% de la IDR[1]**			**>120% de la IDR[1]**		
	Total	***Mujer***	***Hombre***	***Total***	***Mujer***	***Hombre***	***Total***	***Mujer***	***Hombre***
Vitamina A	54	56	50	4	6	0	35	28	50
Vitamina B_1	0	0	0	15	6	38	81	94	50
Vitamina B_2	0	0	0	35	28	50	23	33	0
Vitamina B_6	12	17	0	38	39	38	23	22	25
Vitamina B_{12}	12	17	0	0	0	0	85	78	100
Vitamina C	0	0	0	15	17	13	77	78	75
Vitamina D	58	56	63	19	17	25	8	11	0
Vitamina E	96	100	88	0	0	0	0	0	0
Niacina	12	17	0	8	11	0	62	61	63
Ácido Fólico	15	22	0	8	11	0	54	44	75

[1]Ingesta Diaria Recomendada según las tablas españolas para mayores de 50 años recogidas en el programa ALIMENTACIÓN Y SALUD Versión 0698.046.

La **Tabla 4** muestra los porcentajes de adecuación de la dieta del grupo de ancianos a las ingestas recomendadas de minerales. Las carencias más destacables fueron las de cinc y yodo, al observar que el 100% y 88% de los individuos respectivamente se encuentran por debajo del 70% de la IDR. Estos porcentajes eran de esperar, ya que las cantidades estimadas de cinc y yodo proporcionadas a partir de la dieta fueron bajas, de 6 mg/100 g para el primero y de 55.3 µg/100 g para el segundo **(Tabla 1)**. En general, la población española es deficitaria en cinc (MAPA 2003) por lo que esta deficiencia está relacionada con las propias costumbres dietéticas de nuestro país. La ingesta de calcio resultó significativamente mayor ($P<0.05$) en los hombres (909 mg/100 g, **Tabla 1**) que en las mujeres (804.2 mg/100 g, **Tabla 1**). Según los datos del Estudio Prospectivo Europeo sobre Dieta, Cáncer y Salud (estudio EPIC) el consumo de leche y productos lácteos en Murcia está considerado como un consumo medio-alto con una media de 336 g/día (BERM 2003), lo que representa más de un 90% de la IDR. En cuanto al fósforo, no se encontraron diferencias significativas entre hombres y mujeres, con ingestas estimadas de 1126 y 1136.4 mg/día **(Tabla 1)**, respectivamente, superiores en ambos casos al 120% de la IDR. El cociente Ca/P de los hombres (0.82) fue significativamente superior ($p<0.05$) al de las mujeres (0.72), por lo que sería aconsejable disminuir el consumo de fósforo para adecuar el cociente estimado al valor de 1. Para el magnesio se ha cuantificado una adecuación de la ingesta inferior al 100%, observando que el valor medio de este mineral evaluado en la dieta fue inferior a las cantidades recomendadas (260 frente a 300 mg/100 g). En el caso del hierro, se observó una ingesta de hierro superior al 120% de la IDR en un 53% de los individuos, y solo una pequeña parte de los individuos (8%) mostraron ingestas estimadas inferiores al 70% de la IDR para este mineral. La ingesta media fue de 11.8 y 12.7 mg/100 g de hierro para mujeres y hombres, respectivamente **(Tabla 1)**, determinando un alto porcentaje de individuos por encima del 120% de la IDR.

Tabla 4. ***Porcentajes de adecuación de la ingesta de minerales.***

	<70% de la IDR[1]			**90-110% de la IDR[1]**			**>120% de la IDR[1]**		
	Total	***Mujer***	***Hombre***	***Total***	***Mujer***	***Hombre***	***Total***	***Mujer***	***Hombre***
Calcio	0	0	0	27	22	38	23	28	13
Fósforo	8	11	0	0	0	0	81	78	88
Magnesio	27	28	25	27	39	0	0	0	0
Hierro	8	11	0	12	6	25	54	50	63
Cinc	100	100	100	0	0	0	0	0	0
Yodo	88	89	88	0	0	0	0	0	0

[1]Ingesta Diaria Recomendada según las tablas españolas para mayores de 50 años recogidas en el programa ALIMENTACIÓN Y SALUD Version 0698.046.

Indicadores antropométricos

La **Figura 1** muestra gráficamente la distribución de frecuencias del IMC. El IMC del grupo fue de 30.68 kg/m^2, mostrando valores significativamente superiores ($p<0.05$) superiores el IMC de las mujeres (31.91 kg/m^2) frente al de los hombres (27.90 kg/m^2). En general, se observó una prevalencia de la obesidad en el grupo de ancianos siendo predominante en las mujeres la obesidad de grado II y en los hombres la obesidad de grado I. La obesidad, además de estar relacionada con una ingesta calórica superior al gasto energético, se asocia a una menor actividad física. El estudio EPIC muestra que partir de los 60 años los hombres presentan una mayor actividad física que las mujeres (BERM 2003), lo que contribuye a un mayor gasto energético y favorece un menor IMC. En ningún caso se observaron obesidad grado III ni IMCs inferiores a 20 kg/m^2, indicativos de malnutrición. Diferentes autores han descrito incidencias elevadas de obesidad en grupos de población anciana española (Ortega et al. 1996; Redondo et al. 1996), siendo ésta por lo general más frecuente entre las mujeres lo que contribuye a un mayor riesgo de enfermedad cardiovascular (Falque-Madrid et al. 1997; Gámez et al. 1998).

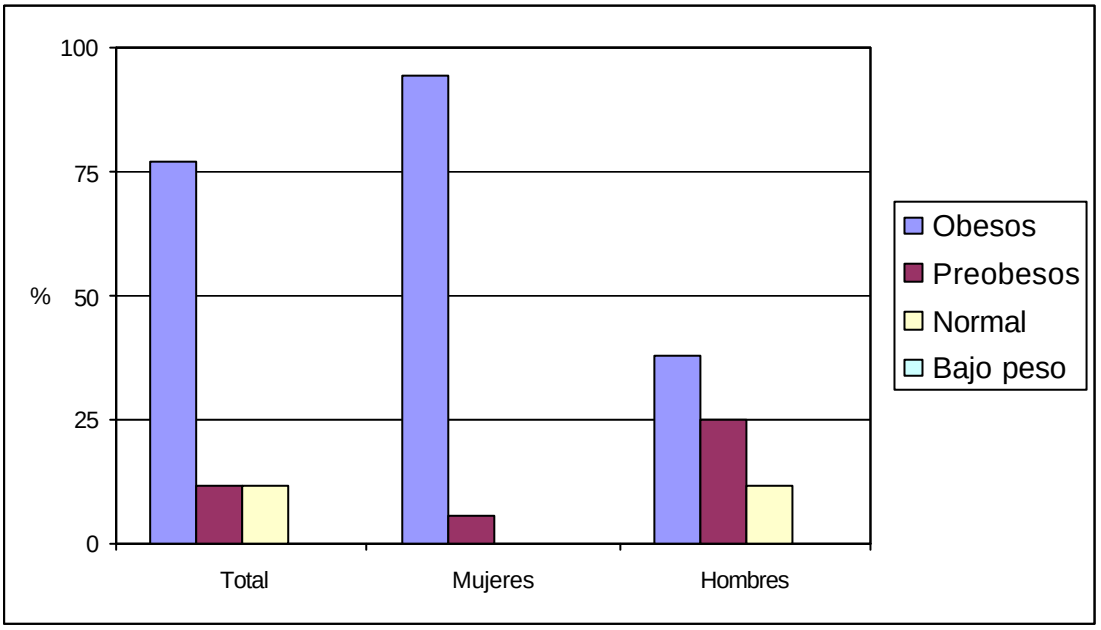

Figura 1. Distribución de frecuencias del IMC (kg/m^2) del grupo de ancianos. Obesos (IMC≥30), preobesos (IMC de 27.0 a 29.9), normal (IMC de 18.5 a 26.9), bajo peso (IMC<18.5)

Indicadores bioquímicos

La **Tabla 5** muestra los valores de actividad antioxidante total plasmática (métodos ABTS y FRAP) y las concentraciones en plasma de albúmina, bilirrubina, hierro, ácido úrico, vitaminas antioxidantes (ácido ascórbico, retinol, tocoferol) y β-caroteno (provitamina A) del grupo de estudio. En general, la mayor parte de los parámetros analizados se encuentran dentro de los valores de referencia considerados como normales en la literatura científica, a excepción de la actividad antioxidante total plasmática determinada por el método del ABTS y el retinol plasmático que se encuentran por debajo de los límites de referencia (Thurnham et al. 1988; Miller et al. 1993). El valor medio del estado antioxidante plasmático fue de 0.62 mM eq Trolox y 0.98 mM eq Fe^{II}, lo que nos muestra un diferente estatus antioxidante de acuerdo al método de análisis utilizado en la evaluación de este parámetro bioquímico. Con la prueba de la *t* de Student se detectaron diferencias significativas ($p<0.05$) en los niveles plasmáticos de capacidad antioxidante total por los métodos ABTS y FRAP, bilirrubina, ácido ascórbico y tocoferol de hombres y mujeres.

Tabla 5. Actividad antioxidante total, parámetros bioquímicos y vitaminas antioxidantes en plasma[1].

Parámetro	*Total*	*Mujeres*	*Hombres*	*Límites de referencia*[2]
ABTS (mM eq Trolox)	0.62±0.12	0.60±0.11*	0.68±0.13*	1.30-1.77 (Miller et al. 1993)
FRAP (mM eq Fe^{II})	0.98±0.16	0.94±0.15*	1.05±0.16*	0.612-1.634 (Benzie y Strain 1996)
Albúmina (g/dL)	3.91±0.43	3.93±0.39	3.86±0.56	3.5-5.0 (Mahan y Escott-Stump 1998)
Bilirrubina (mg/dL)	0.71±0.35	0.58±0.32*	1.02±0.21*	<1.00 (Walters y Gerarde 1970)
Hierro (µmol/L)	13.04±5.60	12.12±3.06	15.13±9.05	9-31 (Mahan y Escott-Stump 1998)
Ácido úrico (mg/dL)	4.72±1.14	4.60±1.19	5.00±1.05	2.7-7.6 (Stocker y Frei 1991)
Ácido ascórbico (mg/L)	8.25±4.52	9.08±4.88*	6.40±3.06*	6.2-14 (Le Grusse y Watier 1993)
Retinol (µmol/L)	0.86±0.19	0.87±0.19	0.82±0.20	>1.05 (Le Grusse y Watier 1993)
Tocoferol (µmol/L)	20.21±4.90	21.35±5.05*	17.64±3.61*	16.31-34.95 (Le Grusse y Watier 1993)
ß-caroteno (µmol/L)	0.27±0.15	0.28±0.16	0.24±0.12	0.25-0.67 (Haller et al. 1996)

[1]Media±desviación estándar; *Diferencias estadísticamente significativas ($p<0.05$). Prueba de la *t* de Student.

[2]Entre paréntesis aparece la referencia bibliográfica de la cuales ha sido tomados los valores.

La **Tabla 6** muestra los porcentajes de individuos incluidos en diferentes categorías de riesgo en función de sus concentraciones plasmáticas de vitaminas antioxidantes. La mayor parte de los individuos (88%) quedaron incluidos en la categoría de bajo riesgo para los niveles plasmáticos de tocoferol, un 65% presentaron bajo riesgo en función de su concentración de vitamina C en el plasma, mientras que un 81% mostraron un riesgo moderado asociado al nivel plasmático de retinol (0.86 μmol/L, **Tabla 5**). Hay que destacar que un pequeño porcentaje presentó un alto riesgo de acuerdo a la concentración de tocoferol y ácido ascórbico, siendo mayoritario en el primer caso el grupo de hombres mientras que en el segundo lo fue el de mujeres.

Tabla 6. ***Evaluación por criterios de riesgo de carencia de los niveles plasmáticos de vitaminas antioxidantes[1]***

	Tocoferol (μmol/L)			**Ácido Ascórbico (mg/L)**			**Retinol (μmol/L)**		
Riesgo	***Alto***	***Medio***	***Bajo***	***Alto***	***Medio***	***Bajo***	***Alto***	***Medio***	***Bajo***
Nivel	*<11.65*	*11.65-16.31*	*>16.31*	*<3.5*	*3.5-6.2*	*>6.2*	*<0.35*	*0.35-1.05*	*>1.05*
Total (%)	8	4	88	15	19	65	0	81	19
Mujeres (%)	6	0	94	17	11	72	0	78	22
Hombres (%)	13	13	75	13	38	50	0	88	13

[1]Según Le Grusse y Watier (1993).

DISCUSIÓN

Las encuestas nutricionales se utilizan como herramienta para estimar el estado nutricional de un grupo de población, aunque sus resultados están íntimamente ligados a las costumbres y hábitos alimenticios de ese grupo. Los datos obtenidos sobre la ingesta de nutrientes en la población anciana estudiada son muy similares a los descritos en la bibliografía para otras áreas españolas, aunque difieren en mayor grado con los encontrados en otros países. En general, el grupo de estudio muestra un desequilibrio nutricional en cuanto a macronutrientes, al presenta un aporte energético mayor al recomendado a partir de las grasas y proteínas. Este comportamiento se describe igual para toda la población española en el estudio de alimentación realizado por el Ministerio de Agricultura, Pesca y Alimentación (MAPA 2003), destacando un bajo consumo de fibra. Aunque en la dieta se observa un gran consumo de frutas y hortaliza, si que hay una pérdida del hábito de comer legumbres, pan, cereales y derivados, ello produce una reducción en el consumo de fibra dietética y en el consumo de hidratos de carbono asimilables, por lo que se produce un desequilibrio a favor de las proteínas y de las grasas. Se observa una deficiencia en la ingesta de vitamina A y E, hecho que puede ser preocupante si consideramos que estas vitaminas intervienen en el estado antioxidante del organismo.

Aunque la ingesta de vitamina D a través de la dieta en el grupo de estudio es baja, se cubren los requerimientos ya que la abundante radiación solar de nuestra región permite su síntesis en el tejido cutáneo a partir de las sustancias precursoras. En cuanto a la vitamina C, el alto consumo de verduras y hortalizas que caracteriza a la dieta española, proporciona con holgura los requerimientos nutricionales de este micronutriente. Incluso, el consumo de este grupo de alimentos puede contribuir a las ingestas de ácido fólico, las cuales se adecuan bastante a las IDR. Esta vitamina tiene una gran importancia en la actualidad para los distintos grupos de edad, y en el caso concreto de los adultos, numerosos estudios experimentales han puesto de manifiesto que una baja ingesta de ácido fólico o vitamina B_9 se encuentra relacionado con altos niveles de homocisteína plasmática, lo cual está considerado como un factor de riesgo cardiovascular (Ros et al. 2002). Son el cinc y el yodo los elementos que más preocupan, ya que aunque el aporte de yodo en la población española se cubre a través de la dieta, no ocurre este hecho con la población de estudio. Relativo al cinc tenemos que decir que el comportamiento que se observa en España es una ingesta un 35% inferior a la IDR de este elemento, aunque en el grupo de ancianos estudiado la deficiencia es del 100. Esta situación es importante desde un punto

de intervención nutricional, ya que el cinc interviene en la síntesis proteica y en el refuerzo de los sistemas inmunitarios (Khoeler y Garry 1993; Schlenker 1994; Sastre-Gallego 1999).

Las deficiencias en vitaminas y minerales, descritas por otros autores, varían en función de la población estudiada. Nuestros datos coinciden con las pautas dietéticas de la población española en general y con los recogidos en otros trabajos, en los que se han descrito aportes superiores a IDR para calcio y vitamina C (Payette y Gray-Donald 1991; Lipski et al. 1993; Falque-Madrid et al. 1997; Redondo et al. 1996; Gámez et al. 1997; Peña et al. 1998). De forma general, podemos decir que la dieta cubre las necesidades de muchos nutrientes, aunque hay que modificar ligeramente las pautas a favor de un mayor aporte de aquellos que se ven disminuidos (hidratos de carbono, fibra dietética, vitaminas A, D y E, y zinc) y con el objeto de reducir aquellos que se ven ligeramente incrementados (grasas y proteínas).

No se encontró correlación entre el IMC y la ingesta estimada de energía, por lo que la alta incidencia de obesidad sería atribuible a la baja actividad física de los individuos, datos que concuerdan con los descritos por Gámez et al. (1996). Sin embargo los valores del IMC son superiores a los determinados por otros autores en poblaciones ancianas españolas y de otros países (Ortega et al. 1990; Collado-Cano et al. 1992; Carbajal et al. 1993; De Groot et al. 1996; Gámez et al. 1998; De Jong et al. 1999; Carroll et al. 2000; Gale et al. 2001), lo que nos muestra una mayor prevalencia de la obesidad en la población murciana, tal y como ocurre en otros grupos de edad. Este hecho puede determinar el mayor riesgo cardiovascular al que están expuestos comparados con personas ancianas de otras áreas geográficas españolas, ya que la población murciana muestra una mayor incidencia de infarto agudo de miocardio comparado con la población española, según los datos del estudio IBERICA (BERM 2002).

Actualmente, la protección del organismo frente a los radicales libres, cuya presencia se asocia con el padecimiento de ciertas patologías y enfermedades degenerativas, depende de la actuación de los distintos sistemas antioxidantes del organismo como son las enzimas y la presencia de moléculas antioxidantes en el plasma (Halliwell 1996). La valoración de las concentraciones plasmáticas de vitaminas antioxidantes así como de los demás parámetros bioquímicos analizados se encuentra estrechamente relacionada con la estado antioxidante del individuo, teniendo en cuenta que las concentraciones de estas vitaminas en el plasma pueden ser modulada por su ingesta en la dieta. Sin embargo, los datos bioquímicos no se relacionan en muchos casos con las estimaciones dietéticas. A pesar de que la gran mayoría de individuos presentaron ingestas estimadas inferiores al

70% de la IDR para las vitaminas E y A, los niveles plasmáticos no se vieron afectados al no observar correlación entre ellos. Para la vitamina E las concentraciones plasmáticas se encontraron dentro de los valores de referencia y sólo ligeramente por debajo de los mismos en el caso del retinol. Los niveles plasmáticos de ácido ascórbico y las ingestas estimadas de vitamina C, sí mostraron una correlación positiva y estadísticamente significativa ($r=0.23$; $p<0.05$). Distintos autores han obtenido resultados similares a los nuestros, no encontrando correlación entre los valores plasmáticos y dietéticos de tocoferol y retinol, pero sí entre las ingestas estimadas de vitamina C y sus niveles en plasma (Picado et al. 2001). Así, Jacques et al. (1995), tampoco encontraron relación entre la ingesta de vitamina E con los carotenoides plasmáticos y si una relación débil con los niveles en plasma de vitamina C. Las ingestas estimadas de vitamina C no se correlacionaron con los niveles en plasma de tocoferol ni de retinol, pero si con los de β-caroteno ($r=0.36$; $p<0.01$). Esta asociación entre la ingesta de vitamina C y los carotenoides plasmáticos ha sido descrita por Jacques et al. (1995), y podría reflejar el consumo frecuente de frutas (principalmente cítricos) y verduras que aportan de forma conjunta vitamina C y carotenoides, con una mayor biodisponibilidad del β-caroteno en el caso de las frutas (De Pee et al. 1998; Rowley et al. 2001).

Los niveles de hierro en plasma no estuvieron correlacionados con las ingestas estimadas, además en la prueba de la *t* de Student no se detectaron diferencias significativas ($p>0.05$) en los valores medios de hierro plasmático, correspondientes a individuos con ingestas estimadas inferiores al 70%, comprendidas entre el 70 y el 110% y superiores al 110% de la IDR (datos no mostrados). La ausencia de correlación estadísticamente significativa entre las ingestas estimadas y los niveles plasmáticos de determinados micronutrientes (en nuestro caso el hierro) y vitaminas antioxidantes puede ser debida a que una analítica sanguínea no siempre refleja los niveles de estas sustancias en sus depósitos orgánicos (células, tejidos y órganos) y en parte también a que una única medida en plasma puede no ser suficiente para indicar una ingesta dietética a largo plazo (Picado et al. 2001), ya que las medidas en plasma están sujetas a fluctuaciones diarias (Tucker et al. 1999).

