Centro de Investigación en Alimentación y Desarrollo, A. C.

EVALUACIÓN DE LA BIOACCESIBILIDAD DE COMPUESTOS FENÓLICOS DE TRES ESPECIES DE ORÉGANO

Por:

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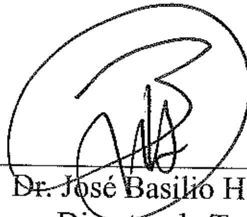

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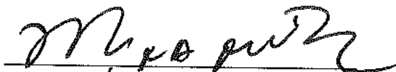

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RESUMEN

Los polifenoles son un grupo de compuestos fitoquímicos que están presentes en una gran variedad de alimentos de origen vegetal. Estudios epidemiológicos y experimentales sugieren una asociación entre el consumo de alimentos ricos en polifenoles y la disminución en la incidencia de enfermedades crónicas. Tradicionalmente se han utilizado diversas hierbas como remedios herbolarios, una de las más comunmente utilizadas ha sido el orégano, que se ha utilizado el orégano en el tratamiento de padecimientos respiratorios, digestivos e inflamatorios. Estos efectos benéficos se han relacionado con el contenido de compuestos fitoquímicos del orégano, específicamente al grupo de los polifenoles.

Sin embargo, aún se conoce poco acerca de los mecanismos exactos a través de los cuales los polifenoles de orégano ejercen su efecto benéfico. Para que los polifenoles dietéticos ejerzan un efecto benéfico es necesario que sean biodisponibles, lo cual implica que sean bioaccesibles y, bioactivos; es decir, que sean liberados de la matriz alimentaria, absorbidos, y distribuidos a órganos y tejidos. En este sentido, una vez ingeridos los polifenoles pueden sufrir transformaciones durante su paso por el tracto gastrointestinal. Una forma que se ha vuelto común para estudiar la bioaccesibilidad de los compuestos fenólicos es la digestión gastrointestinal *in vitro*, generalmente simulando las condiciones fisiológicas y bioquímicas en los compartimentos bucal, gástrico e intestinal.

La primera etapa de esta investigación consistió en evaluar el efecto de una digestión gastrointestinal simulada sobre la estabilidad antioxidante y el contenido de compuestos fenólicos presentes en las especies de orégano: *Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri*. Los resultados demostraron que el proceso de digestión gastrointestinal simulado disminuyó la capacidad reductora total (evaluada por el método Folin-Ciocalteu), el contenido total de flavonoides y la capacidad antioxidante (evaluada por los métodos DPPH, ABTS y ORAC) de extractos polifenólicos de HP, LG y LP. A su vez, se encontró que los polifenoles principales en HP, LG y LP pertenecen al grupo de las flavonas, encontrándose también un derivado del ácido hidroxicinámico y una dihidrochalcona. En este sentido, los polifenoles identificados fueron afectados

significativamente por el proceso digestivo. De particular interés fue que el compuesto fenólico más estable durante la digestión simulada fue el ácido clorogénico encontrado en la especie HP.

En la segunda parte de la investigación se evaluó el efecto de una digestión gastrointestinal simulada sobre las propiedades hipoglucemiantes e hipolipidémicas de los extractos polifenólicos de las especies de orégano *Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri* a través de la inhibición de la actividad enzimática de α-glucosidasa, α-amilasa y lipasa pancreática; además se evaluó la capacidad antioxidante celular en células Caco-2 de los extractos de HP, LG y LP antes y después de una digestión gastrointestinal simulada. Nuestros resultados mostraron que las tres especies tienen potencial inhibitorio de α-glucosidasa en el orden HP > LG > LP, a su vez esta capacidad inhibitoria disminuyó significativamente durante la digestión gastrointestinal. De igual manera los extractos polifenólicos de HP, LG y LP mostraron potencial inhibitorio contra α-amilasa y lipasa pancreática.

Palabras clave: oregano, polifenoles, bioaccesibilidad, *Hedeoma patens*, *Lippia graveolens*, *Lippia palmeri*.

ABSTRACT

Polyphenols are a wide group of phytochemicals present in the vast majority of plant foods, therefore are an important part of human diet. Epidemiological and experimental studies suggest an association between consumption of plant foods rich in polyphenols and a decrease in the incidence from chronic diseases. Traditionally, herbs and species have been used in folk medicine. In this sense, oregano is among the plants with potential health benefits. Oregano has been used in traditional medicine for the treatment of respiratory, digestive disorders and inflammation-related illnesses. These beneficial effects have been linked with the polyphenolic content of oregano, specifically flavonoids.