Los dos métodos aplicados para evaluar la capacidad antioxidante total plasmática (ABTS y FRAP) mostraron una correlación alta ($r=0.60$; $p<0.01$), aunque los valores numéricos obtenidos por el método FRAP son superiores a los obtenidos por el método del ABTS. Esto es debido a que los distintos antioxidantes plasmáticos contribuyen de modo diferente a los valores de actividad antioxidante total en función del método aplicado, ya que el fundamento de los métodos es diferente. Las diferentes contribuciones de estas

sustancias antioxidantes se ponen de manifiesto en el análisis de correlación. Los valores de capacidad antioxidante total plasmática obtenidos por el método del ABTS estuvieron correlacionados con los niveles plasmáticos de ácido úrico ($r=0.57$; $p<0.01$), albúmina ($r=0.33$; $p<0.01$) y con menor intensidad con los niveles de ácido ascórbico ($r=0.29$; $p<0.05$). Al aplicar un análisis de regresión lineal simple, se observó que los porcentajes de varianza explicados por los niveles de ácido úrico, albúmina y con ácido ascórbico fueron, respectivamente 31.4%, 9.6% y 7.1%. Los valores obtenidos por el método FRAP estuvieron correlacionados con los niveles plasmáticos de ácido úrico ($r=0.77$; $p<0.01$), ácido ascórbico ($r=0.32$; $p<0.01$) y en menor medida con los de hierro ($r=0.29$; $p<0.05$) y bilirrubina ($r=0,25$; $p<0,05$). En este caso, las varianzas explicadas fueron respectivamente 59%, 9.2%, 7% y 5.1%. Otros autores han estimado la contribución del ácido úrico a los valores del FRAP en torno al 60% con lo que este compuesto se muestra como principal contribuyente seguido del ácido ascórbico (10-15%) y en menor proporción (5%) de la bilirrubina, tocoferol, proteínas plasmáticas y otros antioxidantes del plasma (Cao y Prior 1998). En el método FRAP, la actividad de la albúmina es muy baja (Benzie y Strain 1996) ya que sus grupos tiol no son capaces de reaccionar en el ensayo (Stocker y Frei 1991; Rice-Evans 2000). En el caso del ABTS, los principales contribuyentes son la albúmina (28%) y el ácido úrico (19%) (Cao y Prior 1998), aunque en nuestro estudio el ácido úrico muestra una mayor contribución al valor del método del ABTS.

CONCLUSIONES

Cabe destacar que las pautas dietéticas de los ancianos institucionalizados deben ser vigiladas con el objeto de mejorar el equilibrio nutricional y aportar aquellos nutrientes que son deficitarios. Teniendo en cuenta que los valores del estado antioxidante son bajos y que en este grupo de edad los mecanismos de protección antioxidante pueden estar disminuidos, la dieta debe aportar en todo momento una amplia variedad de alimentos de origen vegetal que refuerce los niveles de antioxidantes plasmáticos. En este grupo puede existir un mayor riesgo cardiovascular debido al sobrepeso y al mayor consumo de grasas, aunque los niveles plasmáticos de vitaminas antioxidantes pueden ejercer un efecto protector al reducir la oxidación de la LDL. También la ingesta de ácido fólico podría proporcionar cierta prevención, ya que una adecuada ingesta de ácido fólico puede reducir los niveles plasmáticos de homocisteína, factor considerado de riesgo para la enfermedad cardiovascular.

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3.5. Estudio 5

Oxidative stress status in an institutionalised elderly group after the intake of a phenolic-rich dessert

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ABSTRACT

The elderly population undergoes a series of physiological and sociological changes common to old age with a high probability of suffering degenerative illness and malnutrition. A dessert rich in phenolic compounds has been designed by using concentrated juices of grape, cherry, blackberry, blackcurrant and raspberry with the aim of being used as a complementary food in adulthood. In the present study, we investigated the effect of the intake of this dessert, (a jar of 200 g daily for a period of two weeks) with an antioxidant activity equivalent to 10 servings of fruits and vegetables, on several markers of oxidative and antioxidant status in DNA and plasma in a group of elderly individuals. Non-smoking institutionalised elderly subjects were recruited from the pool of volunteers of an old age home in Murcia (Spain). Twenty-two subjects (six men and sixteen women) participated in the study. The study was designed as a randomised intervention trial with a period of 2 weeks. At days 1 and 15, blood samples were collected to analyse total antioxidant capacity, biochemical parameters, antioxidant vitamins, LDL peroxidation and peripheral blood lymphocytes DNA damage. The conclusion of the present study is that a 2-week intervention with our dessert enriched with natural polyphenol compounds in elderly individuals does not give time to find changes in the antioxidant and oxidative status. Also, the view that the marked antioxidant ability of polyphenols *in vitro* does not translate to analogous effects *in vivo* was confirmed. Moreover, a highly oxidative stress status during ageing was confirmed together with the need to follow nutritional studies to improve this situation.

Keywords: Phenolic-rich dessert, ageing, antioxidant status, DNA damage.

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INTRODUCTION

The antioxidant properties of foods in relation to health and particularly on the maintenance and protection from degenerative diseases are of growing interest among scientists, food manufacturers, consumers and health organizations. Since the 1990s, several international organisations have recommended increasing the consumption of fruits and vegetables to five or more daily servings, in order to provide a desirable intake of antioxidants and to improve human health (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997).

However, for certain groups of population, such as the elderly people, it is difficult to consume those daily amounts of fruits and vegetables. The elderly population undergoes a series of physiological and sociological changes common to old age with a high probability of suffering degenerative illness and malnutrition (Tucker and Buranapin 2001). During the ageing process, a more sedentary lifestyle, resulting in less energy expenditure, poor appetite, dental disease, alterations in absorption and metabolism of several nutrients, medication, etc., may lead to a decline of the intake of macro- and micronutrients. Consequently, elderly individuals are at risk of sub-optimal nutritional state or multiple micronutrient deficiencies (De Jong 1999). In a recent study carried out with 10208 participants from eight random population studies and participants in fifty-seven studies included in a meta-analysis, it has been shown that the vitamin status of the Spanish population clearly shows room for improvement, especially with respect to vitamins A, E, D, B_2, B_6, and folates (Ortega et al. 2003). For this reason, there is a trend in the foodstuff industry toward functional foods with healthy effects based, among others, on their antioxidant properties (Karakaya et al., 2001).

Antioxidant vitamins, including vitamin C, vitamin E and a variety of phytochemicals, are important in maintaining effective antioxidant defences against oxidant stress-related diseases, including cancer, cataracts and Alzheimer´s disease. Berries, grapes and cherries are recognised as fruits with a high content of antioxidants. The antioxidant properties of these fruits are believed to be due to the content of anthocyanins and other phenolic compounds. Similarly, concentrated juices with high content of phenolics should probably exhibit an effect (García-Alonso et al. 2002). In this basis, a dessert has been designed by using concentrated juices of grape, cherry, blackberry, blackcurrant and raspberry with the aim of it being used as a complementary food in adulthood. The antioxidant activity of this product is considered similar to that of red wine and higher than that of many fruits and

vegetables. In previous studies, we have observed that the antioxidant capacity of this dessert during storage remained practically invariable for one year at different temperatures (8, 21 and 30ºC) (García-Alonso et al. 2003). In the present study, we investigated the effect of the intake of this dessert (a jar of 200 g daily for a period of two weeks), with an antioxidant activity equivalent to 10 servings of fruits and vegetables, on several markers of oxidative and antioxidant status in DNA and plasma in a group of elderly individuals.

SUBJECTS AND METHODS

Test product

The test product was an experimental dessert prepared by the Department of Research and Development of Hero Spain S.A (Alcantarilla, Murcia, Spain). This product was formulated and designed using the data of total antioxidant activity available in the scientific literature for the fruits used (Cao et al. 1996; Wang et al. 1996). The objective was to reach, per serving (a jar of 200 g), an average antioxidant capacity equivalent to 10 servings of fruits and vegetables, 2-fold higher than the "five a day" recommended by several international organisations (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997). The major ingredient was water, which was mixed with commercially available concentrated juices of grape (26%), cherry (2%), blackberry (0.6%), blackcurrant (0.6%) and raspberry (1%). Pectin was added to jellify the product. The resulting product was pasteurized in order to obtain a microbiologically stable foodstuff and bottled hot in jars to ensure headspace vacuum.

For the characterisation of the dessert, several physical-chemical parameters and nutritional composition were analysed. Total titratable acidity, pH and soluble solids were measured following the procedures described by Shams and Thompson (1987). Proximate composition of the dessert (moisture, ash, total protein, total fat and total dietary fibre) was analysed by the official methods of the AOAC International (1999) and energy was calculated based on the macronutrient composition. Total phenols in the dessert were analysed spectrophotometrically using a Folin-Denis reagent following the AOAC International (1999) method. The major phenolic compounds were analysed by HPLC according to the method described by Cantos et al. (2000) and vitamin C content was measured by HPLC, as described by Esteve et al. (1995). The *in vitro* total antioxidant activity was assessed by the Trolox equivalent antioxidant capacity (TEAC) and by the ferric reducing ability of plasma (FRAP) assays, as described later. All analyses were made in triplicate and results are shown in **Table 1**.

Study design

Non-smoking institutionalised elderly subjects were recruited from a pool of volunteers in an old age home in Murcia (Spain). The protocol was carefully explained to the volunteers and their written informed consent was obtained. Twenty-two subjects (six men and sixteen women) participated in the study. The average age was 78.88 (range 65-92) years and average body mass index (BMI) was 30.98 (range 23.83-37.58) kg/m^2. A control group of elderly volunteers (EC) (n=8) who did not receive the treatment was included in order to establish that any changes in parameters were not merely due to effects of time. A non-smoking healthy group of twelve subjects (six men and six women; age range 25-50 years) recruited from a pool of volunteers of Hero Spain S.A. (Alcantarilla, Murcia, Spain), were also studied to serve as a reference healthy group.

The present study was approved by the local research ethics committee of Murcia University and by the local government (ISSORM, Murcia, Spain) and complied with Helsinki guidelines for clinical studies.

The present study was designed as a randomised intervention trial with a period of 2 weeks. The subjects received the dessert and were instructed to maintain their usual diet and to consume one serving (a jar of 200 g) of test product daily for 2 weeks, at a self-selected time but not replacing a meal. The subjects were also instructed to store the dessert in a refrigerator. The habitual diet of the subjects was checked daily with 24 hours dietary recalls and the content of macronutrients and selected micronutrients in the diet was calculated using the computer program ALIMENTACIÓN Y SALUD 0698.046 (BitASDE General Médica Farmacéutica, Valencia, Spain). At days 1 and 15, blood samples were collected to analyse total antioxidant capacity, biochemical parameters, antioxidant vitamins, LDL peroxidation and DNA damage to peripheral blood lymphocytes. Five women withdrew during study due to dislike of the test product and did not finish all the experiments. Data for twenty-two subjects were available on day 1 (elderly baseline group, EB) and data for seventeen subjects were available at the end of the study (elderly treated group, ET). To compare the results of the analysed parameters, blood samples were also collected from the non-smoking, healthy reference group (HR).

Blood sample collection

At baseline and at the end of the study period, blood samples were collected by venepuncture from fasting subjects. For the analysis of total antioxidant capacity, ascorbic acid, Fe, albumin, bilirubin and uric acid in serum, 10 mL blood were collected into evacuated

glass tubes (Venoject, Terumo, Leuven, Belgium) and allowed to clot at room temperature for 25 minutes. Samples were immediately centrifuged at 1000 *g* for 15 minutes at 4ºC to recover the serum. Serum samples were deproteinised for ascorbate determination before freezing and stored at -80ºC until analysed. For analysis of retinol, α-tocopherol, β-carotene and ubiquinol in plasma, lipid peroxidation markers and DNA damage to peripheral blood lymphocytes, 10 mL blood were collected into evacuated glass tubes containing K_3-EDTA (Venoject, Terumo, Leuven, Belgium). Samples were refrigerated and transported within 4 hours to the Institute of Nutrition and Food Technology (Granada, Spain).

Serum total antioxidant capacity

In order to overcome problems and eliminate tedious determination of individual antioxidants, methods capable of measuring antioxidant activity of all the compounds present in a sample with one simple determination have been developed, including the TEAC and the FRAP assays. These total antioxidant assays are useful in getting a global picture of relative antioxidant activities in foods, body fluids and tissues, and how they change in clinical, physiological and pathological conditions.

Serum TEAC was measured by using the method of Miller et al. (1993) with commercially available kits (Total Antioxidant Status, NX 2332, Randox Laboratories Ltd., Ardmore, UK). This method is based on the inhibition by antioxidants of the absorbance of the radical cations of 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) at 600 nm. ABTS radical cations are formed by incubation of ABTS with metmyoglobin and H_2O_2. The final results are expressed as milimoles of Trolox equivalents/L. The inhibition of 1 Trolox equivalent/L equals the inhibition produced by 1 mmol Trolox/L. Serum FRAP was determined by the method of Benzie and Strain (1996). The FRAP assay measures the ferric-reducing ability of plasma or serum. At low pH, when a ferric (Fe^{3+})-tripyridyltriazine complex is reduced by antioxidants to the ferrous (Fe^{2+}) form, an intense blue colour with an absorption maximum at 593 nm develops. In the FRAP assay, Fe^{2+} was used as a standard. The final results were expressed as mmol Fe^{2+} equivalents/L. The unit of 1 Fe^{2+} equivalent/L equals the amount of Fe^{2+}/L required to give the same absorbance change.

Biochemical parameters

Fe, albumin, bilirubin and uric acid were measured in serum by using a Cobas Mira Plus Chemistry Analyser (ABX Diagnostics, Montpellier, France) with reagent kits purchased from ABX Diagnostics.

Co-enzyme Q_{10} and antioxidant vitamin determination

For ascorbic acid (vitamin C) determination, serum samples were added to one volume of 10% (w/v) of metaphosphoric acid containing 0.54 mmol of Na_2-EDTA, agitated in a vortex mixer and centrifuged at 7200 *g* for 5 minutes to pellet the precipitated proteins. The supernatant fraction was removed and stored at -80ºC until analysed. Serum ascorbic acid was assayed by using reagent kits for colorimetric determinations (Böehringer-Mannheim, 409677, Mannheim, Germany). Analyses of coenzyme Q_{10}, retinol, ß-carotene and a-tocopherol were assayed according to MacCrehan (1990) by reversed-phase HPLC using a Spherisorb S5 ODS1 (Merck, Darmnstadt, Germany) column and ethanol-purified water (97:3; v/v) as the mobile phase. The HPLC system was a Beckman in-line dode array detector; model 168 (Fullerton, CA, USA) connected to a Water (Milford, MA, USA) 717 plus autosampler. The column was maintained at a constant temperature of 22ºC. Coenzyme Q_{10}, retinol, ß-carotene and a-tocopherol were identified by predetermining the retention times of individual pure standards.

Determination of low-density lipoprotein oxidation susceptibility

LDL was isolated as described Chung et al. (1981). LDL protein was measured by the Bradford (1979) method. To study the susceptibility to oxidation of LDL, two determinations were performed; thiobarbituric-acid-reactive substances (TBARS) and conjugated dienes. LDL protein (100 mg/L) was oxidised in the presence of Cu^{2+} (10 and 20 µmol/L) in PBS for 6 hours at 37ºC (Jialal and Grundy, 1991). The lipid peroxide content of oxidised LDL was determined as TBARS according to Buege and Aust (1978). Conjugated dienes in LDL were carried out according to Puhl et al. (1994) in a Perkin Elmer UV-VIS Lambda 40 spectrometer equipped with an auto-cell holder and controlled by a Peltier element at a temperature of 37ºC. The lag phase and slope were calculated using the Perkin Elmer UV-WINLAB software.

DNA oxidative damage (Comet assay)

Peripheral blood was collected and the 'buffy coat', enriched in erythrocytes, was removed, diluted 1:1 with RPMI-1640 medium, layered onto an equivalent volume of Histopaque to obtain peripheral blood lymphocytes. The comet assay was used to measure DNA strand breaks in the cells (Collins et al. 1996).

Statistical analyses

Before any statistical analysis, all variables were checked for normality and homogeneous variance using the Kolmogorov-Smirnoff and the Levene tests, respectively. When a variable was found not to follow normality, it was log-transformed and reanalysed. A Student's *t* test was performed to evaluate differences between baseline and after-treatment parameters in the elderly individuals. All parameters for the elderly baseline (EB), elderly treated (ET) and healthy reference (HR) groups were analysed by a one-way ANOVA; to evaluate mean differences from EB and ET groups versus HR group a multiple comparison test adjusted by Bonferroni corrections was performed. A *P* value of less than 0.05 was considered significant. Data were analysed using a statistical software package (SPSS for Windows, 11.0.1., SPSS Inc. Chicago, IL, USA).

RESULTS

Compositional indices, total antioxidant capacity, phenolic profile and vitamin C content in the dessert made of grapes, cherries and berries are shown in **Table 1**. The product showed a high water content, whilst the concentrations of the nutrients such as protein, fibre and minerals were very low, with levels under 0.5% of total weight. Fat was not detected in the compositional analysis. Total phenol content was 1904.21 mg/kg, whereas the main phenolics were anthocyanins, followed by hydroxycinnamic acids, stilbenoids, flavonols and ellagic acids. This product showed a high antioxidant capacity with a mean activity of 18.22 mmol TEAC/L and 23.65 mmol Fe^{2+}/L, for both methods assayed.

Table 1. Compositional indices, total antioxidant activity, phenolics profile and vitamin C content of the dessert[1].

	Mean	SD
Energy *(kcal/100g)* ♠	81.00	0.50
Energy *(kJ/100g)* ♠	338.00	2.10
Moisture (*%*)	79.18	0.10
Total protein (*%*)	0.50	0.02
Total fat (*%*)	nd*	
Total dietary fiber (*%*)	0.28	0.00
Ash (*%*)	0.34	0.02
Total soluble solids (*ºBrix*)	21.00	1.12
pH	3.65	0.01
Total titratable acidity (*% citric acid*)	0.62	0.02
TEAC (*mmol/L*)	18.22	1.35
FRAP (*mmol/L*)	23.65	0.85
Total phenols (*mg/kg*)	1904.21	89.58
Anthocyanins (*mg/kg*)	224.50	1.10
Hydroxycinnamic acids (*mg/kg*)	71.97	2.97
Stilbenoids (*mg/kg*)	4.70	0.45
Flavonols (*mg/kg*)	30.73	1.40
Ellagic acids (*mg/kg*)	3.24	0.14
Vitamin C (*mg/kg*)	138.80	0.50

[1]Mean±Standard Deviation; *nd, not detected; ºBrix, beverage such as juice; TEAC, Trolox-equivalent antioxidant capacity; FRAP, ferric-reducing ability of plasma; ♠Calculated based on the macronutrient composition.

The average daily intake of energy and nutrients **(Table 2)** was compared with the Recommended Dietary Allowances (RDA) according to the National Research Council (1989). Total energy intakes for women and men were below the RDA, but the subjects showed a stable weight (body-weight changes were less than 1 kg), during the intervention period. As regards the macronutrients, there were high intakes of protein and fat, which represented 16% and 36% of the total energy intake, respectively. The intake of micronutrients showed a high variability, depending on the subjects. Vitamin A and vitamin C intakes met the RDA but showed a large standard deviation. The diet provided only vitamin D and E in amounts below the RDA, showing the same pattern as the total intake of Zn and I. No statistical differences were found in all parameters analysed between elderly subjects after the intake of the high antioxidant dessert and the elderly control group at the end of the study.

Table 2. Average daily intake of energy and nutrients in the group of elderly individuals[1].