Nonetheless, the mechanisms of action through which the phenolic compounds exert their beneficial effects are yet not fully known, nonetheless are related, in part, to their bioavailability. In this sense, once ingested, polyphenols undergo physicochemical transformations during their passage through the gastrointestinal tract. Thus, a common strategy to evaluate polyphenol's bioaccessibility is through the use of *in vitro* simulated gastrointestinal digestion models that simulate the physiological and biochemical conditions in the mouth, stomach and small intestine. In this sense, at the time when this work was made, there is little information related to the bioaccessibility of oregano polyphenols and the effect of an *in vitro* gastrointestinal digestion process on its potential antioxidant and bioactive properties.

The present work is one of the first of its kind and aims to assess comprehensively identifying phenolic compounds in three species of oregano, namely *Hedeoma patens*, *Lippia graveolens*, and *Lippia palmeri* and their bioaccessibility. To achieve the goal, the experimental work of this thesis was divided in two sections. The first section aimed to evaluate the effect of a simulated gastrointestinal digestion process on the stability of the antioxidant capacity and polyphenolic content from three oregano species, namely *Hedeoma patens*, *Lippia graveolens*, and *Lippia palmeri*. Our results showed that the *in vitro* digestion decreased the total reducing capacity (by the Folin-Ciocalteu method), the total flavonoid content and the antioxidant capacity (evaluated by the DPPH, ABTS and ORAC chemical assays) of the polyphenolic-rich extracts from HP, LG and LP.

Moreover, the UPLC-PDA analysis showed that flavones are the main constituents of the evaluated oregano species, and that its content was negatively affected by the gastrointestinal process, with the exception of chlorogenic acid in HP with around a bioaccessibility of around 97%.

The second section of this research aimed to evaluate the effect of the *in vitro* digestion process on the potential hypoglycemic, hypolipidemic and cellular antioxidant properties of the polyphenolic-rich extracts from *Hedeoma patens*, *Lippia graveolens*, and *Lippia palmeri*. To assess the goal, we evaluated the inhibitory potential of oregano polyphenols on α-glucosidase, α-amylase and pancreatic lipase and their cellular antioxidant activity against Caco-2 cells. Our results exhibited that the polyphenols from HP, LG and LP are good inhibitors of the evaluated enzymes, thus are potential anti-obesogenic agents. Furthermore, the polyphenolic extracts exhibited prominent cellular antioxidant activity. Additionally, the evaluated bioactive properties are maintained during the gastrointestinal digestion process.

Keywords: oregano, polyphenols, bioaccessibility, *Hedeoma patens*, *Lippia graveolens*, *Lippia palmeri*.

I. SINOPSIS

Los compuestos polifenólicos son un grupo heterogéneo de compuestos que están presentes en la gran mayoría de los alimentos de origen vegetal. La importancia de su estudio radica en la evidencia epidemiológica y experimental que relaciona el consumo de alimentos ricos en compuestos polifenólicos con una baja incidencia de enfermedades crónicas, las cuales son la principal causa de muerte a nivel mundial. Sin embargo, para que los polifenoles dietéticos ejerzan un efecto benéfico es necesario que sean biodisponibles, lo cual implica que sean bioaccesibles y, bioactivos; es decir, que sean liberados de la matriz alimentaria, absorbidos, y distribuidos a órganos y tejidos.

En este sentido, una vez ingeridos, los polifenoles dietéticos pasan por el tracto gastrointestinal en donde pueden sufrir modificaciones estructurales debido a las condiciones fisiológicas y bioquímicas del tracto digestivo. Por lo tanto, las propiedades bioactivas de los polifenoles pueden modificarse positiva y negativamente.

Algunas plantas, como las distintas especies de orégano (*Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri*) han sido utilizadas tradicionalmente para tratar malestares relacionados a padecimientos crónico-degenerativos. Las propiedades benéficas del orégano son atribuidas a sus compuestos bioactivos, sin embargo, de particular interés es el estudio de su contenido de polifenoles. Los mecanismos de acción de los compuestos polifenólicos están relacionados con sus propiedades antioxidantes y a su papel como regulador sobre cascadas de señalización relacionadas con el desarrollo de enfermedades crónicas. Sin embargo, a la fecha, no existe información referente a la bioaccesibilidad de los compuestos fenólicos de orégano (Gunawardena et al., 2014).

Las enfermedades crónicas como artritis reumatoide, diabetes, aterosclerosis, enfermedades cardiovasculares, Alzheimer, Parkinson y cáncer son la principal causa de muerte en México y en el mundo. En México, estas enfermedades provocan el 75% de las muertes al año (Globocan, 2014; World Health Organization, 2018).

El orégano además de su uso como condimento, se ha utilizado como remedio casero para el tratamiento de padecimientos respiratorios, digestivos, dolores de cabeza, reumatismo, entre otros (Laferriere et al., 1991). El orégano ha sido motivo de estudio debido a potenciales efectos benéficos a la salud que se le atribuyen a su contenido de polifenoles (Santos-Buelga et al., 2014; Viveros-Valdez et al., 2008). En México, se conocen cerca de 40 especies de orégano, de las cuales 29 son endémicas y forman parte de los géneros Lamiaceae, Verbenaceae, Compositae y Leguminoceae. La familia Verbenaceae es la de mayor relevancia, ya que a ella pertenece el "orégano mexicano" (*Lippia palmeri* Watson y *Lippia graveolens* H.B.K). Algunas otras especies consideradas como orégano son *Origanum vulgare*, *Poliomintha longiflora*, *Hedeoma floribunda* y *Hedeoma patens* (Aguilar-Murillo et al., 2013; Martínez-Gordillo et al., 2013; Villavicencio-Gutiérrez et al., 2007).

Para que los polifenoles dietéticos ejerzan un efecto benéfico es necesario que estén biodisponibles, y para ello se requiere que sean bioaccesibles y bioactivos, es decir que sean absorbidos, alcancen circulación sistémica y sean distribuidos a los órganos y tejidos (Crozier et al., 2010). En este sentido se define biodisponibilidad como la fracción de una dosis administrada de un compuesto que alcanza la circulación sistémica y que por lo tanto está disponible para su distribución a los tejidos y producción de un efecto. A su vez, bioaccesibilidad se define como la cantidad de cualquier constituyente alimentario que está presente en el intestino, como consecuencia de su liberación de la matriz alimentaria y que está disponible para ser absorbido a través de la barrera intestinal (Velderrain-Rodríguez et al., 2014).

No obstante, existen diversos factores que afectan la bioaccesibilidad y bioactividad de estos compuestos. Entre los factores que influyen tanto en la bioaccesibilidad como en la biodisponibilidad de los compuestos fenólicos se encuentran el grado de polaridad, glicosilación, esterificación, grado de polimerización e interacciones con la matriz alimentaria (Chen et al., 2001; Srinivasan, 2001).

Asimismo, es importante considerar que, una vez ingeridos, los compuestos bioactivos sufren una serie de modificaciones digestivas y metabólicas. Durante su paso por el sistema digestivo, los compuestos fenólicos son sometidos a diferentes cambios mecánicos, enzimáticos y de pH. A la fecha, permanecen poco estudiados los cambios

digestivos de los compuestos fenólicos y su bioactividad durante el proceso. Estos procesos afectan la estructura parental de los compuestos fenólicos y por lo tanto su biodisponibilidad (Kroon et al., 2004; Wootton-Beard et al., 2011). Existen actualmente diversos métodos *in vitro* para estudiar la bioaccesibilidad de compuestos bioactivos dietéticos. Uno de los más frecuentemente utilizados, por su practicidad y bajo costo, es el uso de métodos de digestión gastrointestinal *in vitro*, en los cuales se simulan las condiciones fisiológicas en el tracto gastrointestinal utilizando mezclas de electrolitos y enzimas digestivas (Oomen et al., 2003; Versantvoort et al., 2004; Alminger et al., 2014).

El presente trabajo el primero en el estudio de la evaluación de la bioaccesibilidad de los compuestos polifenólicos presentes en diversas variedades de orégano mexicano. Los resultados obtenidos representan el primer paso para dilucidar el potencial uso farmacológico de diversas especies de orégano mexicano (*Hedeoma patens*, *Lippia graveolens, Lippia palmeri*) o como auxiliar en la prevención de enfermedades crónico degenerativas.