Nutrient	RDA*		Average daily intake (n=17)	
	Men	Women	Mean	SE
Energy (*kJ*)	9614	7942	7610	1009
Energy (*kcal*)	2300	1900	1821	242
Proteins (*g*)	63	50	76	13
Carbohydrates (*g*)	--	--	213	37
Lipids (*g*)	--	--	76	14
Saturated fatty acids (*g*)	--	--	19	4
Monounsaturated fatty acids (*g*)	--	--	29	8
Polyunsaturated fatty acids (*g*)	--	--	10	4
Cholesterol (*mg*)	<310	<310	264	50
Dietary fiber (*g*)	25	25	18	5
Vitamin A (*mg*)	1000	800	1526	1183
Vitamin B1 (*mg*)	1.2	1	1.4	1.1
Vitamin B2 (*mg*)	1.4	1.2	1.57	0.61
Vitamin B6 (*mg*)	2	1.6	1.8	0.2
Vitamin B12 (*mg*)	2	2	5.3	2.8
Vitamin C (*mg*)	60	60	157.6	85
Vitamin D (*mg*)	5	5	3.5	2.2
Vitamin E (*mg*)	10	8	5.7	1.6
Niacin (*mg*)	15	13	20.1	3.9
Folic acid (*mg*)	200	180	248.3	76.1
Sodium (*mg*)	--	--	1443	342
Potassium (*mg*)	--	--	2955	524
Calcium (*mg*)	800	800	859	151
Phosphorus (*mg*)	800	800	1144	168
Ratio Ca/P	1	1	0.76	0.14
Magnessium (*mg*)	350	280	272.5	51.6
Iron (*mg*)	10	10	12.4	2.5
Zinc (*mg*)	15	12	6.3	1.5
Iodine (*mg*)	150	150	59.3	17.1

[1]Mean±Standard Deviation; *According to the National Research Council (1989) for men and women over 50 years old.

Serum antioxidant capacity and biochemical measures obtained from the subjects after treatment compared with the baseline and the healthy reference groups are shown in **Table 3**. There were no significant differences in the antioxidant activity and the biochemical parameters measured in the serum of elderly individuals after the intervention study period. In addition, the data of TEAC and FRAP of the elderly individuals were not different than those obtained in the healthy reference group. Only uric acid was significantly lower ($P<0.05$) in the healthy reference group compared with data from elderly individuals.

Table 3. Serum total antioxidant capacity and biochemical measures in elderly volunteers consuming a phenolic-rich dessert (Mean values and standard deviations).

Variable	EB (n=22)		ET (n=17)		EC (n=8)		HR (n=12)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TEAC (*mmol/L*)	0.62[a]	0.14	0.66[a]	0.16	0.58[a]	0.21	0.65[a]	0.14
FRAP (*mmol/L*)	0.96[a]	0.16	1.01[a]	0.17	0.82[a]	0.09	0.95[a]	0.19
Albumin (*g/L*)	39.4[a]	5.7	40.1[a]	3.9	36.0[a]	4.4	46.2[b]	3.1
Bilirubin (*mmol/L*)	11.11[a]	5.59	12.73[a]	5.93	11.12[a]	5.5	10.7[a]	3.9
Iron (*µmol/L*)	12.9[a]	6.8	15.30[a]	6.2	13.1[a]	3.1	15.0[a]	5.9
Uric acid (*µmol/L*)	281[ab]	72	300[a]	77	240[a]	70	251[a]	88
Ascorbic acid (*mmol/L*)	51[a]	26	53[a]	15	38[a]	11	55[a]	22

EB, elderly baseline group; ET, elderly after treatment group; EC, elderly control group; HR, healthy reference group; TEAC, Trolox-equivalent antioxidant capacity; FRAP, ferric-reducing ability of plasma.

[a,b]Within a row, mean values with unlike superscript letters are significantly different ($p<0.05$).

No differences were found in plasma lipid antioxidant vitamins (a-tocopherol, ß-carotene and retinol) and coenzyme Q_{10} values in elderly subjects after the intake of the high antioxidant dessert and **(Table 4)**. It is important to emphasise that plasma antioxidant vitamin levels (a-tocopherol, retinol, ß-carotene) and coenzyme Q_{10} in the elderly subject were below those considered as normal values (Human healthy averages are: retinol 2.27 µmol/L; a-tocopherol 23.5 µmol/L; ß-carotene 0.35 µmol/L and coenzyme Q_{10} 0.60 µmol/L; Cutler and Mattson, 2003). However, there were significant differences for these vitamins between the elderly and healthy groups. Susceptibility of LDL to oxidation did not change with the intake of the dessert in elderly volunteers but the LDL TBARS levels and LDL lag phase were significantly different from the healthy group, showing more LDL oxidation in the elderly subjects **(Table 4)**.

Table 4. Plasma lipid antioxidant vitamins and co-enzyme Q_{10} values; and susceptibility of low-density lipoproteins to oxidation in elderly volunteers consuming a phenolic-rich dessert (Mean values and standard deviation).

Variable	**EB (n=22)**		**ET (n=17)**		**EC (n=8)**		**HR (n=12)**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma								
a-tocopherol (µmol/L)	19.0[a]	6.3	16.7[a]	4.36	17.7[a]	5.4	20.5[a]	3.8
Retinol (µmol/L)	0.85[a]	0.21	0.74[a]	0.21	0.80[a]	0.30	1.5[b]	0.4
ß-carotene (µmol/L)	0.29[a]	0.1	0.29[a]	0.1	0.41[a]	0.2	0.8[b]	0.4
Coenzyme Q_{10} (µmol/L)	0.18[a]	0.08	0.26[a]	0.15	0.15[a]	0.03	0.52[b]	0.19
Low-density lipoproteins (LDL)								
CONJUGATE DIENES								
Phase-lag (minutes)	53.8[a]	12	55.1[a]	11	53.3[a]	13.3	81.3[b]	27
Slope	0.02[a]	0.09	0.02[a]	0.009	0.014[a]	0.004	0.02[a]	0.01
TBARS (nmol/mg LDL protein)								
10 µM (Cu^{2+})	27.7[b]	10.8	34.16[b]	10.5	27.4[b]	11.5	19.2[a]	4.7
20 µM (Cu^{2+})	33.9[b]	12.0	30.05[b]	11.1	30.5[b]	11	20.3[a]	3.0

EB, elderly baseline group; ET, elderly after treatment group; EC, elderly control group; HR, healthy reference group; TBARS, thiobarbituric-acid reactive substances

[a,b]Within a row, mean values with unlike superscript letters are significantly different ($p<0.05$).

DNA strand breakage did not decrease in elderly institutionalised individuals after the intake of the high antioxidant dessert but their values were significantly higher compared with the healthy reference group **(Figure 1)**.

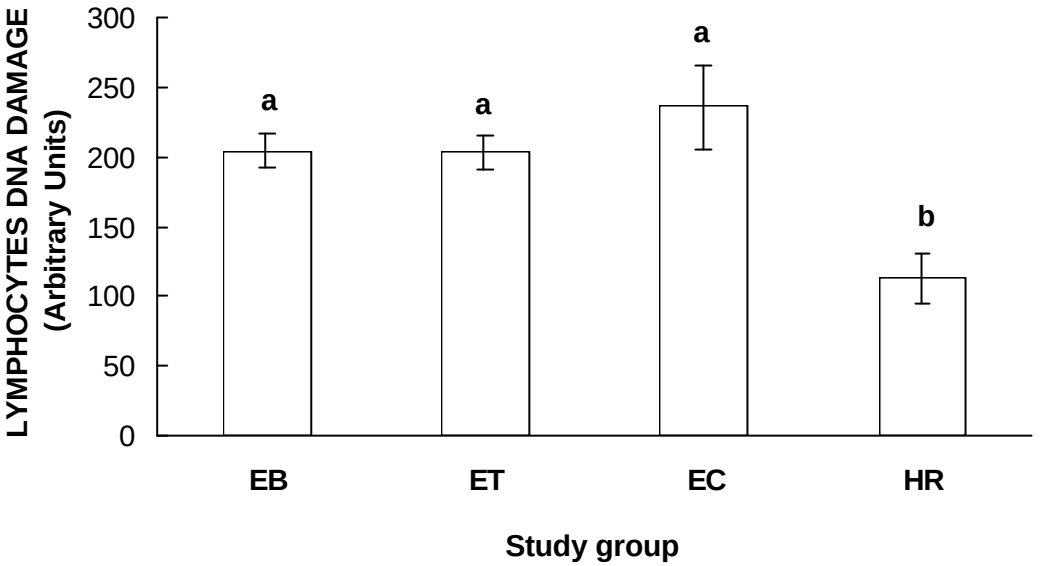

Figure 1. Peripheral lymphocyte DNA strand breakage after the intake of a high antioxidant dessert. Values are means, with standard deviations represented by vertical bars. EB, elderly baseline group (n=22); ET, elderly after treatment group (n=17); EC, elderly control group (n=8); HR, healthy reference group (n=12). Mean values with unlike superscript letters were significantly different ($p<0.05$).

DISCUSSION

Ageing is usually defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality with advancing age (Kirkwood and Austad, 2000). The importance of ageing is based, first, in the high percentage of people over 65 years (close to 20%) and the raise in the number of individual over 80 years, and second, in the growing incidence of ageing-related chronic diseases such as Alzheimer's disease, Parkinson's disease, diabetes and cancer. Moreover, a leading cause of death among older individuals worldwide is vascular disease and associated chronic conditions. The impact of the diet and dietary components on ageing and age-associated degenerative diseases has been widely recognized in recent years (Ames et al. 1993; Meydani, 2001). Accordingly, there is great potential for the prevention of these diseases through healthy lifestyles that include physical activity and well-balanced diets (Tucker and Buranapin, 2001).

There is increasing evidence that the oxidation of biomolecules (DNA, proteins and lipids) may play a role in susceptibility to disease, especially ageing-related conditions such as cancer and heart disease, and in the ageing process itself (Halliwell and Chirico, 1993; Pryor, 1987). Animal data have shown that DNA damage accumulates with age (Richter et al. 1988) and, as Harman (1956) suggested almost a half a century ago in his free radical theory of ageing, oxidative damage is related to the debilities associated with ageing. Consequently, it would seem that the oxidative stress status and antioxidant status of the elderly population should be of considerable interest and importance. The present results are in agreement with those of Trevisan et al. (2001). These authors demonstrated that ageing is associated with increased oxidative stress and reduced antioxidant potentials, as has been reported in the present study for the low TEAC, FRAP, plasma antioxidant vitamin values and the higher susceptibility of LDL to Cu-induced oxidation in elderly volunteers at the beginning of the study compared with the healthy group.

In the last few years, much attention has been focused on the antioxidant properties of flavonoids, a large class of polyphenolic compounds derived from plants. Evidence suggests that these compounds may protect tissues against damage caused by oxygen free radicals and lipid peroxidation (Bub et al. 2003). The antioxidant capacity of the dessert used in the present study was higher than that observed in many fruits and vegetables, being similar to that of antioxidant beverages, such as tea and red wine (García-Alonso et al. 2003). The antioxidant activity of the product seems to be related to its phenolic compounds, since there was a positively and significant correlation between anthocyanin content and total antioxidant activity as assessed by TEAC and FRAP assays.

We thought that maybe supplementation with flavonoids in our elderly volunteers would help to improve their antioxidant status and consequently attenuate their oxidative status. The main flavonoid used in our dessert was anthocyanin (224 mg/kg). We have previously described anthocyanin as endowed with antioxidant effects because it decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats (Ramirez-Tortosa et al. 2001). Furthermore, other studies have reported that anthocyanins are absorbed in elderly women, finding a high level of these compounds in plasma and urine after their intake (Cao et al. 2001). However, we did not find a significant effect after supplementation with our dessert on biological markers of oxidative stress, plasma antioxidant defence and LDL oxidisability in the elderly volunteers and, maybe, to get effects as antioxidants requires doses far in excess of that which is nutritionally relevant. Therefore, the present results have confirmed the view that the marked antioxidant ability of polyphenols *in vitro* does not translate to analogous effects *in vivo*.

The present results are in accordance with Young et al. (2002), who found that an intervention in healthy human subjects with 18.6 mg catechin/day for 6 weeks did not affect markers of oxidative stress and antioxidant status, including plasma or haemoglobin protein oxidation, plasma oxidation and plasma lipid antioxidant vitamins. Furthermore, Hininger et al. (2001) did not find any effect of lutein (15 mg), lycopene (15 mg) or beta-carotene (15 mg) supplementation on biochemical indices of oxidative status in healthy adult males after 3 months of intervention. In all these studies, the intervention period was longer than in the present research. However, it is reasonable to think that since the product tested here is much richer in antioxidants than the amounts used in those mentioned studies, a study period of 2 weeks should be enough to find an improvement in the oxidative stress status of the subjects.

Oxidative DNA damage accumulates with age and is related to lifespan of the particular organism, being also associated with premature ageing (Beckman and Ames, 1998). Antioxidants protect the cellular system from oxidative damage (Krinsky, 1992) and consumption of foods rich in antioxidants such as vitamin E, vitamin C and polyphenols is associated with a decreased risk for cancer and coronary disease (Byers and Perry, 1992; Hertog et al. 1995). The study of biomarkers of DNA damage (for example, the comet assay) and biochemical markers (for example, plasma antioxidants) as putative indicators of ageing is used increasingly to provide a focused and mechanistic approach to the study of diet, health and disease. Using this approach, we decided to measure the potential effect of our

dessert rich in flavonoids on DNA damage. Unfortunately, we did not find any effect on DNA strand breakage. The reason for this, maybe, is the short intervention period (2 weeks) and the poor antioxidant status of the elderly volunteers confirmed by the high endogenous DNA damage at the beginning of the study. However, Pool-Zobel et al. (1997) showed that endogenous DNA strand breakage was reduced in human lymphocytes isolated from subjects given supplemental vegetable juice (tomato juice with lycopene, carrot juice with β-carotene and spinach with lutein in water) for 2 week each. Other studies showed that prolonged supplementation (80 days) with a commercially available fruit and vegetable extract also decreased DNA strand breakage in elderly volunteers (Smith et al. 1999). Lymphocytes isolated from human volunteers fed a diet supplemented with lycopene (16.5 mg) for 21 days were more resistant to *ex vivo* H_2O_2 treatment compared with lymphocytes from the untreated controls (Riso et al. 1999).

It can be considered that any change in the parameter were not due to the effect of time because no differences between elderly subjects after the intake of the high-antioxidant dessert and the elderly control group at the end of the study were found. Finally, it is important to keep in mind that elderly subjects have a risk for marginal deficiency of lipid antioxidant vitamins as a-tocopherol, retinol and ß-carotene. Dietary components with a high antioxidant activity have to receive particular attention because of their potential role in modulating oxidative stress associated with ageing and chronic conditions (Meydani, 2001).

The conclusion of the present study is that an intervention of 2 weeks with our dessert enriched with natural polyphenol compounds in elderly individuals does not provide enough time to find changes in the antioxidant and oxidative status. We have confirmed the view that the marked antioxidant ability of polyphenols *in vitro* does not translate to analogous effects *in vivo*. Moreover, a highly oxidative stress status during ageing is confirmed together with the need to conduct follow-up nutritional studies to improve this situation.

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3.6. Estudio 6

Short-term effect of the intake of a phenolic-rich juice on biomarkers of antioxidant protection in healthy subjects

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ABSTRACT

In the present study, we evaluated the short-term effect of a phenolic-rich juice made from grape and berries on biomarkers of antioxidant protection. After a 48-hours washout period, 12 fasting subjects consumed 400 mL of the juice. No significant changes were detected in serum total antioxidant capacity (FRAP). A slight increase was observed in serum lipid-bound polyphenols from 2 to 6 hours, which was associated with a significant decrease in lipid peroxidation products (TBARS). The urinary excretion of phenolics peaked 2 hours post intake. Our data suggest that phenolics from the juice were bioavailable and able to bind with the lipid fraction of serum, therefore reducing lipid peroxidation. In contrast, the serum protein carbonyl content increased between 1 and 4 hours post intake, and later fell back to baseline levels at 6 hours. This indicates a transient prooxidant effect of juice consumption, which could be due to a sugar-mediated glycoxidation and/or glycosylation mechanism.

Keywords: Berries; phenolic-rich juice; Antioxidant; Polyphenols; FRAP; TBARS; Biomarkers; Carbonyl protein; Urinary excretion.

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INTRODUCTION

Nutritional guidelines (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997) indicate that an increase in the consumption of foods rich in antioxidant nutrients may decrease or prevent the risk of many diseases caused by oxidative stress. Therefore, increasing the plasma/serum antioxidant status has been proposed as a method for preventing the development of cancer, cardiovascular disease, diabetes and other diseases (Kay and Holub 2002). Significant and possibly relevant effects on total plasma/serum antioxidant capacity have been reported for phenolic-rich foods and beverages, such as wine, tea, different fruits and their derivatives, both in short- and long-term human studies (Cao et al., 1998; Duthie et al., 1998; Serafini et al., 1998; Mazza et al., 2002).

For phenolic compounds to exert a beneficial effect on human health, it is reasonable to assume that they have to be absorbed. Although data on polyphenol bioavailability and its metabolic fate is scarce and considered controversial (Williamson et al., 2000; Karakaya 2004), several human studies have reported direct evidence supporting the absorption and urinary excretion of these compounds after the intake of phenolic-rich foods (Pedersen et al., 2000; Kay and Holub, 2002; Netzel et al., 2002; Prior 2003). However, due to the great variability in the chemical structure of polyphenols and the several metabolites than can be formed during digestion, such studies require specific analytical techniques, such as HPLC-MS to provide reliable data (Cerdá et al., 2004; Ichiyanagi et al., 2005; Manach et al., 2005). A voluminous body of research on the potential role of antioxidant nutrients in the prevention of chronic disease is available in the scientific literature, but despite these efforts, much remains unknown. Biomarker research in this field has the potential to help fill the gaps in current knowledge concerning the role of phenolic compounds in the prevention of diseases (Mayne 2003).

In a previous study, we evaluated the effect of the intake of a dessert made from grape and berries on the overall antioxidant status a group of institutionalized elderly people (Ramírez-Tortosa et al., 2004). Although the test product was rich in phenolic compounds and showed a high antioxidant activity (García-Alonso et al., 2003), no significant effect was observed on the antioxidant status or on any biomarkers of oxidative stress (lipid and DNA damage). These results were attributed to the low degree of phenolic absorption from the dessert, to the short intervention period (2 weeks) or to the age of the elderly subjects, since age-related physiological changes impair antioxidant status and nutrient absorption. In fact, in the elderly group the baseline antioxidant status was lower than that of a group of young

healthy subjects who also participated in the study, therefore supporting the role of oxidative stress in the aging process.

Based on this knowledge, the question arises whether the intake of a high dose of natural polyphenols can really exert an effect on the functional biomarkers of antioxidant protection and/or oxidative stress in healthy subjects. For this reason, the purpose of the present study was to evaluate the short-term effect of the intake of a single dose of a phenolic-rich juice made from grape and berries on the oxidative stress status of healthy adult subjects. This study design was aimed at evaluating the kinetics of absorption of any antioxidants from the juice, as well as the effect of such intake on biomarkers of lipid and protein oxidation.

SUBJECTS AND METHODS

Test product

The test product was an experimental juice prepared by the Research and Development Department of Hero Spain S.A (Alcantarilla, Murcia, Spain). This product was formulated and designed using the data concerning total antioxidant activity available in the scientific literature for the fruits used (Cao et al., 1996; Wang et al., 1996). The objective was for each serving (a bottle of 200 mL) to have the average antioxidant capacity equivalent to 10 servings of fruits and vegetables, double the "Five a day" recommended by several international organizations (World Health Organization 1990; World Cancer Research Foundation and American Institute for Cancer Research 1997). The major ingredient was water, which was mixed with commercially available concentrated juices of grape (26%), cherry (2%), blackberry (0.6%), blackcurrant (0.6%) and raspberry (1%). The resulting product was pasteurized in order to obtain a microbiologically stable foodstuff and bottled hot to ensure headspace vacuum.

The chemical composition and antioxidant properties of the functional juice (Table 1) were assessed as suggested by García-Alonso et al. (2003). The main groups of phenolic compounds in the juice were HPLC-analyzed on a L-7100 liquid chromatograph equipped with a Merck-Hitachi 7455 UV diode array detector and a Licrochart RP-18 column of 25 x 0.4 cm, 5-mm particle size (Merck, Darmstadt, Germany). Total phenolics in the juice were analyzed by a colorimetric assay using Folin-Ciocalteu's phenol reagent and the total antioxidant activity of the juice was evaluated as its iron reducing capacity. Ascorbic acid in the juice was analyzed by reversed phase HPLC in a 2690 Waters system equipped with a Waters 996 UV diode array detector (Waters, Milford, MA, USA).

Subjects

Twelve healthy volunteers (6 men and 6 women) were enrolled in this study. The subjects were aged 23–43 years, with a body mass index ranging from 23 to 25 kg/m^2. They took no vitamin/mineral supplements. Smokers and subjects with inflammatory disease or taking any medication were excluded from the study. All participants were in good health based on their medical history, a medical examination, and normal results from clinical laboratory tests. The protocol was carefully explained to the volunteers and their written informed consent was obtained. The study was approved by the Clinic Research Ethics Committee of the University of Murcia (Murcia, Spain), and complied following the Helsinki guidelines for clinical studies.

Study design

To partially standardize and limit the intake of antioxidants during the study, the subjects were asked to refrain from consuming fruits and vegetables or their juices, tea, coffee or wine for 2 days before taking the juice dose, as well as throughout the study. After an overnight fast, blood baseline samples were obtained before drinking 400 mL of the phenolic-rich juice, which was consumed by all participants within 30 minutes. Blood samples were withdrawn again 1, 2, 4 and 6 hours post intake. Blood was collected by venipuncture into evacuated plastic tubes containing clotting activator and Gel Z serum separator (Vacuette, Greiner Bio-One, Kremsmuenster, Austria), and centrifuged at 5000 rpm for 5 min at 4°C, to recover serum. Urine was collected over 24 hours the day before the juice intake (baseline) and cumulative urine samples were collected 1, 2, 4, 6 and 24 hours after the intake of the juice. Urine was collected in tared plastic containers and the total weight of the each urine collection was recorded. Both serum and urine samples were aliquoted and stored at -80ºC until analysis. For vitamin C analysis, serum samples were stabilized with 5% (w/v) trichloroacetic acid (TCA) before freezing. Aliquots (200 µL) of serum were transferred into 1.5 mL microcentrifuge tubes, together with 300 µL of 5% TCA, and the resulting mixture kept frozen at -80ºC until analyzed.

Blood, serum and urine assays

Standard biochemical determinations. Baseline data collection included complete blood count and blood analyses. Among others, liver function tests, such as glutamic pyruvic transaminase (GPT), glutamic oxalacetic transaminase (GOT), gamma glutamyl transpeptidase (gamma-GT), alkaline phosphatase, bilirrubin, and albumin, hematocrit, haemoglobin, plasma glucose, triglycerides and cholesterol levels, were measured by standard clinical laboratory tests. C-reactive protein (CRP) was also measured as an index of inflammatory processes **(Table 2)**.

Measurement of total polyphenols. The total polyphenol content in the juice, urine and serum samples was quantified with a colorimetric assay using Folin-Ciocalteau's phenol reagent (Sigma, St. Louis, USA) according to the method described by Singleton and Rossi (1965). Before the colorimetric analysis, the juice and urine samples were diluted 50-fold and 10-fold in water, respectively, whereas total phenolic compounds in serum were determined after a procedure of extraction/hydrolysis, as described by Serafini et al. (1998). In order to remove protein interferences, serum protein was precipitated with 0.75 M meta-phosphoric acid (MPA). For hydrolyzing the conjugated forms of polyphenols, 200 µL of 1 M HCl was added to 100 µL of the serum, vortexed for 1 minute and incubated at 37°C for 30 minutes. Later, 200 µL of 2 M NaOH in 75% methanol was added, and the resulting mixture was vortexed for 2 minutes and incubated at 37°C for 30 minutes. This step breaks down the bonds of polyphenols with lipids and enables the extraction of lipid-bound phenolic compounds. Then, 200 µL of 0.75 M MPA was added after vortexing for 2 minutes to remove serum proteins, and the sample was centrifuged at 10000 rpm for 10 minutes. The supernatant was removed and diluted 5-fold in distilled water before the colorimetric analysis. For the assay, 500 µL of 0.2 N Folin-Ciocalteu's phenol reagent and 400 µL of a 2 M Na_2CO_3 solution were added to 100 µL of the diluted sample. After 2 hours of incubation in darkness at room temperature, absorbance at 750 nm was measured with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Gallic acid monohydrate (Sigma, St. Louis, USA) was used as the standard to prepare the calibration lines. The results were expressed as milligrams of gallic acid equivalents (GAE) per litre.

Measurement of total antioxidant activity. The antioxidant potential of the juice, and serum and urine samples was estimated from their ability to reduce ferric iron, using the FRAP assay (Benzie and Strain 1996). Prior to the assay, juice and urine samples were diluted 10-fold in water, whereas serum samples were assayed undiluted. The FRAP reagent was freshly prepared by mixing 25 mL of 300 mM acetate buffer, pH 3.6 (3.1 g $C_2H_3NaO_2 \cdot 6H_2O$ and 16 mL acetic acid glacial/L), 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine in 40 mM HCl solution, and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ in water. For the assay, 750 µL of freshly prepared FRAP reagent was warmed to 37ºC and a reagent blank reading was taken at 593 nm. Then, 25 µL of sample was added along with 75 µL water and the reaction was monitored up to 4 minutes. The 4-minutes absorbance readings were used for calculation of FRAP values. Aqueous solutions of known Fe^{2+} concentration in the range of 100 to 2000 µM ($FeSO_4 \cdot 7H_2O$) were used for calibration, and the results were expressed as µM of Fe^{2+}

equivalents. One Fe^{2+} equivalent per litre equals the amount of Fe^{2+} per litre required to give the same absorbance change.

Measurement of vitamin C levels. The concentrations of ascorbic acid in serum were measured by the 2,4-dinitrophenylhydrazine (DNPH) method (Roe and Kuether 1943), in which ascorbic acid is oxidized to dehydroascorbic acid through the catalytic action of copper. After that, dehydroascorbic acid is coupled with 2,4-dinitrophenylhydrazine and the resulting derivative is treated with sulphuric acid to produce a colour which is measured photometrically at 520 nm. The 2,4-dinitrophenylhydrazine (DNPH) working reagent was freshly prepared as follows. Firstly, a 2.2% (w/v) DNPH solution in 9 N H_2SO_4 was prepared and filtered through Whatman paper No. 1 (Whatman International, Maidstone, Kent, UK). Then, to 20 volumes of the above solution, we added 1 volume of an aqueous 5% (w/v) thiourea solution together with 1 volume of an aqueous 0.6% (w/v) $CuSO_4 \cdot 5H_2O$ solution. For the assay, thawed TCA-stabilized serum extracts were centrifuged at 12000 rpm for 5 minutes at 4ºC. Then, 300 µL of supernatant was mixed with 100 µL of DNPH working reagent, vortexed and incubated at 60ºC for 1 hour in a water bath. After this, samples were cooled in an ice bath for 5 minutes and 400 µL of 85% (v/v) H_2SO_4 solution was added and vortexed. Absorbance readings at 520 nm were taken after incubating the samples at room temperature in the darkness for 20 minutes. For calibration, aqueous solutions of L-(+)-ascorbic acid (Merck, Darmstadt, Germany), in the range 2 to 20 mg/L, were prepared by diluting in water a stock solution of 1 g/L ascorbic acid in 5% (w/v) TCA. Then, 200 µL of water (blank) or standard was mixed with 300 µL of 5% TCA and the resulting mixture vortexed. Finally, 300 µL of the latter mixture was assayed as described above. The results were expressed as mg/L of ascorbic acid.

Measurement of serum uric acid. Uric acid was measured by an enzymatic colorimetric test using a commercially available uricase/PAP kit (Spinreact SA, Girona, Spain) in a Cobas Mira plus (ABX Diagnostics, Montpellier, France) and following the manufacturer's instructions. In this assay, uric acid is oxidized by uricase to allantoine and hydrogen peroxide, which, under the influence of peroxidase, oxidizes sulphonated 2,4-dichlorophenol (2,4-DCPS) and 4-aminophenazone (4-AP) to form a red quinoneimine compound. The red dye formed is measured at 520 nm, and its quantity is proportional to the uric acid concentration. The working reagent was prepared by dissolving a lyophilized enzyme mixture containing 60 U/L uricase, 660 U/L peroxidase, 200 U/L ascorbate oxidase and 1 mM 4-AP, with a phosphate buffer 50 mM, pH 7.4 containing 4 mM 2,4-DCPS. For the assay, 50 µL of sample or standard (6 mg/dL uric acid solution) was mixed with 2 mL of working reagent, incubated at room temperature for 10 minutes, and absorbance readings

were taken at 520 nm against a reagent blank. Results were calculated by using the following equation, and expressed as mg/dL.

$$\text{Uric Acid (mg/dL)} = \frac{\text{Absorbance Sample}}{\text{Absorbance Standard}} \times \text{Standard Concentration}$$

Determination of serum thiobarbituric acid reacting substances (TBARS). For the measurement of malondialdehyde and related aldehydes, 100 μL of serum samples were mixed with 1000 μL of 0.67% (w/v) thiobarbituric acid (TBA) and 500 μL of 20% TCA, and incubated at 100°C for 20 minutes. After centrifugation at 5000 rpm for 10 minutes, the absorbance of the supernatants was measured at 532 nm. The total content of aldehydes capable of reacting with TBA to form chromophores absorbing at 532 nm was estimated using a molar absorption coefficient for the malondialdehyde–TBA complex of 1.56×10^5 $M^{-1}\cdot cm^{-1}$ (Buege and Aust 1978; Nourooz-Zadeh et al., 1994).

Carbonyl protein measurement. Protein carbonyl content was measured by forming labelled protein hydrazone derivatives, using 2,4-dinitrophenylhydrazine (DNPH), which were then quantified spectrophotometrically (Levine et al., 1994; Reznick and Packer 1994; Evans et al., 1999). Briefly, after precipitation of protein with an equal volume of 1% TCA, the pellet was resuspended in 1 mL of DNPH 10 mM in 2 N HCl. Separate blanks were prepared by adding 1 mL of 2 N HCl without DNPH. Samples were left at room temperature for 1 hour in the dark and vortexed every 15 minutes. An equal volume of 20% TCA was added and, after centrifugation at 12000 *g* for 15 minutes at 4ºC, pellets were washed three times with 1 mL of ethanol:ethylacetate mixture (1:1) to remove the free DNPH and lipids contaminants. The final pellet was dissolved in 1 mL of 6 M urea and kept at 37ºC for 1 hour in a water bath with mixer. The solution was centrifuged for 15 minutes at 12000 *g*. The carbonyl content was determined from the absorbance at 370 nm with the use of a molar absorption coefficient of 22000 $mol\cdot L^{-1}\cdot cm^{-1}$.

Determination of urine creatinine. In order to normalize all urine determinations, urine creatinine was determined by the Jaffè picric acid spectrophotometric method (Helger et al., 1974). The creatinine present in samples directly reacts with alkaline picrate, resulting in the formation of a red colour, which is measured at 510 nm. The alkaline picrate solution was freshly prepared by mixing equal volumes of a 1.6 M NaOH (64 g/L) aqueous solution and a 35 mM picric acid (8 g/L) aqueous solution. Creatinine (2 mg/100 mL) was used for calibration. For the assay, urine samples were diluted 50-fold in distilled water and assayed as described next. One millilitre of alkaline picrate solution was added to 0.5 millilitres of

diluted sample, standard or water (blank). The reaction mixtures were incubated at room temperature in the darkness for 20 minutes, and absorbance readings were taken at 510 nm. Results were expressed as mg creatinine/mL by using the following equation:

$$\text{Creatinine (mg/mL)} = \frac{\text{? Absorbance Sample} \times \text{Standard Concentration}}{\text{? Absorbance Standard} \times 100} \times \text{Dilution Factor}$$

Statistical analysis

Data were analyzed using a statistical software package (SPSS 11.0.1; SPSS Inc., Chicago, IL, USA). A Student's t test was performed to evaluate differences between baseline and post intake parameters, and relationships between variables were examined using Pearson correlation coefficients. *P* values <0.05 were considered statistically significant. Results are presented as mean±SEM.

RESULTS

Table 1 shows the compositional indexes and antioxidant properties of the juice. **Table 2** shows the baseline haematological and biochemical parameters of the volunteers, compared with reference values for adults. The mean values of the parameters analyzed were all within normal reference range. **Table 3** shows mean time trends of serum FRAP, uric acid, vitamin C, lipid-bound phenolic compounds and protein carbonyl content, as well as the changes in cumulative urinary excretion of phenolics and reductant substances (measured as FRAP), after the intake of the juice. **Figures 1-3** illustrate the relative changes in the above mentioned serum and urine parameters following consumption of the juice and throughout the study. Increments were calculated for each subject by subtracting its baseline level from each post intake-value. The urine parameters were expressed relative to the urinary creatinine concentrations.

Table 1. Compositional indexes and antioxidant properties of the juice.

Parameter	Value	Dose (400 ml)
Total protein	0.30 %	1.2 g
Total fat	ND[5]	0
Total dietary fibre	ND	0
Ash	0.40 %	1.6 g
Total soluble solids	19.30 ºBrix	-
pH	3.57	-
Total titratable acidity (citric acid)	0.54 %	-
Total sugars[1]	15.50 %	62 g
Fructose	9.00 %	36 g
Glucose	6.50 %	26 g
Vitamin C	ND[3]	--
Total phenols[3]	2000 mg GAE/L	800 mg
Anthocyanins	670 mg/L	286 mg
Catechins	425 mg/L	170 mg
Hydroxycinnamic acids	200 mg/L	80 mg
Stilbenoids	9.5 mg/L	3.8 mg
FRAP	32 mM Fe^{2+} equivalents	13 mM Fe^{2+} equivalents

[1]Calculated as the sum of fructose plus glucose content.

[2]GAE, gallic acid equivalents

[3]ND, not detected

Table 2. Fasting baseline characteristics of the participants in the study.

Parameter	Units	Mean	SEM	Reference Range
Blood analyses				
Albumin	g/dL	4.75	0.12	3.5-5
Bilirrubin	mg/dL	0.82	0.15	0.2-1.4
Uric acid	mg/dL	4.33	0.32	2.2-7
Urea	mg/dL	37.92	1.76	10-50
Creatinin	mg/dL	0.99	0.04	0.4-1.4
Glucose	mg/dL	82.08	3.25	60-120
Triglycerides	mg/dL	90.92	15.18	35-200
Cholesterol	mg/dL	213.40	8.40	125-230
HDL-Cholesterol	mg/dL	55.63	2.71	35-110
LDL-Cholesterol	mg/dL	147.10	8.64	30-150
VLDL-Cholesterol	mg/dL	19.88	2.56	7.5-45
Atherogenicity index	None	3.90	0.24	1.2-5.5
GPT	U/L	20.75	2.16	5-40
GOT	U/L	19.42	0.97	5-40
gamma-GT	UL	37.33	10.25	5-50
Alkaline phosphatase	U/L	69.92	9.72	30-120
C-reactive protein (CRP)	mg/L	< 0.5	--	0-5
Complete blood count				
Hematocrit	%	42.18	1.51	37-52
Haemoglobin	g/dL	13.84	0.51	12-18
Erythrocyte count	$10^6/mm^3$	4.92	0.15	4.3-6
Mean corpuscular volume	μm^3	85.76	2.08	80-98
Mean corpuscular haemoglobin	pg	28.14	0.80	17-34
Mean corpuscular haemoglobin concentration	$10^3/mm^3$	32.77	0.22	31-37
Platelet count	$10^3/mm^3$	297.67	22.15	120-450
Leukocyte count	$10^6/mm^3$	6.30	0.46	4.5-12
Leukocytes formula				
Neutrophyls	%	58.41	1.45	40-70
Eosinophyls	%	2.61	0.40	0-7
Basophyls	%	1.17	0.07	0-2
Lymphocytes	%	32.18	1.41	20-45
Monocytes	%	5.64	0.19	3-10

Table 3. Changes in serum and urine parameters after the intake of a single dose of the phenolic-rich juice[1].

	Time after juice intake (hours)					
Serum	**0**	**1**	**2**	**4**	**6**	**24**
FRAP (μM Fe^{2+})	1052±45	1096±46	1084±46	1031±46	1022±45	--
Uric acid (mg/dL)	4.33±0.32	4.54±0.32	4.40±0.33	4.12±0.31	3.99±0.33	--
Vitamin C (mg/L)	10.61±0.73	10.92±0.71	10.58±0.74	10.36±0.76	10.38±0.79	--
Polyphenols (mg GAE/L)	227±5	223±5	238±6	230±4	234±6	--
TBARS (nM MDA)	559±11	538±10	512±5**	516±6**	515±3**	--
Carbonyl Protein (mg/mg protein)	3.14±0.61	7.38±0.33**	5.94±0.71**	7.29±0.52**	3.43±0.59	--
Urine						
Polyphenols (mg GAE/mg creatinine)	0.31±0.03	2.95±0.48**	4.62±0.93*	2.31±0.81**	2.29±0.64**	0.41±0.04*
FRAP (μM Fe^{2+}/mg creatinine)	5.6±0.5	52.5±7.7**	80.3±18.2**	36.6±15.4*	35.1±10.4**	6.9±0.7

[1]Results are expressed as mean±SEM, *p<0.05, **p<0.01 compared with baseline (0 hours) (Student's t-test).

In general, no significant changes were detected in the mean values of serum FRAP, uric acid, vitamin C or serum lipid-bound polyphenols after the intake of the juice or during the study. The lack of statistical significance can be attributed to the high interindividual variations observed for these parameters. Although mean FRAP values did not show significant differences during the study, consumption of the juice resulted in significant changes ($p<0.05$; Student's t-test) in FRAP values for each volunteer compared with the individual's baseline level. FRAP values showed similar behaviour in each individual, increasing from 1 to 2 hours and decreasing later to values sometimes below baseline levels (data not shown).

Changes in serum uric acid levels showed a similar trend to that of FRAP values. Thus, consumption of the juice caused a slight but not significant increase in uric acid levels from 1 to 2 hours post intake. Since ascorbic acid was not detected in the juice, no significant increases were observed in the serum levels of this vitamin, which showed mean values of between 10 and 11 mg/L during the study **(Table 3)**. FRAP is a measure of total antioxidant protection in plasma or serum and is generally correlated with uric acid concentrations and, to a lesser extent, with ascorbic acid concentrations (Benzie and Strain 1996). Our results are in agreement with this premise since both serum uric acid and vitamin C paralleled FRAP values, and a positive and significant correlation was observed between mean FRAP and mean uric acid levels ($r=0.964$, $p<0.01$), and between FRAP and vitamin C ($r=0.884$, $p<0.05$). Consequently, uric acid and vitamin C levels also showed strong correlation ($r=0.918$, $p<0.05$).

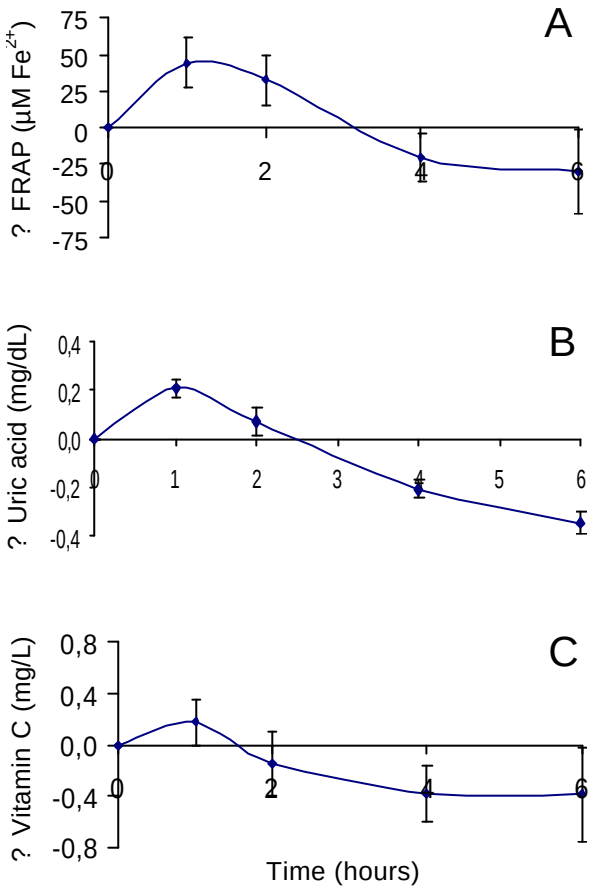

Figure 1. Relative changes in (A) total serum antioxidant capacity (FRAP), (B) serum uric acid levels, and (C) serum vitamin C content after the intake of the phenolic-rich juice. Data are expressed as mean±SEM. Increments were calculated for each subject by subtracting its baseline level from each post intake value.