Con la finalidad de evaluar la bioaccesibilidad de extractos ricos en polifenoles de tres especies de orégano, se diseñó una investigación del tipo descriptivo que consta de 5 etapas. La primera etapa consistió en someter a un proceso simulado de digestión gastrointestinal a los extractos polifenólicos de *Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri*. La segunda etapa consistió en evaluar el efecto de la digestión simulada en el perfil antioxidante y la capacidad reductora total de los extractos. La tercera etapa consistió en la identificación y cuantificación de los polifenoles presentes en HP, LG y LP y el efecto de la digestión simulada sobre el contenido de los mismos, esto se realizó utilizando herramientas cromatográficas (UPLC-PDA, LC-MS). La cuarta y quinta etapa consistieron en la evaluación del efecto de la digestión *in vitro* sobre las propiedades anti-obesogénicas (hipoglucémicas e hipolipemiantes) y antioxidantes de los extractos.

Integración del Manuscrito de Tesis

La información presente en este manuscrito está dividida en secciones denominadas capítulos y son presentados de la siguiente manera:

Capítulo I "Sinopsis". Consiste en una introducción concisa del problema de investigación, mencionando a grandes rasgos la problemática a tratar, así como el enfoque central de esta investigación.

Capítulo II "Review: Dietary Phenolic Compounds, Health Benefits, and Bioaccessibility". Consiste en una revisión exhaustiva y comprensiva de la literatura relacionada a la biodisponibilidad de compuestos polifenólicos dietéticos. Esta sección coadyuvó en la definición del problema de investigación, el enfoque que se tomaría durante la investigación y en el planteamiento de las preguntas de investigación e hipótesis.
Artículo publicado, referencia: Gutiérrez-Grijalva, E. P., Ambriz-Pérez, D. L., Leyva-López, N., Castillo-López, R. I., & Heredia, J. B. 2016. Review: dietary phenolic compounds, health benefits and bioaccessibility. Archivos Latinoamericanos de Nutrición, 66(2), 87-100.

Capítulo III "Review: Bioavailability of Dietary Phenolic Compounds". Consiste en una revisión exhaustiva y comprensiva de la literatura relacionada a la biotransformación de compuestos polifenólicos dietéticos. La información recopilada fortaleció la definición del problema de investigación, el enfoque que se tomaría durante la investigación y en el planteamiento de las preguntas de investigación e hipótesis. A su vez, la escritura de este capítulo nos permitió delimitar la investigación hacia el estudio *in vitro* de la bioaccesibilidad de los compuestos polifenólicos de orégano.
Artículo publicado, referencia: Gutiérrez-Grijalva, E. P., Ambriz-Pérez, D. L., Leyva-López, N., Castillo-López, R. I., & Heredia, J. B. 2015. Biodisponibilidad de

compuestos fenólicos dietéticos: Revisión. Revista Española de Nutrición Humana y Dietética, 20(2), 8. doi: 10.14306/renhyd.20.2.184

Capítulo IV "Effect of *In Vitro* Digestion on the Total Antioxidant Capacity and Phenolic Content of 3 Species of Oregano (*Hedeoma patens*, *Lippia graveolens*, *Lippia palmeri*)". Describe el trabajo en la evaluación del efecto de la digestión gastrointestinal *in vitro* sobre la estabilidad de la capacidad antioxidante y el contenido de los compuestos polifenólicos de tres especies de orégano (*Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri*). La investigación presente en este capítulo nos permitió dilucidar que el proceso gastrointestinal simulado tiene un efecto negativo en los compuestos fenólicos identificados en las tres especies de orégano, el cual se atribuye principalmente a los cambios de pH durante cada fase digestiva. A su vez, el compuesto con mayor bioaccesibilidad fue el ácido clorogénico presente en *Hedeoma patens*, con un porcentaje de bioaccesibilidad de alrededor del 97% al final de la fase intestinal. Es interesante mencionar que, a pesar de la disminución del contenido de compuestos fenólicos, el potencial reductor y la capacidad antioxidante de los extractos de orégano se conservó al final del proceso digestivo. Estos resultados sirven de pauta para futuros estudios de bioaccesibilidad y bioactividad.