The serum levels of lipid-bound polyphenols showed a mean value of around 230 mg GAE/L, with no significant differences during the time of the study **(Table 3; Figure 2 A)**. Nevertheless, serum lipid-bound polyphenols slightly increased 2 hours post intake and this effect persisted for the duration of the experiment. As shown in **Table 3** and **Figure 2B**, serum TBARS levels significantly decreased 2 hours after juice intake from 559 to 512 nM MDA, and this effect was still apparent 6 hours post intake. These results might be due to a reduction in the lipid oxidation associated with the intake of the phenolic-rich juice. Interestingly, the decrease in TBARS levels seem to be consistent with the weak variations observed in serum lipid-bound polyphenols. In fact, TBARS levels and lipid-bound phenolic compounds showed a negative but non-significant correlation ($r=-0.725$, $p=0.166$). This observation suggests that the amount of phenolics able to bind serum lipids might be sufficient to cause a significant reduction in lipid oxidation.

Contrary to expectation, consumption of the juice caused a significant increase in serum protein oxidation during the first 4 hours post intake, when it increased from 3.14 to 7.29 mg of carbonyl protein/mg protein **(Table 3; Figure 2 C)**. Following this and at the last sampling time of the study (6 hours after the intake) the serum carbonyl protein dropped back to baseline. This illustrates the prooxidant effect of juice consumption on protein oxidation, which might be attributable to the digestion process, as discussed later.

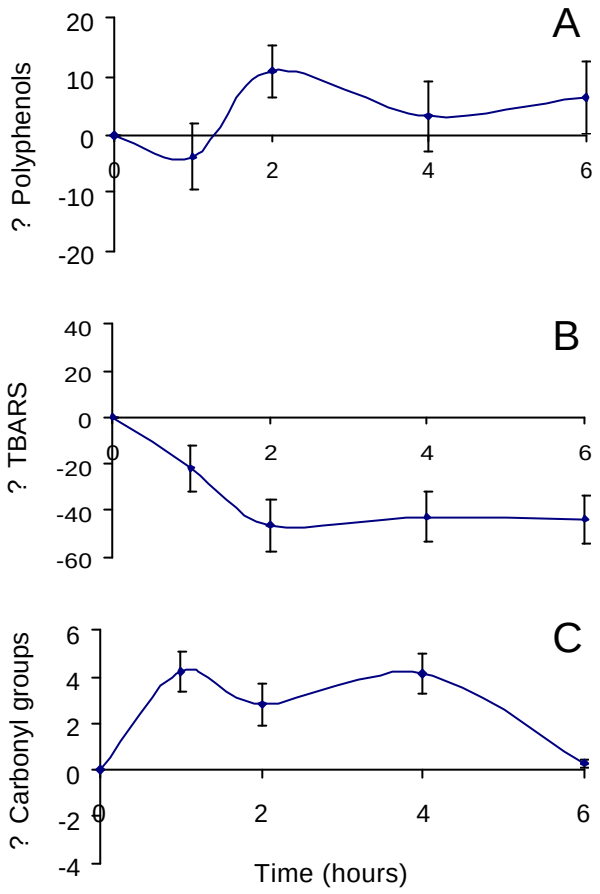

Figure 2 Relative changes in (A) total serum lipid-bound phenolic compounds (mg GAE/L), (B) serum TBARS levels (nM MDA), and (C) serum protein carbonyl content (mg/mg protein) after the intake of the phenolic-rich juice. Data are expressed as mean±SEM. Increments were calculated for each subject by subtracting its baseline level from each post intake value.

Figure 3 illustrates the relative changes in cumulative urinary excretion of phenolics and reductant substances (measured as FRAP) after the intake of the phenolic-rich juice. Urinary excretion of phenolics **(Figure 3 A)** increased and peaked at 2 hours after the intake ($p<0.01$), indicating that phenolics from the juice were readily absorbed and excreted in urine. Increased urinary excretion of phenolics persisted to a lesser extent from 4 to 6 hours after the intake of the juice ($p<0.01$), and finally fell back to baseline levels 24 hours post intake. The cumulative urinary excretion of reductant substances was also analyzed as an indicator of the bioavailability of antioxidants. As can be seen in **Figure 3 B**, changes in cumulative urinary FRAP closely reflected the changes in cumulative urinary excretion of polyphenols. Hence, statistical analysis revealed a strong correlation between urinary FRAP values and total polyphenols ($r=0.996$, $p<0.01$).

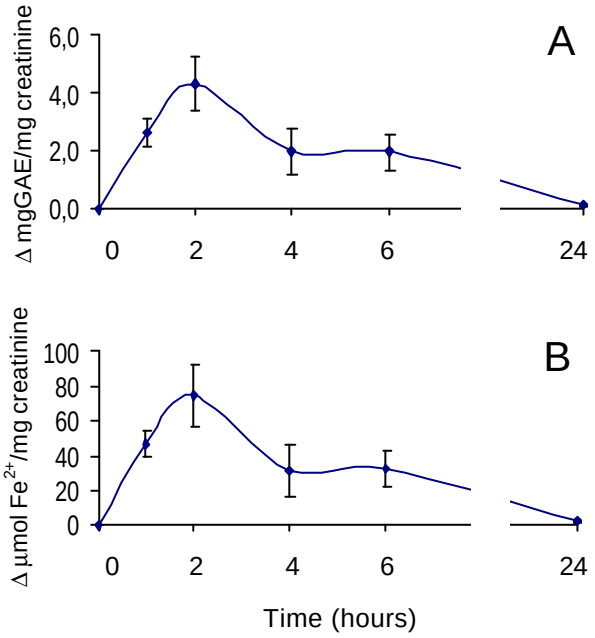

Figure 3. Relative changes in urinary parameters after the intake of the phenolic-rich juice. (A) Cumulative urinary excretion of phenolics. (B) Cumulative urinary excretion of reductant substances measured as FRAP values. Data are expressed as mean±SEM. Increments were calculated for each subject by subtracting its baseline level from each post intake value.

DISCUSSION

Antioxidant activity and absorption of phenolic compounds

Short-term studies involving the consumption of berry juices have reported acute increases in the antioxidant capacity of plasma or serum, which have usually been attributed to the high levels of polyphenolic antioxidants provided by berries (Pedersen et al., 2000; Kay and Holub, 2002; Netzel et al., 2002). However, Cao and Prior (2000) observed a significant postprandial increase in total serum antioxidant capacity in elderly healthy women after the consumption of low antioxidant meals. In their study, uric acid was the only individual antioxidant that they could identify as being responsible for part of the increased serum antioxidant capacity. In agreement with this observation, Lotito and Frei (2004) observed a transient increase in plasma FRAP one hour after the consumption of either apples or an aqueous solution of fructose, which was matched by increases in plasma urate and vitamin C. Furthermore, they assessed the contribution of ascorbate to FRAP by treating plasma with ascorbate oxidase prior to analyzing FRAP, and finding that vitamin C from apples did not make a significant contribution to the changes observed in FRAP. Consequently, urate was considered to be mainly responsible for the increase in plasma FRAP. Hence, these authors reported that many of the previously described effects of phenolic-rich foods on overall *in vivo* antioxidant capacity are not due to the phenolic compounds. Instead, they suggested that food intake *per se* had an effect on plasma urate, which may partly be explained by the metabolic effect of fructose on urate. Indeed, fructose has been found to increase uric acid production by accelerating the degradation of AMP to urate (Mayes 1993). Accordingly, the hyperuricemic effect of D-fructose and its stereoisomer D-tagatose was observed in subjects consuming an aqueous solution of D-fructose or D-tagatose (30 g/400 mL), with serum uric acid levels peaking at 50 minutes after consumption of both sugars (Buemann et al., 2000).

Consistent with these findings, we observed a slight and transient but non-significant rise in total antioxidant capacity, which peaked 1 hour after the intake of the juice. This increase was concomitant with an increase in serum uric acid and, to a lesser extent, vitamin C. This observation is in agreement with the notion that the major contributors to FRAP values are uric acid (60%) and vitamin C (15%), followed by protein (10%), bilirubin (5%) and a-tocopherol (5%). Other antioxidants, including plasma/serum phenols and polyphenolic compounds, have been thought to contribute about 5% of the FRAP values (Benzie and Strain 1996; Baines and Shenkin 2002). For this reason, the contribution of polyphenols to FRAP could be considered negligible. In our study, each subject consumed 36 g of fructose (36 g/400 mL) (the fructose content in the juice was

9%, **Table 2**), which is sufficient to cause a rise in serum uric acid levels (Buemann et al., 2000). In addition, other sugars from fruits, such as sucrose or sorbitol, may well contribute to urate rise after fruit consumption (Bode et al., 1973).

Taken together, the above data suggest that the response, albeit weak, observed in total serum antioxidant capacity after the intake of the juice is not due to polyphenols in the juice and could be better explained by a sugar-mediated rise in serum uric acid levels. As shown in **Table 1**, the major phenolic compounds in the juice were anthocyanins, which have been reported to be poorly absorbed from the diet (Murkovic et al., 2000; Milbury et al., 2002; Mülleder et al., 2002; Netzel et al., 2002). This fact, together the high variability between individuals, may explain why no significant differences were observed in the study.

However, our data suggested that phenolics were absorbed from the juice, as revealed by the slight increase in serum lipid-bound phenolic compounds and the rise in the urinary excretion of total phenolics. In our study, the maximum concentration of phenolics in serum and urine was reached 2 hours post intake. This agrees with data reported by other researchers after the intake of anthocyanin-rich foods, with mean plasma anthocyanin levels peaking at 1.5 hours, and a mean time to reach maximum urinary excretion of 2.5 hours (Manach et al., 2005). These findings suggest that phenolics from the juice were readily absorbed, entered the bloodstream and became bound to the lipid fraction of serum. Later, they were almost completely excreted in urine within 6 hours. However, it should be noted that some individuals could be better absorbers than others, possibly because of particular polymorphisms of intestinal enzymes or transporters.

Biomarkers of antioxidant status

When evaluating the effect of the present intervention study on markers of oxidative status, it should be borne in mind that the juice selected in this study contains not only anthocyanins but also several polyphenolic antioxidants such as catechins, hydroxycinnamic acids and stilbenoids **(Table 1)**. All these compounds have been recognised as antioxidants in the prevention of the oxidation of macromolecules such as lipids and proteins in the human body (Griffiths et al., 2002).

The oxidation of LDL is believed to be mainly responsible for the pathogenesis of atherosclerosis in humans (Steinbrecher 1987). The ability of dietary phenolic compounds

to prevent LDL oxidation has been reported by several researchers in human studies, suggesting that the antioxidant properties of dietary phenolics may explain the inverse association with cardiovascular disease observed in some epidemiological studies (Hollman and Katan 1997; Nigdikar et al., 1998; Aviram et al., 2000; Hyson et al., 2000; Serafini et al., 2000; O'Byrne et al., 2002). Phenolics that bind LDL are good candidates for preventing lipid peroxidation and atherosclerotic processes because they are able to prevent the autocatalytic chain reaction of fatty acid peroxidation in LDL directly and/or by preservation of other chain-breaking antioxidants, such as tocopherol (Zhu et al., 2000). Our finding that MDA levels decreased by around 8% after the intake of 400 mL of juice suggests an improvement in the antioxidant status and indicates that even low amounts of absorbed phenolic compounds from the juice might decrease lipid oxidation.

Other authors have described similar results after the intake of anthocyanin-rich juices (Young et al., 1999; Netzel et al., 2002). Nigdikar et al. (1998) observed an increase in the total phenol content of low-density lipoproteins (LDL), as measured by the Folin-Ciocalteau method, after red wine consumption. Lamuela-Raventós et al. (1999) reported the presence of individual dietary phenolic compounds bound to human LDL, even in subjects taking a non-supplemented diet. Based on this observation, we could reasonably hypothesize that diets rich in phenolic compounds might be used to increase the levels of polyphenols bound to lipoproteins, thereby achieving a prolonged protection of the plasma/serum lipid fraction against oxidation. In fact, our results showed that overall serum lipid peroxidation significantly decreased after the intake of the juice, an effect that lasted up to at least 6 hours post intake. During this time, both serum and urine phenolics remained above their respective baseline levels. Nevertheless, since we only evaluated serum parameters up to 6 hours after juice consumption, we cannot affirm for how long this serum lipid oxidation-lowering effect might last.

A strong prooxidant action of juice consumption on protein seemed to be directly related to its intake. As reviewed by Griffiths et al. (2002), human studies involving supplementation with phenolic-rich foods have reported contradictory results regarding protein oxidation. However, some authors have shown similar results to those obtained in this study. For example, Young et al. (1999) reported that the intake of apple and blackcurrant juice for one week, with three daily doses of juice (750, 1000 and 1500 mL) increased the protein oxidation biomarker 2-amino adipic semialdehyde (AAS) in a dose-dependent manner, suggesting a prooxidant effect. The same authors also observed that supplementation of 15 subjects with a grape seed extract rich in polyphenols for 1 week had no effect on plasma AAS (Young et al., 2000). Van den Berg et al. (2001) tested a

vegetable and a fruit concentrate in male smokers and found no change in plasma carbonyl content after a 3-week treatment. Dragsted et al. (2004) described an increase in AAS in a 25-day intervention study performed in 43 healthy non-smokers, and involving the daily intake of 600 g of fruits and vegetables, or a pill matching the mineral and vitamin content of the fruit and vegetable dose. In general, in all of these studies, a direct association between plasma ascorbate and the protein oxidation product, AAS, was observed. For this reason, it has been suggested that ascorbic acid and other plant-derived antioxidants might be involved in protein oxidation, most probably via Fenton-type reactions (Young et al., 1999; Dragsted et al., 2004).

Nevertheless, in our study the juice tested did not contain vitamin C in the form of ascorbic acid. Furthermore, serum vitamin C levels and serum protein carbonyl content showed a different trend, with no significant correlation between them. So, it could be reasonable to think that in the absence of L-ascorbic acid, the sugar content of the juice may have played an important role in the transient increase observed in serum protein oxidation. In fact, Breinholt et al. (2003) reported that sugars present in juices were involved in the increase observed in AAS levels in female rats, because the same effect was observed in rats fed different fruit juices, and in a control group after consuming sucrose, fructose, and glucose in the drinking water, at concentrations similar to those of the assayed fruit juices.

It is well known that carbohydrates play a significant role in oxidative processes *in vivo*. Diabetes is associated with an increased oxidative stress status, which is strongly related to hyperglycemia (Odetti et al., 1999). Hyperglycemia has been reported to cause increased production of oxygen free radicals through glucose autooxidation and non-enzymatic glycation (Maillard reaction) processes (Wolff 1993; Domínguez et al., 1998), and consequently may damage cellular and tissue components. The advanced glycation end-products (AGEs), which accumulate with age and at an accelerated rate in diabetes, cause irreversible chemical modifications of proteins (Dills 1993; Bierhaus et al., 1998). High levels of plasma carbonyl groups have been reported in diabetic patients (Dominguez et al., 1998; Odetti et al., 1999), and an increase in carbonyl groups has been shown to be related to increased haemoglobin glycation (Odetti et al., 1999).

In addition, an increase in the dietary intake of fructose might result in changes in the tissue concentrations of fructose and its metabolites (e.g. fructose-1-phosphate and D-glyceraldehyde), which could, in turn, potentiate the Maillard reaction and the accumulation of AGEs *in vivo* (Dills 1993). Indeed, fructose has been shown to induce

protein oxidation and to accelerate the formation of AGEs *in vitro*, to a greater extent than glucose (Tagaki et al., 1995; Liggins and Furth 1997; Sakai et al., 2002). Moreover, interrelations between sugar-mediated protein oxidation and lipid peroxidation have also been reported. In this context, an *in vitro* study carried out by Burcham and Kuhan (1996) reported time- and concentration-dependent increases in carbonyl groups formation in bovine serum albumin (BSA) incubated with 1-10 mM MDA for 2 hours at different pH values. Carbonyl formation, as measured by the DNPH method, increased in a pH- and concentration dependent manner, while the lowest carbonyl formation was observed at a physiologically relevant pH. The results obtained by Burcham and Kuhan (1996) raise the possibility that MDA adducts may contribute to the carbonyl content of proteins and provide further evidence of the possible link between lipid peroxidation and protein oxidation. Nevertheless, in our opinion, our results do not agree with the possibility of an MDA-mediated rise in carbonyl groups for two reasons. First, in our study, the time courses of carbonyl formation and MDA decrease were different. Furthermore, serum carbonyls dropped back to baseline levels 6 hours after the intake of the juice, whereas the MDA lowering effect was still apparent 6 hours post intake. Second, in Burcham and Kuhan's study (1996), even the lowest MDA concentration tested (1 mM) was far higher than the concentrations in our study, which were in the nanomolar range.

For this reason, in order to explain our results, we suggest that the acute and transient rise in protein carbonyl groups observed after the intake of the juice was probably induced by the metabolization of sugars from the juice, via a glycosylation or glycoxidation mechanism. Unlike our study, the above mentioned intervention trials involved long supplementation periods because both AGEs formation and protein oxidation were expected to proceed very slowly, and thus did not provide information of postprandial variations in protein oxidation biomarkers after the intake of foods rich in phenolic compounds. In our study, the oxidized proteins seemed to be removed from the bloodstream after 6 hours, supporting rigid control of the injury. However, the carbonyl assay measures generic oxidation products and thus might does not differentiate between those arising directly from protein oxidation and those formed by adduction of another oxidised product, such as glucose or malondialdehyde (Griffiths et al., 2002).

Conclusions

Based on our results and on previous observations, it is reasonable to suggest that the increase, albeit weak, in total antioxidant capacity observed after the ingestion of the juice might be caused by an increase in serum uric acid due to the rapid metabolism of fructose and/or other sugars in the juice, and not by serum polyphenols levels. Nevertheless, the phenolic compounds in the juice appeared to be absorbed in sufficient amounts to decrease lipid peroxidation, which is thought to play a pivotal role in cardiovascular disease. These findings raise the possibility that phenolics in the juice may protect the lipid fraction of serum against oxidation and may therefore be natural anti-atherosclerotic components of the diet. However, this will largely depend on their pharmacokinetics, as well as on their ability to bind serum lipids and lipoproteins. Our observations also suggest that natural carbohydrates from fruits are capable of mediating oxidative stress *in vivo,* by increasing carbonyl proteins after intake. Future studies should take into account the role that carbohydrates from fruits and derived products play in human nutrition and in the modulation of antioxidant defences.

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4

Discusión General

General Discussion

4. DISCUSIÓN GENERAL

Estudios epidemiológicos han mostrado que dietas ricas en alimentos vegetales reducen de forma significativa la incidencia y tasas de mortalidad de enfermedades degenerativas causadas por el estrés oxidativo (Tibble 1998). Este efecto protector ha sido atribuido principalmente a los compuestos fenólicos y a otros tipos de antioxidantes naturales presentes en dichos alimentos (Ames et al., 1993). Las frutas pigmentadas tales como las uvas, cerezas y bayas (fresas, frambuesas, zarzamoras, etc.) constituyen una de las fuentes más importantes de compuestos fenólicos de la dieta, aportando fundamentalmente derivados de los ácidos hidroxibenzoicos e hidroxicinámicos, antocianos, flavonoles, catequinas y taninos, que además permanecen en los productos elaborados a partir de estas frutas (García-Viguera et al., 1997; Heinonen et al., 1998; Cantos et al., 2000).

En base a los datos disponibles en la literatura científica sobre el contenido en compuestos fenólicos de estas frutas, se elaboró un postre gelificado empleando en la formulación zumos concentrados de uva, cereza, frambuesa, zarzamora y grosella. De acuerdo a las expectativas del diseño, el contenido y perfil de compuestos fenólicos del postre de frutas estuvo determinado por el la naturaleza y contenido de los compuestos fenólicos presentes en los zumos concentrados empleados como ingredientes. Así, los compuestos fenólicos mayoritarios en el postre recién elaborado fueron los antocianos, seguidos de los ácidos hidroxicinámicos, flavonoles, estilbenos y ácido elágico. Durante el almacenamiento, el contenido en compuestos fenólicos se vio afectado significativamente por el tiempo y temperatura de almacenamiento, exceptuando el caso de los estilbenos totales. La disminución más acusada que se observó fue la del contenido en antocianos totales, mientras que esta disminución fue menos acusada en el caso de los ácidos hidroxicinámicos y los flavonoles totales. Por el contrario, el contenido en estilbenos totales se vio incrementado durante el almacenamiento. Este comportamiento, que ha sido descrito por Zafrilla el al. (2001) en mermeladas de frambuesa, podría deberse a la liberación de ácido hexahidroxidifénico de los elagitaninos durante el procesado, el cual se transforma posteriormente en ácido elágico.

La actividad antioxidante total del postre de frutas a penas se vio afectada por las condiciones de almacenamiento. Así, sus valores se mantuvieron prácticamente constantes a la temperatura de 8ºC y mostraron sólo un leve descenso en las muestras almacenadas a 21 y 30ºC. Aunque el contenido de la mayor parte de los grupos de compuestos fenólicos del postre disminuyó durante el almacenamiento, la actividad

antioxidante total se mantuvo prácticamente invariable. Este hecho sugiere que otros compuestos fenólicos, como por ejemplo productos de degradación de los antocianos, podrían contribuir al mantenimiento de la actividad antioxidante durante el almacenamiento. Este proceso podría asemejarse al que ocurre durante el envejecimiento de los vinos, en el cual se ha sugerido que complejos polifenólicos y productos de condensación que se forman durante el proceso de envejecimiento de los vinos contribuyen de forma significativa a la actividad antioxidante (Burns et al., 2001). Un proceso similar podría producirse durante el almacenamiento del postre ya que el concentrado mayoritario es el de uva, el cual muestra a su vez un contenido elevado de antocianos.

Nuestros resultados muestran con claridad que el uso de concentrados de uvas, cerezas y bayas como materia prima es una estrategia adecuada para la producción de alimentos ricos en antioxidantes naturales con potenciales efectos beneficiosos para la salud del consumidor. Además, el estos productos experimentales mantienen sus propiedades antioxidantes durante el almacenamiento. No obstante, a fin de evaluar sus efectos beneficiosos, en la presente investigación se han llevado a cabo estudios *in vitro* en cultivos celulares y estudios de intervención en humanos.

Los ensayos *in vitro* en cultivos celulares son una herramienta útil en el estudio de procesos fisiológicos, bioquímicos y toxicológicos modulados por compuestos bioactivos puros de los alimentos o por el alimento en conjunto (Glei et al., 2003). Numerosos estudios desarrollados con diferentes líneas celulares en cultivo han mostrado la capacidad de los compuestos fenólicos para inhibir la proliferación de células tumorales, así como para proteger las células en cultivo frente al daño oxidativo inducido por diversos agentes (Sestili et al. 1998; Aherne y O'Brien 1999; 2000; Johnson y Loo 2000; Feng et al. 2002; Sestili et al. 2002; Jimenez-López y Cederbaum, 2004). En particular, la línea de células tumorales hepáticas humanas HepG2 ha sido ampliamente empleada en estudios bioquímicos y nutricionales ya que está considerada como unos de los modelos experimentales que mejor se asemeja al hepatocito humano en cultivo (Alía et al., 2005).

Para los ensayos en cultivos celulares, se empleó el producto experimental en el formato de zumo con la finalidad de facilitar la preparación de las muestras. El perfil de compuestos fenólicos y la actividad antioxidante del zumo fueron similares a las del postre de frutas. El lo relativo a la actividad antiproliferativa del zumo rico en compuestos fenólicos, en nuestro estudio observamos una inhibición de la proliferación de las células HepG2 en función de la dosis, tras la incubación de las células con extractos del zumo

conteniendo diferentes concentraciones de compuestos fenólicos. Esto indica que los compuestos fenólicos del zumo fueron capaces de iniciar una serie de procesos celulares que conducen a la inhibición de la proliferación y/o a la inducción de la muerte celular. Nuestro resultados están de acuerdo con los obtenidos por otros investigadores empleando extractos de frutas y verduras de consumo habitual, particularmente en el caso de extractos de frutas pigmentadas como uvas, frambuesas, arándanos o fresas (Chu et al., 2002; Liu et al., 2002; Sun et al., 2002; Meyers et al., 2003).

Además de la actividad antiproliferativa, numerosos estudios han puesto de manifiesto la capacidad de los compuestos fenólicos para proteger las células cultivadas frente a diversos agentes oxidantes. No obstante, cuando se evalúa el efecto protector de extractos de alimentos o compuestos puros, no existe un criterio consensuado en lo referente a los diseños experimentales. De este modo, aunque actualmente existe una gran cantidad de información publicada al respecto, la comparación de resultados se hace a menudo complicada debido a la gran variedad de diseños experimentales, la gran diversidad líneas celulares empleadas (e.j. células de diferentes órganos y tejidos, diferentes especies, etc.) o al empleo de diferentes agentes oxidantes para inducir daños celulares.

En el presente estudio, las células HepG2 fueron preincubadas con extractos del zumo de frutas conteniendo diferentes concentraciones no tóxicas de compuestos fenólicos, y posteriormente expuestas a la acción del *tert*-butil hidroperóxido (tB-OOH) o del peróxido de hidrógeno (H_2O_2). Se ha comprobado que el H_2O_2 puede causar daños en lípidos, ADN y otras macromoléculas, provocando por tanto daño oxidativos a la célula. De un modo similar, el tB-OOH induce una serie de alteraciones celulares que incluyen la peroxidación de lípidos de membrana, la depleción de glutatión y proteinas tiol, alteración de la homeostasis del calcio y daño al ADN, que conduce finalmente a la muerte celular (Alía et al., 2005). De acuerdo a los datos obtenidos por otros investigadores (Nardini et al., 1998; Feng et al., 2002; Yau et al., 2002; Lazzè et al., 2003; Hong y Liu 2004; Sohn et al., 2005), las células preincubadas con extractos del zumo rico en compuestos fenólicos mostraron una mayor resistencia al estrés oxidativo inducido tanto por tB-OOH como por H_2O_2. Este hecho se pone de manifiesto como una mayor tasa de viabilidad celular y como una menor formación de productos de la peroxidación lipídica (malondialdehido), tras la exposición a ambos oxidantes. Además, este efecto protector se observó tras un periodo breve de incubación con los extractos del zumo, lo cual sugiere que los compuestos fenólicos del zumo fueron rápidamente absorbidos por las células y previnieron eficazmente frente a la muerte celular y la peroxidación lipídica.

Los cambios en la actividad de los sistemas enzimáticos celulares se consideran un biomarcador sensible de la respuesta celular frente al estrés oxidativo. En este estudio, empleamos el tB-OOH como agente oxidante ya que se ha comprobado su eficacia para incrementar la actividad de los sistemas enzimáticos de la línea celular HepG2 (Alía et al., 2005). Así, se han descrito aumentos en la actividad de los enzimas glutatión peroxidasa (GPx) y glutatión-*s*-trasferasa (GST) en células cultivadas tras la exposición a tB-OOH, asociados además con una disminución de los niveles de glutatión reducido (GSH) intracelular (Aniya y Daido 1994; Alía et al., 2005). Así, en nuestro estudio, la exposición de las células al tB-OOH produjo un aumento de la actividad de los enzimas GPx y GST. La preincubación de las células con el extracto del zumo de frutas a la concentración de 150 μM suprimió eficazmente el incremento de la actividad del enzima GPx inducido por tB-OOH, pero no así en el caso del enzima GST. Nuestro resultados están de acuerdo con los obtenidos por Murakami et al. (2002), quienes describieron la eficacia de las catequinas del té para disminuir la actividad del enzima GPx inducida por tB-OOH, en la línea celular HepG2. Por tanto, en lo referente a la actividad del enzima GPx, los compuestos fenólicos del zumo ejercerían su efecto protector retrasando el consumo de GSH y/o de otros antioxidantes celulares, durante el proceso de detoxificación del tB-OOH. De hecho, otros autores han observado que compuestos fenólicos y extractos vegetales con alta actividad antioxidante son capaces de prevenir de la depleción de GSH causada por tB-OOH en hepatocitos cultivados (Yau et al., 2002; Sohn et al., 2005).

Para caracterizar mejor el efecto protector del zumo rico en compuestos fenólicos, llevamos a cabo un estudio empleando la línea celular de leucemia mieloide humana U937. En dicho estudio evaluamos la capacidad del zumo para proteger las células U937 frente al estrés oxidativo causado por el tB-OOH. De modo similar al los resultados obtenidos con la línea celular HepG2, un periodo breve de incubación con extractos del zumo incrementó la resistencia de las células frente al estrés oxidativo. Esto se puso de manifiesto mediante disminuciones en muerte celular, daño al ADN, generación de especies reactivas del oxígeno (ROS) a nivel mitocondrial, así como por una menor incidencia de la apertura de poros de transición de permeabilidad en la membrana mitocondrial (MPT). En concordancia con nuestros resultados, otros estudios realizados en células U937 han mostrado la capacidad de diversos flavonoides y ácidos hidroxicinámicos (derivados del ácido cafeico) para proteger frente a la muerte celular y el daño al ADN causados por tB-OOH (Sestili et al., 1998; 2002). Además, la capacidad protectora de los antocianos frente al la muerte celular y el daño al ADN inducidos por tB-

OO ha sido descrita en la línea de células de músculo liso de rata SMC y en la línea tumoral hepática de rata MH1C1 (Lazzè et al., 2003). Otros estudios desarrollados el la línea celular humana de tumor de colon Caco-2 han mostrado la capacidad de flavonoides tales como la quercetina, miricetina y rutina, para prevenir el daño al ADN causado por agentes como el tB-OOH, el H_2O_2 o la menadiona (Aherne y O'Brien 1999; 2000). Además, los compuestos fenólicos del té han mostrado también su eficacia en la protección frente a la muerte celular, daño al ADN y generación mitocondrial de ROS provocados por la exposición al tB-OOH, en la línea de células de epitelio hepático normal de rata RL-34 (Feng et al., 2002).

La apertura de poros de transición de permeabilidad en la membrana mitocondrial (MPT) es un proceso de permeabilización de la membrana interna de la mitocondria, que precede a la muerte celular tanto necrótica como apoptótica. La incidencia de MPT ha sido relacionada con un cambio en el estado redox de la mitocondria y/o con un incremento en la generación mitocondrial de ROS (Castilho et al., 1995; Vercesi et al., 1997). Además, se piensa que el proceso de apertura de poros inducido por tB-OOH depende de la generación intramitocondrial de ROS, que como consecuencia inicia la apertura de poros y conduce finalmente a la muerte celular (Kim et al., 2003). Por lo tanto, la muerte celular en condiciones en las que está implicado el proceso de MPT pueden ser prevenidas por antioxidantes, ya que estas sustancias son capaces de prevenir procesos previos a la apertura de los poros, en particular en la fase de generación de ROS (Kowaltowsky et al., 2000; Kim et al., 2003). De acuerdo con esta afirmación, la prevención de la generación de ROS ejercida por el zumo favorecería la capacidad del mismo para prevenir el inicio del proceso de MPT, y por tanto su capacidad para prevenir la muerte celular. Además, tal y como sugieren Feng et al. (2002) en el caso de las flavinas del té, la capacidad de los compuestos fenólicos para prevenir la generación intracelular de ROS favorecería su capacidad para proteger el ADN del daño oxidativo.

Los datos obtenidos indican claramente que los compuestos fenólicos del zumo fueron capaces de proteger las células cultivadas frente al estrés oxidativo inducido. Sin embargo, la cuestión que nos planteamos es cómo los compuestos fenólicos del zumo ejercen su efecto protector. Desde un punto de vista químico, los compuestos fenólicos tienen una estructura que les permite tanto captar radicales libres como quelar iones metálicos. Esta última actividad es de interés ya que previene la formación de radicales libres en reacciones de Fenton catalizadas por metales de transición, principalmente hierro y cobre (Rice-Evans et al., 1997). En relación con esta actividad, la presencia de 2

grupos hidroxilo en posición *orto* ha sido descrita como una característica molecular crucial para la actividad quelante de hierro y la eficacia protectora de los compuestos fenólicos (Sestili et al., 2002). Por este motivo, ya que tanto los flavonoides (e.j. quercetina, catequinas y antocianos) como el ácido cafeico comparten esta característica estructural, es razonable pensar que la protección ejercida por estos compuestos podría deberse en parte a una actividad quelante de hierro. Además, como el zumo de frutas muestra un contenido importante de antocianos, derivados del ácido cafeico y catequinas, compuestos que presentan esta característica estructural, no debe descartarse la posibilidad de la implicación de la actividad quelante de hierro en la protección ejercida por el zumo. Por esta razón, con la finalidad de aclarar si la protección ejercida por el zumo rico en compuestos fenólicos es dependiente de la actividad captadora de radicales libres o de la actividad quelante de hierro, en los experimentos con células HepG2 y U937 se ensayaron además el captador de radicales libres *N,N'*-difenil-1,4-fenilendiamina (DPPD) y el quelante de hierro intracelular *o*-fenantrolina (*o*-phe).

En el estudio realizado en células HepG2, observamos que la peroxidación lipídica inducida por tB-OOH o por H_2O_2 es prevenida tanto por el DPPD como por la *o*-phe. Esto indica que, en la prevención de la peroxidación lipídica causada por ambos peróxidos, están implicadas tanto la actividad captadora de radicales libres como la actividad quelante de hierro. Sin embargo, estos datos no nos permiten afirmar cual de estos dos mecanismos es el predominante en la prevención de la peroxidación lipídica, ni si este efecto protector depende de una combinación de ambas actividades. No obstante, nuestros datos relativos a las actividades enzimáticas sugieren que los compuestos fenólicos del zumo podrían haberse comportado cómo quelantes de hierro, más que como captadores de radicales libres. Como se ha mencionado anteriormente, tras la exposición al tB-OOH, tanto el zumo como la *o*-phe disminuyeron la actividad del enzima GPx, pero no la del enzima GST. Esta última sólo disminuyó en células tratadas previamente con el DPPD. Por tanto, en base a estas observaciones, parece razonable pensar que las propiedades antoxidantes de los compuestos fenólicos del zumo dependieron más de su actividad quelante de hierro, que de su capacidad captadora de radicales libres. A este respecto, nuestros resultados obtenidos en células U937 parecen apoyar la hipótesis de la actividad quelante de hierro, ya que el zumo, el DPPD y la *o*-phe previnieron la muerte celular causada por tB-OOH, y sólo el zumo y la *o*-phe fueron capaces de prevenir el daño al ADN inducido por el tB-OOH. Esta observación está de acuerdo con la idea de que el daño al ADN inducido por el tB-OOH es prevenido por quelantes de hierro, y no por captadores de radicales, mientras que la muerte celular causada por el tB-OOH es prevenida tanto por quelantes de hierro, como por captadores

de radicales libres (Sestili et al., 1998; 2002). Además, en nuestro estudio con células U937, la generación intramitocondrial de ROS fue prevenida por el zumo y la *o*-phe, pero no por el DPPD.

En conjunto, estos datos sugieren que la actividad predominante en la protección ejercida por el zumo rico en compuestos fenólicos sería la quelación de hierro, la cual es potencialmente relevante desde un punto de vista biológico. Aunque generalmente la actividad quelante de hierro ha sido relegada a un segundo plano en lo referente a la actividad antioxidante de los compuestos fenólicos, la presencia en los alimentos de compuestos con actividad quelante y su eficacia en el secuestro de hierro podría explicar en parte la eficacia protectora y los efectos beneficiosos para la salud atribuidos a los compuestos fenólicos (Duthie et al., 2000; Prior y Cao 2000).

En general, se considera que los ensayos *in vitro* mediante pruebas químicas y el empleo de cultivos celulares proporcionan información valiosa acerca de la eficacia y los mecanismos de acción de determinados antioxidantes o de alimentos ricos en dichas sustancias. Sin embargo, la extrapolación de los efectos observados a la situación *in vivo* debe hacerse con precaución, ya que en estos ensayos no se consideran la absorción en el tracto gastrointestinal ni el metabolismo. Por esta razón, cuando se evalúa la eficacia de un alimento rico en sustancias antioxidantes, la estrategia más adecuada es la realización de estudios de intervención en humanos. Aunque en las últimas décadas se ha incrementado el interés por el estudio del impacto de la dieta en el envejecimiento y las enfermedades degenerativas asociadas con el mismo (Ames et al., 1993; Meydani 2001), la relación entre el estrés oxidativo y el envejecimiento fue ya sugerida por Harman en los años 50. Este autor sugirió, en su teoría del envejecimiento por radicales libres, que la disminución de las funciones vitales y los trastornos asociados al envejecimiento estás causados por un daño oxidativo que se acumula durante toda la vida (Harman 1956). Por tanto, un estilo de vida saludable, que implique la práctica de ejercicio físico moderado y una dieta equilibrada, podría prevenir las enfermedades degenerativas asociadas con el envejecimiento y retrasar incluso el propio proceso de envejecimiento (Tucker y Buranapin 2001). Por este motivo, se diseñó un postre de frutas rico en compuestos fenólicos con el objetivo de ser empleado como alimento complementario en la edad adulta. Como se ha mencionado anteriormente, el postre proporcionaría un alto contenido de antioxidantes naturales en la dieta. Como parte del estudio del efecto de la ingesta de este postre por un grupo de ancianos, se realizó una evaluación nutricional del grupo, en la cual se determinó la adecuación de la dieta de los individuos y se evaluaron parámetros bioquímicos y antropométricos de los ancianos.

La evaluación nutricional del grupo de ancianos reveló ingestas excesivas de proteínas y lípidos, y deficiencias en la de hidratos de carbono, fibra, cinc, yodo, así como en las ingestas de las vitaminas A, E y D. Resultados similares han sido obtenidos en estudios llevados a cabo en otros grupos de población anciana en España (Ortega et al., 1996; Gámez et al., 1997; 1998), los cuales pueden atribuirse a las pautas dietéticas de la población española (MAPA 2003). La actividad antioxidante total sérica del grupo resultó baja cuando en comparación con los valores normales de referencia (Miller et al., 1993: Benzie y Strain 1996), particularmente en el caso de las mujeres. Los niveles plasmáticos de retinol también estuvieron por debajo de los valores de referencia (Legrusse y Watier 1993). En este estudio se evaluó además el riesgo oxidativo de los individuos, en base a los intervalos de nivel establecidos por Legrusse y Watier (1993) para los niveles plasmáticos de las vitaminas C, A y E. Así, en el 80% de los individuos se observó un riesgo moderado de estrés oxidativo en relación con los niveles plasmáticos de retinol, y se observó un menor riesgo en el caso del tocoferol y el ácido ascórbico. En cuanto a la relación entre la ingesta de vitaminas y sus niveles en plasma/suero, se observó una correlación positiva y significativa entre la ingesta de vitamina C y sus niveles en suero, mientras que no se observó correlación positiva entre la ingesta de tocoferol y retinol y sus niveles en plasma. Resultados similares han sido descritos por otros autores (Jacques et al., 1995; Picado et al., 2001). De acuerdo con Benzie y Strain (1996) y Prior y Cao (1998), la capacidad antioxidante total sérica estuvo correlacionada con los niveles séricos de ácido úrico y en menor medida con los de albúmina, ácido ascórbico, hierro y bilirrubina, pero no con los niveles del resto de antioxidantes de la dieta.

Con el objetivo de evaluar el efecto de la ingesta del postre de frutas se realizó un estudio de intervención de dos semanas en este grupo de ancianos institucionalizados, en el cual se evaluaron cambios en la capacidad antioxidante total sérica, en los niveles de antioxidantes en plasma/suero y en diferentes biomarcadores del daño oxidativo, antes y después de la intervención. Tras la intervención no se observaron cambios significativos en la capacidad antioxidante total, parámetros bioquímicos, niveles de antioxidantes (retinol, a-tocoferol, β-caroteno y coenzima Q_{10}), analizados en el plasma/suero de los ancianos. Además, la capacidad antioxidante total de los ancianos fue similar a la del grupo de individuos sanos que sirvió como grupo de referencia. No obstante, los niveles de retinol, a-tocoferol, β-caroteno y coenzima Q_{10} de los ancianos estuvieron por debajo de los valores normales y fueron significativamente inferiores a los del grupo de referencia. La intervención tampoco produjo cambios en los biomarcadores

de la oxidación de las lipoproteínas plasmáticas de baja densidad (LDL) ni en los del daño al ADN de linfocitos. De modo similar, Young et al. (2002) tampoco observaron cambios en biomarcadores del estrés oxidativo en individuos que consumieron diariamente 18.6 mg de catequina, durante 6 semanas.

El hecho de que no se haya observado ningún efecto tras la ingesta del postre de frutas podría atribuirse a la corta duración del período de intervención y/o al deficiente estado antioxidante total de los ancianos. De hecho, nuestro resultados ponen de manifiesto que el grupo de ancianos muestra un daño oxidativo elevado en comparación con el grupo de referencia, ya que presenta una mayor susceptibilidad a la oxidación de las LDL y un mayor grado de daño en el ADN de linfocitos. No obstante, ya que en general se estima que compuestos fenólicos de la dieta (en particular los antocianos) se absorben de manera poco eficaz (Manach et al., 2005), la falta de efectividad de la intervención podría atribuirse en parte a la baja biodisponibilidad de los compuestos fenólicos.

Por este motivo, para evaluar si un producto rico en compuestos fenólicos puede en realidad producir cambios en biomarcadores del estado antioxidante, llevamos a cabo un estudio de intervención a corto plazo en un grupo de 12 individuos sanos. Para este estudio empleamos el zumo de frutas rico en compuestos fenólicos ya que el diseño experimental implicó la ingesta de una dosis única de 400 mL. Previamente a la ingesta del zumo, los participantes siguieron una dieta pobre en antioxidantes durante 2 días y el día del estudio consumieron la dosis de zumo en ayunas. Se tomaron muestras de sangre antes de la ingesta y en las 1, 2, 4 y 6 horas posteriores a la misma. Se evaluaron en suero la capacidad antioxidante total, el ácido úrico, la vitamina C, los compuestos fenólicos unidos a lípidos, los productos de peroxidación lipídica (malondialdehido) y el contenido en proteínas carbonilo. Además, la capacidad antioxidante total y los fenoles totales se analizaron en orina a los mismos tiempos y a las 24 horas tras la ingesta.

En concordancia con los resultados obtenidos en el grupo de ancianos, la ingesta del zumo rico en compuestos fenólicos no produjo cambios significativos en la capacidad antioxidante total sérica del grupo de individuos sanos. Esta falta de significación estadística puede atribuirse a las grandes variaciones observadas entre los individuos participantes para dicho parámetro. No obstante, cuando se examinan las variaciones observadas en la capacidad antioxidante sérica de cada individuo si se observan cambios significativos a lo largo del estudio. Así, se observó para cada individuo un aumento

significativo en la capacidad antioxidante durante las primeras 2 horas que siguen a la ingesta, acompañado por un aumento similar en los niveles de ácido úrico. Esta variación de la capacidad antioxidante en paralelo con el ácido úrico podría deberse en parte al efecto metabólico de la fructosa sobre el ácido úrico (Lotito y Frei 2004). De este modo, estos autores sugieren que los efectos que previamente se habían descrito tras la ingesta de alimentos ricos en compuesto fenólicos no son debitos a los compuestos fenólicos sino al efecto hiperuricémico de la fructosa. Sin embargo, nuestros datos sugieren que los compuestos fenólicos del zumo fueron absorbidos, tal y como indica el leve aumento en los niveles séricos de compuestos fenólicos unidos a lípidos y el aumento en la excreción urinaria de fenoles totales. Esta observación concuerda con las de otros autores quienes han descrito que, tras la ingesta de alimentos ricos en antocianos, la concentración máxima de antocianos en plasma se alcanza por término medio 1.5 horas después de la ingesta, mientras que la concentración máxima en orina se observa a las 2.5 horas (Manach et al., 2005).

Además, nuestros datos indican que los compuestos fenólicos del zumo fueron absorbidos, se unieron a la fracción lipídica del suero y redujeron los niveles de peroxidación lipídica a corto plazo. Otros autores han descrito resultados similares tras la ingesta de zumos ricos en antocianos (Young et al., 1999; Netzel et al., 2002), lo cual sugiere que la capacidad de los compuestos fenólicos para unirse a la fracción lipídica del plasma/suero, fundamentalmente a las lipoproteínas, es crucial para la prevención de la peroxidación lipídica (Nigdikar et al., 1998; Lamuela-Raventós et al., 1999).

Por el contrario, la ingesta del zumo rico en compuestos fenólicos mostró un efecto prooxidante transitorio con respecto a la oxidación proteica. Tal y como se recoge en el artículo de revisión de Griffiths et al. (2002), los estudios de intervención en humanos que implican la suplementación con alimentos ricos en antioxidantes han mostrado efectos contradictorios en lo relativo a la oxidación proteica. Así, los aumentos en biomarcadores de la oxidación proteica derivados del consumo de alimentos vegetales han sido atribuidos generalmente a la acción del ácido ascórbico y/u otros compuestos antioxidantes, los cuales podrían mostrar efectos prooxidantes, probablemente a través de reacciones de Fenton (Young et al., 1999; Dragstedt et al., 2004). No obstante, la posible implicación de los azucares del zumo en el aumento de la oxidación proteica no debe descartarse. A este respecto, un estudio en ratas mostró que los azúcares presentes en varios zumos de frutas estuvieron implicados en el incremento de la oxidación proteica observada en plasma (Breinholt et al., 2003). Además, se sabe que la hiperglucemia es capaz de inducir un aumento en la generación de radicales libres, a

través de la autooxidación de la glucosa y procesos no enzimáticos de glicosilación (Reacciones de Maillard), y en consecuencia producir daños en componentes de las células y tejidos (Wolf 1993; Domínguez et al., 1998; Odette et al., 1999).

En general, los resultados obtenidos en los estudios de intervención en humanos indican que la ingesta de productos ricos en compuestos fenólicos no modifica la capacidad antioxidante total ni los niveles de antioxidantes del plasma/suero. No obstante, los compuestos fenólicos presentes en estos alimentos podrían proporcionar efectos beneficiosos específicos relativos a la disminución de la peroxidación lipídica en individuos sanos. Por el contrario, en el caso de individuos ancianos, el alto grado de daño oxidativo asociado con el proceso de envejecimiento podría dificultar la mejora del estado antioxidante total a través del consumo de alimentos ricos en antioxidantes. Sin embargo, desde nuestro punto de vista, el consumo de este tipo de alimentos en la edad adulta puede ser interesante, particularmente en el caso de los ancianos, ya que estos productos proporcionan cantidades elevadas de antioxidantes naturales. A este respecto, se sabe que durante el envejecimiento se producen cambios fisiológicos que pueden conducir a una disminución en la ingesta de antioxidantes en la dieta debido a un menor consumo de frutas y verduras. Esto se debe principalmente a problemas dentales que dificultan la masticación y a la disminución general del apetito. Por tanto, estos alimentos elaborados a base de frutas pigmentadas podrían ser adecuados para este grupo de la población, ya que se trata de alimentos atrayentes que proporcionan unas cantidades elevadas de compuestos fenólicos antioxidantes, y que además presentan una textura adecuada para personas con problemas de masticación. Además, estos productos podrían considerarse adecuados para la población en general ya que, debido al estilo de vida actual, muchas personas no consumen las cantidades recomendadas de frutas y verduras. Por tanto, estos productos ricos en compuestos fenólicos podrían constituir un complemento adecuado en la dieta y proporcionar efectos saludables.

4. GENERAL DISCUSSION

Epidemiological studies have shown that diets rich in plant foods significantly reduce the incidence and mortality rates of degenerative diseases caused by oxidative stress (Tibble 1998). This protective effect has been attributed mainly to the phenolic compounds and other natural antioxidants (Ames et al., 1993). Berries (such as strawberry, blueberry and raspberry), grapes and cherries are among the most important sources of phenolic compounds in our diets, especially hydroxybenzoic and hydroxycinnamic acid derivatives, anthocyanins, flavonols, catechins and tannins, which continue to be present in their processed products (García-Viguera et al., 1997; Heinonen et al., 1998; Cantos et al., 2000).

Based on this knowledge, as well as on the data available in the scientific literature regarding the antioxidant properties of fruits, a dessert was prepared using concentrated juices of grape, cherry, raspberry, blackberry and blackcurrant. As expected from the juices used as ingredients, the phenolic composition of the dessert was determined by the phenolic content of the fruit concentrates. The main phenolic compounds detected immediately after manufacture were anthocyanins followed by hydroxycinnamic acids, flavonols, stilbenoids and ellagic acid. During storage, the phenolic composition significantly depended on the temperature and time of storage, with the exception of total stilbenoids. The main losses were detected in total anthocyanins as have been described by other authors in raspberry and strawberry jams (García-Viguera et al., 1998). Minor losses were observed in total hydroxycinamic acids and total flavonols. In contrast, the content of total ellagic acid increased during storage as have been reported by Zafrilla et al. (2001) in raspberry jams. This effect could be explained by a release of hexahydroxydiphenic acid from ellagitannins during processing, which is transformed to ellagic acid.

The total antioxidant activity of the dessert was only slightly modified by storage conditions, values remaining more or less constant when samples were stored at 8ºC, and decreasing slightly in samples stored at 21 and 30ºC. Although individual phenolic compounds decreased, the total antioxidant activity remained more or less constant. These findings suggest that other phenolics, such as the products of anthocyanins degradation, contribute to maintaining the total antioxidant activity of the dessert. This process could be similar to that which occurs in red wines during aging, in which it has been suggested, the larger polyphenolic complexes and condensation products that

appear during aging make a sizable contribution to overall antioxidant activity (Burns et al., 2001). Such an effect is not surprising in the dessert because of the high anthocyanin content of grape concentrate and because of the high proportion of this fruit in the final formulation.

Our results clearly indicate that the use of selected concentrated juices of grapes and berries as raw material is a feasible strategy to produce foods rich in natural antioxidants with potential health benefits for consumers. In addition, these experimental products maintain their antioxidant properties during shelf-life. However, in order to ascertain the health benefits, further research involving cell culture studies and human intervention studies has been carried out.

Cell culture is a powerful technique for studying physiological, biochemical and toxicological processes modulated by pure phytochemicals or by whole foods *in vitro* (Glei et al., 2003). Several studies developed with different cell lines have reported the ability of plant phenolics to inhibit the tumor cell proliferation and to protect against oxidant-induced damage (Sestili et al. 1998; Aherne and O'Brien 1999; 2000; Johnson and Loo 2000; Feng et al. 2002; Sestili et al. 2002; Jimenez-López and Cederbaum, 2004). In particular, the hepatoma cell line (HepG2) has been widely used in biochemical and nutritional studies because it is considered one of the experimental models that more closely resembles the human hepatocyte in culture (Ramos et al., 2005).

For the cell culture assays, we used the experimental product in a juice format in order to facilitate the sample preparation. The phenolic compounds profile and the total antioxidant properties of the juice were rather similar to those mentioned above. As regard the antiproliferative activity of the juice, we observed a dose-dependent inhibitory effect on HepG2 cell growth after incubation with different phenolics concentrations. This indicates that the polyphenols in the juice initiated a series of cellular events leading to the inhibition of cell proliferation and/or the induction of cell death. These results are in agreement with those reported by other researchers for extracts of commonly consumed fruits and vegetables, especially those of pigmented fruits such as grapes, raspberries, cranberries or strawberries (Chu et al., 2002; Liu et al., 2002; Sun et al., 2002; Meyers et al., 2003).

In addition to their antiproliferative activity, phenolic compounds have been shown to protect cell cultures from different oxidants. However, there is a lack of consensus regarding the experimental design when evaluating the protective effects of both food extracts and pure phenolic compounds in cell culture systems. Although a vast amount of

information in this subject is currently available in the scientific literature, it is often difficult to compare results due to the large variety of cell lines (e.g. cells from different organs and tissues) and to the different oxidant agents used. In the present study, HepG2 cells were preincubated with juice extracts at different sub-toxic concentrations of phenolic compounds and challenged with *tert*-butyl hydroperoxide (*t*-BOOH) or hydrogen peroxide (H_2O_2). It is well known that H_2O_2 can directly damage DNA, lipids, and other macromolecules, causing oxidative injury to the cell. Similarly, the organic hydroperoxide tB-OOH induces an array of cellular dysfunctions, including peroxidation of membrane lipids, glutathione and protein thiol depletion, alteration of calcium homeostasis, and DNA damage, eventually leading to cell death (Alia et al., 2005). In agreement with other previously reported data (Nardini et al., 1998; Feng et al., 2002; Yau et al., 2002; Lazzè et al., 2003; Hong and Liu 2004; Sohn et al., 2005), cells preincubated with extracts of the phenolic-rich juice showed an increased resistance to oxidative challenge, as revealed by the increase in cell survival and the decrease in malondialdehyde production after exposure to both H_2O_2 and tB-OOH. This protective effect was observed after a short preincubation period, suggesting that phenolics compounds were rapidly absorbed by cells and effectively prevented cell death and lipid peroxidation.

Changes in endogenous enzyme activities are considered a sensitive biomarker of the cellular response to oxidative stress. In this study, tB-OOH was chosen as oxidative stressor because it has been shown to efficiently increase the activity of endogenous enzymes in HepG2 cells (Alía et al., 2005). Increases in the activities of the glutathione-related enzymes glutathione peroxidase (GPx) and glutathione-*s*-transferase (GST) have been reported in cell systems after exposure to tB-OOH, and concomitant with a decrease in reduced glutathione (GSH) levels (Aniya and Daido 1994; Alía et al., 2005). A significant increase in both GPx and GST activity after exposure to tB-OOH was observed. Preincubation with the 150 µM-juice extract effectively suppressed the increase in GPx activity, but not that of GST. Consistent with our results, Murakami et al. (2002) reported the ability of tea catechins to suppress the tB-OOH-induced increase in GPx activity in HepG2 cells. Thus, as regards GPx activity, phenolics in the juice might exert their protective effect by delaying the consumption of GSH and/or other cellular antioxidants, during the process of tB-OOH detoxification. Accordingly, the plant polyphenolic compounds, panduratin A and sylibin, as well as plant extracts showing high antioxidant activity have been shown to reverse the GSH-depleting effect of tB-OOH in hepatocyte systems (Yau et al., 2002; Sohn et al., 2005).

To further characterize the protective effect of the phenolic-rich juice, we carried out a study with the human myeloid leukaemia (U937) cell line, in which we evaluated the ability of the juice to protect cells against tB-OOH-induced oxidative stress. Similarly to the above results, a short-term preincubation period with the phenolic-rich juice increased the resistance of cells to oxidative challenge in terms of preventing cell death, DNA damage, the intramitochondrial generation of reactive oxygen species (ROS) and the mitochondrial permeability transition (MPT) pore opening. Accordingly, studies performed in U937 cells have shown that flavonoids and several hydroxycinnamic acids (caffeic acid esters) afford protection against *tert*-butylhydroperoxide (tB-OOH)-induced cell death and DNA damage (Sestili et al. 1998; 2000). In addition, anthocyanins have been shown to protect against tB-OOH-induced cell death and DNA damage in the rat smooth muscle (SMC) and rat hepatoma (MH1C1) cell lines (Lazzè et al. 2003). Other studies developed with the Caco-2 and HepG2 cell lines have reported the ability of flavonoids, such as quercetin, myricetin and rutin, to protect against DNA damage caused by tB-OOH, H_2O_2 and menadione (Aherne and O'Brien 1999; 2000). Furthermore, phenolics from tea have demonstrated a protective effect against tB-OOH-induced cell death, DNA damage and intramitochondrial ROS generation in the rat normal liver epithelium (RL-34) cell line (Feng et al. 2002).

MPT pore opening is an inner mitochondrial membrane permeabilization process that may precede necrotic and apoptotic cell death. The occurrence of MPT has been linked to an oxidized shift in the mitochondrial redox state and/or increase in mitochondrial generation of ROS (Castilho et al. 1995; Vercesi et al. 1997). Furthermore, it is generally thought that tB-OOH-induced MPT pore opening depends on the intramitochondial generation of ROS, which in turn initiates the MPT pore opening, and eventually leads to cell death (Kim et al. 2003). Therefore, cell death under conditions involving MPT can be prevented by antioxidants, since they are able to prevent events prior to opening of MPT pores, particularly at the ROS generation stage (Kowaltowsky et al. 2002; Kim et al. 2003). In agreement with this notion, the suppression of the intramitochondrial ROS generation by the juice might favour its protective effect in terms of preventing MPT pore opening and cell death. In addition, the suppressive effect of phenolics compounds on ROS generation might facilitate the prevention of DNA damage, as suggested by Feng et al. (2002) in the case of theaflavins.

The data we report above clearly indicate that phenolics in the juice were able to protect cultured cells against induced oxidative damage. However, the question arises as to how the phenolics in the juice exert their cytoprotective effect. In addition to its radical scavenging activity, polyphenols possess an ideal structural chemistry for metal chelation,

supporting the role of polyphenols as preventative antioxidants in terms of inhibiting transition-metal catalyzed free radical formation (Rice-Evans et al. 1997). In this context, it has been reported that the presence of two hydroxyl groups in the *o*-position is critical for the chelation of iron and the protection afforded by phenolic compounds (Sestili et al., 2002). Since this essential structural feature is shared by flavonoids (e.g. quercetin, catechins and anthocyanins) and caffeic acid, it is reasonable to think that the protective effects exerted by these polyphenols might partly be explained by an iron-chelating activity. Importantly, anthocyanins, caffeic acid derivatives and catechins were well represented in the juice. Since these polyphenols possess ideal structural features for iron chelation, the possible role of iron-chelating activity in the protection afforded by the juice should not be ruled out. For this reason, in an attempt to elucidate whether the protective effects exerted by the phenolic-rich juice are dependent on radical scavenging or iron chelating activities, the radical scavenger *N,N'*-diphenyl-1,4-phenylene-diamine (DPPD) and the intracellular iron chelator *o*-phenanthroline (*o*-phe) were also assayed in both HepG2 and U937 cells.

In the study carried out in HepG2 cells, we observed that the lipid peroxidation caused by either H_2O_2 or tB-OOH was prevented by both DPPD and o-phe. This indicates that both radical scavenging and iron chelation play a role in the prevention of the lipid peroxidation caused by H_2O_2 and tB-OOH but, however, do not allow us to conclude whether these effects are mediated by a radical scavenging or an iron chelating mechanism, or by a combination of these two activities. Nevertheless, our data regarding GSH-related enzyme activities suggest that the phenolics in the juice might have acted more like iron chelators than radical scavengers. As previously mentioned, both juice and *o*-phe decreased the tB-OOH-induced GPx activation, but not that of GST, whereas the tB-OOH-induced GST activation was only suppressed by DPPD. Based on these observations, we could reasonably hypothesize that the antioxidant properties of the phenolics in the juice relied more on their iron-chelating ability than on radical scavenging activity. In this regard, our results obtained in U937 cell might support this hypothesis, since juice, DPPD and o-phe prevented tB-OOH-induced cell death, and only the juice and o-phe were able to protect cells from DNA damage caused by tB-OOH. This observation is in agreement with the notion that tB-OOH-induced DNA damage is prevented by iron chelators and insensitive to radical scavengers, whereas tB-OOH-induced cell death is prevented by both iron chelators and radical scavengers (Sestili et al., 1998; 2002). Furthermore, in our study with U937 cells, the intramitochondrial ROS generation was also prevented by both juice and o-phe, but not by DPPD.

Taken together, these data suggest a more prominent iron-chelating activity, potentially of biological relevance, in the protection afforded by the phenolic-rich juice. Although iron chelation has generally been regarded as playing a minor role in the antioxidant activity of polyphenols, the presence of iron chelating groups in food and their efficiency in iron chelation may partly explain the health protective role of specific phenolics in the human diet (Duthie et al. 2000; Prior and Cao 2000).

It is now well accepted that *in vitro* chemical tests and *in vitro* cell culture assays provide useful information about the efficacy and mechanisms of action of particular antioxidants or antioxidant-rich foods. However, extrapolation to effects of dietary antioxidants *in vivo* should be carefully done, because uptake from the gastrointestinal tract and metabolism are not considered. For this reason, supplementation of human subjects is the best approach to be taken when evaluating the effectiveness of antioxidant-rich foods. The impact of the diet and dietary components on ageing and age-associated degenerative diseases has been widely recognized in recent years (Ames et al. 1993; Meydani 2001). Harman suggested almost a half a century ago, in his free radical theory of ageing, oxidative damage is related to the debilities associated with ageing (Harman 1956). Therefore, a healthy lifestyle, that include physical activity and well balanced diets, may prevent the age-associated degenerative diseases and slow the ageing process (Tucker and Buranapin 2001). For this reason, a phenolic-rich dessert was designed with the aim to be used as a complementary food in adulthood. As described above, this dessert provides a high content of natural antioxidant to the diet. Prior to investigate the intake effect of this dessert, nutritional status, anthropometrics and biochemical indices were evaluated in an institutionalised elderly group.

The dietary evaluation showed excessive intakes of proteins and lipids, and deficiencies in those of carbohydrates, dietary fibre, zinc, iodine and vitamins A, E and D. These results are in agreement with those reported in other studies for other Spanish elderly groups (Ortega et al., 1996; Gámez et al., 1997; 1998), which can be attributed to the dietary patterns of Spanish population (MAPA 2003). Serum total antioxidant activity was low compared with reference values (Miller et al., 1993; Benzie and Strain 1996), particularly in women. Plasma retinol levels were also below the reference values (Le Grusse and Watier 1993). Furthermore, the oxidative risk in this group was evaluated on the basis of their plasma levels of vitamins C, A and E, according to Le Grusse and Watier (1993). A moderate oxidative risk was observed for retinol in 80% of the individuals. Lower risk was observed for tocopherol and ascorbic acid levels. Plasma levels of tocopherol and retinol were not correlated with their estimated dietary intake, whereas serum

ascorbic acid levels showed a positive correlation with its estimated dietary intake. Similar results have been reported by Jacques et al. (1995) and Picado et al. (2001). In agreement with Benzie and Strain (1996) and Cao and Prior (1998), overall antioxidant capacity was strongly correlated with serum uric acid, and in a lesser extent with albumin, ascorbic acid, iron and bilirubin, but not with the dietary antioxidant levels.

In order to evaluate the effect of the intake of this phenolic-rich dessert, a two-week intervention study was carried out with this institutionalized elderly group. Changes in the serum total antioxidant capacity, plasma/serum levels of antioxidants and several biomarkers of oxidative damage were evaluated before and after the intervention period. There were no significant differences in the antioxidant activity, in the biochemical parameters and antioxidant vitamins (a-tocopherol, ß-carotene and retinol) and coenzyme Q_{10} measured in serum/plasma of elderly people after the intervention study period. In addition, the data of total antioxidant capacity of the elderly people were not different than those obtained in the healthy reference group. However, plasma antioxidant vitamin levels (a-tocopherol, retinol, ß-carotene) and coenzyme Q_{10} in elderly subject were below those considered as normal values, showing significant differences between elderly and healthy reference groups. As regards biomarkers of oxidative damage, the susceptibility of LDL to oxidation and lymphocyte DNA damage did not change with the intake of the dessert. Similarly, Young et al. (2002) did not find significant changes in markers of oxidative stress and antioxidant status after supplementation with 18.6 mg catechin per day for 6 weeks.

The lack of effect observed in our study after consumption of the dessert could be attributed to the short intervention period and/or the poor antioxidant status of the volunteers. The latter was confirmed by the high endogenous DNA damage and the highest susceptibility of LDL oxidation observed in the elderly group, compared to the healthy reference group. However, since phenolics compounds (particularly anthocyanins) are believed to be poorly absorbed from the diet, the lack of effect observed could partly be due to the low bioavailability of these compounds (Manach et al., 2005).

For this reason, we carried out a short-term intervention study in order to know whether phenolic-rich products could modify biomarkers of the antioxidant status in healthy volunteers. In this study, we used the juice format instead of the jellified dessert because the experimental design involved the intake of a single oral dose of 400 mL. After a 48-hours washout period, 12 fasting subjects consumed the juice dose, and the total

antioxidant capacity, uric acid, vitamin C, lipid-bound polyphenols, lipid peroxidation products and protein carbonyl content were analyzed in serum 0, 1, 2, 4 and 6 hours post intake. Total phenolics and total antioxidant activity were also analyzed in urine at the same times and 24 hours post intake.

In agreement with the results obtained in the elderly group, no significant changes were detected in serum total antioxidant capacity after the intake of the phenolic-rich juice. The lack of statistical significance can be attributed to the high interindividual variations observed for these parameters. Although the mean total antioxidant activity did not show significant differences during the study, consumption of the juice resulted in significant changes in this parameter increasing for each volunteer, from 1 to 2 hours post-intake, concomitant with an increase in uric acid. These changes could partly be explained by the metabolic effect of fructose on urate, as suggested by Lotito and Frei (2004). Hence, these authors reported that many of the previously described effects of phenolic-rich foods on overall *in vivo* antioxidant capacity are not due to the phenolic compounds. However, our data suggested that phenolics were absorbed from the juice, as revealed by the slight increase in serum lipid-bound phenolic compounds and the rise in the urinary excretion of total phenolics. This agrees with data reported by other researchers after the intake of anthocyanin-rich foods, with mean plasma anthocyanin levels peaking at 1.5 hours, and a mean time to reach maximum urinary excretion of 2.5 hours (Manach et al., 2005). In addition, our data indicate that phenolics from the juice were bioavailable and able to bind with the lipid fraction of serum, therefore reducing lipid peroxidation. Other authors have described similar results after the intake of anthocyanin-rich juices, (Young et al., 1999; Netzel et al., 2002), suggesting a key role of the binding of phenolics to lipoproteins, in the prevention of lipid peroxidation (Nigdikar et al., 1998; Lamuela-Raventós et al., 1999).

In contrast, a strong but transient prooxidant action on serum protein was observed after the intake of the juice. As reviewed by Griffiths et al. (2002), human studies involving supplementation with phenolic-rich foods have reported contradictory results regarding protein oxidation. The prooxidant effect of plant-foods on protein oxidation has been generally attributed to ascorbic acid and other plant-derived antioxidants, which could enhance protein oxidation, most probably via Fenton-type reactions (Young et al., 1999; Dragsted et al., 2004). Nevertheless, the possible role of sugars on protein oxidation should not be ruled out. In this context, a study in female rats reported that sugars present in juices were involved in the increase observed in protein oxidation (Breinhold et al., 2003). Furthermore, hyperglycemia has been reported to cause

increased production of oxygen free radicals through glucose autooxidation and non-enzymatic glycation (Maillard reaction) processes and consequently may damage cellular and tissue components (Wolff 1993; Domínguez et al., 1998; Odetti et al., 1999).

Overall, the results obtained in the *in vivo* studies indicate that the intake of phenolic-rich foods do not modify the total antioxidant capacity of plasma/serum and the antioxidant levels. However, phenolic compounds from these foods might provide specific health benefits in terms of reducing lipid oxidation in healthy volunteers. In contrast, the increased oxidative damage associated with ageing might hinder the improvement of the antioxidant status of the elderly population through the intake of phenolic-rich foods. Nevertheless, from our point of view, the use of these products as a complementary food in the adulthood, in particular for the elderly, could be of interest, because they provide a high amount of natural antioxidants. It is well established that physiological changes common to old age might lead to a low intake of dietary antioxidants as a consequence of a lower intake of fruits and vegetables, mainly due to the loss of appetite, oral and dental problems. In this context, these processed foods made from pigmented fruits could be optimal for this group of population, because they are appealing foods providing high levels of natural polyphenols and at the same time are the appropriate textures for people suffering from oral problems. In addition, since in today's lifestyle many people do not consume the recommended amounts of fruits and vegetables, these phenolic-rich foods could also be considered as appropriate to complement the diet of the general population and to provide health benefits.

5

Conclusiones

Conclusions

5. CONCLUSIONES

1. El postre y el zumo elaborados a partir de uvas, cerezas y bayas muestra una alta actividad antioxidante, la cual está asociada a su alto contenido en compuestos fenólicos.

2. Los resultados del estudio de envejecimiento del postre de frutas rojas rico en compuestos fenólicos indican que el almacenamiento a temperatura ambiente es adecuado para el mantenimiento de sus propiedades antioxidantes. Este dato es de interés para la industria ya que estas condiciones de almacenamiento permiten reducir costes de distribución y almacenamiento del producto.

3. La proliferación de las células en cultivo fue inhibida por los extractos del zumo de frutas rojas rico en compuestos fenólicos en función de la dosis ensayada. Los resultados indican que los compuestos fenólicos del zumo iniciaron una serie de procesos celulares que conducen a la inhibición de la proliferación celular y/o a la inducción de la muerte celular.

4. Los ensayos en cultivos celulares mostraron que un periodo corto de incubación con los extractos del zumo rico en compuestos fenólicos ejerce un efecto protector frente al estrés oxidativo inducido, a través de la prevención de la muerte celular, peroxidación lipídica y daño al ADN. Además, los extractos del zumo ejercieron un efecto significativo sobre procesos oxidativos celulares, modulando la actividad del enzima glutatión peroxidasa y disminuyendo la generación intracelular de especies reactivas del oxígeno. Esto indica que los compuestos fenólicos del zumo fueron rápidamente absorbidos por las células y contribuyeron a sus defensas antioxidantes.

5. Los resultados del estudio realizado en el grupo de ancianos institucionalizados mostraron que la ingesta diaria del postre de frutas rojas durante dos semanas no mejoró el débil estado antioxidante del grupo de acianos. De hecho, los participantes mostraron un nivel de daño oxidativo elevado que confirma el incremento del estrés oxidativo durante el proceso de envejecimiento.

6. La ingesta de una dosis única del zumo rico en compuestos fenólicos produjo un aumento transitorio, aunque no significativo, de la capacidad antioxidante total sérica en individuos sanos. Esta leve variación podría deberse, más que a los

compuestos fenólicos del zumo, al aumento del ácido úrico sérico como consecuencia del rápido metabolismo de la fructosa y/u otros azúcares del zumo.

7. Aunque la ingesta del zumo no aumentó de forma significativa la capacidad antioxidante total sérica, los resultados ponen de manifiesto la absorción de compuestos fenólicos, su unión a la fracción lipídica del suero y la disminución de la peroxidación lipídica sérica. Esta observación sugiere que el consumo del zumo podría ejercer efectos beneficiosos mediante la disminución de la peroxidación lipídica del suero, y por lo tanto reduciendo el riesgo de enfermedad cardiovascular.

8. Por contra, la ingesta del zumo produjo un aumento transitorio de la oxidación de proteínas séricas, que podría ser debido a procesos oxidativos mediados por los azúcares del zumo. Por este motivo, sugerimos que en estudios futuros se considere el efecto de los hidratos de carbono de las frutas y derivados ejercen sobre los procesos oxidativos que tienen lugar en el organismo.

6. CONCLUSIONS

1. The dessert and juice made from grapes, cherries, and berries have high antioxidant activity associated to their high content in phenolic compounds.

2. As regards the stability of the phenolic-rich dessert during storage, our results indicate that storage at room temperature is appropriate in the maintenance of the antioxidant properties during shelf-life. This is of great interest for the industry since room temperature storage allows the reduction of both distribution and storage costs.

3. The proliferation of cultured cells was inhibited in a dose-dependent manner by juice extracts, indicating that polyphenols in the juice initiated a series of cellular events leading to the inhibition of cell proliferation and/or the induction of cell death.

4. Cell culture assays showed that short-term preincubation with juice extracts affords protection against induced oxidative stress, in terms of preventing cell death, lipid peroxidation and DNA damage. In addition, juice extracts exerted a significant effect on oxidative reactions in cellular systems, by modulating the activity of the enzyme glutathione peroxidase, and decreasing the generation of intracellular reactive oxygen species. This indicates that phenolics from the juice were rapidly absorbed by cells and contributed to the cellular antioxidant defences.

5. The results of the study carried out in the group of institutionalised elderly people showed that an intervention study for two weeks with a daily intake of the phenolic-rich dessert did not improve the low antioxidant status of the elderly group. In fact, the participants showed a high endogenous oxidative stress status, which confirms the increased oxidative stress associated with the ageing process.

6. The intake of a single dose of the phenolic-rich juice caused a non significant transient increase in the total serum antioxidant capacity of healthy subjects. This could be caused by an increase in serum uric acid due to the rapid metabolism of fructose and/or other sugars in the juice, and not by polyphenols in the juice.

7. Although juice intake did not significantly raise the overall serum antioxidant capacity, the phenolic compounds in the juice appeared to be absorbed and capable of binding the serum lipid fraction, and able to decrease serum lipid peroxidation. These findings raise the possibility that consumption of the juice might provide health benefits in terms of reducing serum lipid oxidation, and reducing the risk of cardiovascular disease.

8. In contrast, a transient increase in serum protein oxidation was observed after the intake of the juice. This prooxidant effect could be due to sugar-mediated oxidative processes. For this reason, future studies should take into account the role that carbohydrates from fruits and fruit-derived products play in in vivo oxidative processes.

6

Resumen

Summary

6. RESUMEN

El estrés oxidativo está implicado en el desarrollo de numerosas enfermedades crónicas y en el proceso de envejecimiento. Debido a que la dieta desempeña un papel importante en la prevención del daño oxidativo, la industria alimentaria muestra hoy en día un interés creciente por el desarrollo de alimentos funcionales ricos en antioxidantes que puedan proporcionar beneficios para la salud del consumidor. El objetivo de la presente investigación ha sido evaluar la eficacia de dos productos ricos en compuestos fenólicos, elaborados por el Departamento de Investigación y Desarrollo de Hero España. S.A. Ambos productos, un postre gelificado y un zumo, fueron elaborados a partir de zumos concentrados de uva, cereza y bayas. Para la evaluación de la eficacia antioxidante, se realizaron pruebas químicas, estudios *in vitro* empleando cultivos celulares y estudios de intervención en humanos.

Ambos productos mostraron una alta actividad antioxidante total, en pruebas químicas basadas en la captación de radicales libres y en la capacidad reductora de hierro férrico. Las propiedades antioxidantes de los productos estuvieron determinadas por sus respectivos perfiles de compuestos fenólicos, principalmente compuestos por antocianos y ácidos hidroxicinámicos. En el caso del postre gelificado se realizó un estudio de envejecimiento, en el cual se evaluó la estabilidad de las propiedades antioxidantes durante un año a diferentes temperaturas (8, 21 y 30ºC). De modo interesante, aunque se observó una disminución en el contenido de los compuestos fenólicos individuales, la actividad antioxidante total del producto se mantuvo prácticamente invariable a las tres temperaturas de almacenamiento. Esto sugiere que diferentes productos de degradación de los compuestos fenólicos podrían contribuir al mantenimiento de la actividad antioxidante total.

En los estudios en cultivos celulares se emplearon dos líneas celulares humanas; la línea de carcinoma hepático HepG2 y la línea de leucemia mieloide U937. Para facilitar la preparación de las muestras, en estos estudios con células cultivadas se empleó el producto experimental en el formato de zumo. Los extractos del zumo rico en compuestos fenólicos inhibieron de forma eficaz la proliferación de la línea celular HepG2, de un modo dependiente de la dosis. Por otra parte, los extractos del zumo a concentraciones no tóxicas ejercieron un efecto protector frente al estrés oxidativo inducido por *tert*-butil hidroperóxido y por peróxido de hidrógeno. Esto se tradujo en un aumento de la viabilidad celular y en la disminución de la peroxidación lipídica, daño al ADN y generación

intracelular de especies reactivas del oxígeno, en las células preincubadas con los extractos del zumo.

En la presente investigación se realizaron dos estudios de intervención en humanos con la finalidad de evaluar la eficacia *in vivo* de los productos. El primer estudio se realizó en un grupo de ancianos y consistió en la ingesta diaria durante dos semanas de una ración del postre de frutas gelificado (200 g). El consumo del postre no produjo cambios en la capacidad antioxidante sérica de los individuos, ni tampoco en biomarcadores del estrés oxidativo. El hecho de que no se observase ningún efecto tras la ingesta podría deberse en parte al deficiente estado antioxidante que mostraron los ancianos, el cual se caracterizó principalmente por un alto nivel de daño en el ADN y una elevada susceptibilidad de las lipoproteínas plasmáticas a la oxidación.

El segundo estudio se realizó en un grupo de individuos sanos, los cuales consumieron una dosis única de 400 mL del zumo de frutas, tras seguir durante dos días una dieta pobre en antioxidantes. Así, en este estudio se evaluó durante las 6 horas posteriores a la ingesta el efecto a corto plazo derivado del consumo del zumo. De modo similar al estudio en ancianos, el consumo del zumo no produjo cambios significativos en la capacidad antioxidante total sérica. De modo interesante, se observó un aumento leve de los niveles de compuestos fenólicos unidos a lípidos entre las 2 y las 6 horas posteriores a la ingesta, el cual estuvo asociado con una disminución de la peroxidación lipídica sérica durante el mismo intervalo de tiempo. Esto sugiere que los compuestos fenólicos del zumo fueron absorbidos y mostraron un efecto antioxidante *in vivo*. Por el contrario, la ingesta del zumo se observó un efecto prooxidante transitorio que incrementó la oxidación proteica sérica. Este efecto podría ser debido a procesos oxidativos mediados por azúcares del zumo, que implicarían reacciones de Maillard.

En conjunto, nuestros resultados indican que el empleo de zumos concentrados de uva, cereza y bayas constituye una estrategia adecuada para el desarrollo de alimento ricos en antioxidantes, cuyas propiedades se mantengan estables durante el almacenamiento. Además, los datos obtenidos en los ensayos en cultivos y en los estudios en humanos sugieren que los alimentos ricos en compuestos fenólicos podrían proporcionar efectos beneficiosos específicos, particularmente mediante la prevención de la peroxidación lipídica.

6. SUMMARY

Oxidative stress is a factor in many human chronic diseases and in the aging process itself. Since the diet plays a key role in the protection against oxidative injuries, there is a trend in the food industry toward the development of antioxidant-rich functional foods with potential health benefits. The objective of the present research was to evaluate the antioxidant effectiveness of two phenolic-rich products, a jellified dessert and a juice, made from grapes, cherries and berries, which were prepared by the Research and Development Department of Hero Spain S.A. For that purpose, *in vitro* chemical tests, cell culture assays and human intervention studies were carried out in order to generate scientific evidence supporting any beneficial effect.

Both products showed a high total antioxidant activity in different *in vitro* chemical tests, in terms of scavenging of free radicals and reducing ferric iron. The antioxidant properties of the products were related to their phenolic compounds profile, mainly composed by anthocyanins and hydroxicinnamic acids. In the case of the jellified dessert, a one-year storage trial was carried out involving different temperatures (8, 21 and 30ºC), with the aim of evaluating the stability of its antioxidant properties. Interestingly, although individual phenolic compounds decreased, the total antioxidant activity of the dessert remained practically invariable at all of the storage temperatures. This suggests that products of polyphenols degradation might contribute to the maintenance of the antioxidant activity.

The *in vitro* cell culture assays were carried out in the human hepatoma cell line HepG2 and in the human myeloid leukaemia cell line U937. In order to facilitate the sample preparation, for the cell culture experiments, we used the product in the juice format. Extracts of the phenolic-rich juice effectively inhibited the proliferation of HepG2 cells in a dose-dependent manner. Furthermore, sub-toxic concentrations of juice extracts protected the cultured cells from either *tert*-butyl hydroperoxide- or hydrogen peroxide-induced oxidative damage, as revealed by an increase in cell viability, and the decreases in lipid peroxidation, DNA damage and the intracellular generation of reactive oxygen species.

In the present research, two human intervention studies were carried out in order to test the *in vivo* efficacy of the experimental products. The first human study was performed in elderly subjects and involved the daily intake of one serving (200 g) of the dessert for two weeks. Consumption of the dessert had no effect on biomarkers of

oxidative stress or antioxidant protection. This lack of effect could be partly attributed to the high oxidative stress level observed in the elderly group, which was revealed by a high endogenous DNA damage and a high susceptibility of plasma lipoproteins to oxidation.

The second intervention study was carried out in a group of healthy subjects. After a two-day washout period, the subjects consumed a single oral dose of the phenolic-rich juice (400 mL), and the short-term effect of the juice consumption was evaluated within 6 hours post-intake. Juice consumption did not significantly raised the serum total antioxidant capacity of the subjects, and only a slight increase in serum lipid-bound phenolic compounds was observed from 2 to 6 hours post-intake. Interestingly, the weak increase in lipid-bound polyphenols was associated with a significant decrease in serum lipid peroxidation, suggesting that phenolics from the juice were absorbed and showed *in vivo* antioxidant activity. In contrast, a transient prooxidant effect was observed with regard to serum protein oxidation, which could be attributed to sugar-mediated oxidative processes involving Maillard reactions.

Overall, our results indicate that the use of selected concentrated juices of grapes, cherries and berries is a feasible strategy to produce stable antioxidant-rich foodstuffs, and highlight the fact that phenolic-rich foods might provide specific health benefits, particularly by preventing lipid peroxidation.

7

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