Artículo publicado, referencia: Gutiérrez-Grijalva, E. P., Angulo-Escalante, M. A., León-Félix, J., & Heredia, J. B. 2017. Effect of *In vitro* Digestion on the Total Antioxidant Capacity and Phenolic Content of 3 Species of Oregano (*Hedeoma patens*, *Lippia graveolens*, *Lippia palmeri*). Journal of Food Science, 82(12), 2832-2839. doi: 10.1111/1750-3841.13954

Capítulo V "Flavonoids and Phenolic Acids from Oregano: Occurrence, Biological Activity and Health Benefits". Este capítulo corresponde a una revisión exhaustiva de la literatura referente a la presencia y distribución de flavonoides y ácidos fenólicos en las diferentes especies de plantas conocidas como orégano; así como las propiedades antioxidantes, antiinflamatorias y anticancerígenas que se les han atribuido. La información recopilada mostró que la mayoría de los compuestos fenólicos presentes en las diferentes especies de orégano pertenecen a la categoría de las flavonas, flavonoles,

derivados del ácido hidroxicinámico e hidroxibenzoico, y flavanonas. A su vez, diversos estudios epidemiológicos sugieren que la ingesta regular de alimentos ricos en estos compuestos contribuye a la disminución del riesgo de padecer diversas enfermedades crónico-degenerativas como cáncer, diabetes, síndrome metabólico, etc.

Artículo publicado, referencia: Gutiérrez-Grijalva, E. P., Picos-Salas, M. A., Leyva-López, N., Criollo-Mendoza, M. S., Vazquez-Olivo, G., & Heredia, J. B. (2018). Flavonoids and Phenolic Acids from Oregano: Occurrence, Biological Activity and Health Benefits. Plants, 7(1), 2. doi:10.3390/plants7010002

Capítulo VI "Cellular Antioxidant Activity and Anti-Obesity Properties of Oregano Polyphenols Under Simulated Gastrointestinal Digestion". Se demostró que los extractos polifenólicos de *Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri* son inhibidores de las enzimas α-glucosidasa, α-amilasa y lipasa pancreática, lo cual sugiere el potencial uso de extractos polifenólicos de orégano como agentes hipoglucémicos e hipolipemiantes. La capacidad inhibitoria de α-amilasa y lipasa pancreática se potencia durante un proceso de digestión gastrointestinal simulada, mientras que la inhibición de α-glucosidasa disminuye. Además, según las evaluaciones químicas y mediante líneas celulares (Caco-2), los extractos polifenólicos de *Hedeoma patens*, *Lippia graveolens* y *Lippia palmeri* son fuente de antioxidantes. El potencial antioxidante de estos extractos se mantiene estable durante el proceso de digestión gastrointestinal simulada.

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II. REVIEW: BIOAVAILABILITY OF DIETARY PHENOLIC COMPOUNDS

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Abstract

Phenolic compounds are ubiquitous in plant-based foods. High dietary intake of fruits, vegetables and cereals is related to a decreased rate in chronic diseases. Phenolic compounds are thought to be responsible, at least in part, for those health effects. Nonetheless, phenolic compounds bioaccessibility and biotransformation is often not considered in these studies; thus, a precise mechanism of action of phenolic compounds is not known. In this review we aim present a comprehensive knowledge of the metabolic processes through which phenolic compounds go after intake.

Keywords: phenolic, biotransformation, metabolism

Resumen

Los compuestos fenólicos son ubicuos en alimentos de origen vegetal. La alta ingesta de frutas, vegetales y cereales está relacionada con un bajo índice en padecimientos crónicos. Se cree que los compuestos fenólicos son, en parte, responsables de este efecto benéfico. Sin embargo, la bioaccesibilidad y biotransformación de los compuestos fenólicos generalmente no es considerada en este tipo de estudios. Por lo tanto, no se ha podido obtener un mecanismo de acción de los compuestos fenólicos. En este trabajo, presentamos una revisión de literatura de los procesos metabólicos a través de los cuales los compuestos fenólicos son sometidos después de ser ingeridos.

Palabras clave: fenólicos, biotransformación, metabolismo

Introduction

Phenolic compounds constitute a large and an important group of phenylpropanoids produced by plants as secondary metabolites. Phenolic compounds have an aromatic ring and several hydroxyl groups attached to it. Phenolic compounds can classified into different groups. They are grouped as a function of the number of phenolic rings that they contain and the radicals that bind these rings to another one [1,2].

Phenolic compounds have received considerable attention because their dietary intake is related to lower incidence of chronic diseases, such as cancer, diabetes, Alzheimer's disease and cardiovascular diseases. Cereals, fruits, and vegetables are rich sources of phenolic compounds. In fact, the health benefits of their dietary intake have been related, at least in part, to their phenolic compounds content [3].

Factors Affecting The Bioavailability of Dietary Phenolic Compounds

In nutrition science, bioavailability is defined as the fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological action. Bioavailability depends on proper absorption, release of a dosage form and presystemic elimination. Therefore bioavailability also depends on the route of administration and dosage form used, but can vary from one individual to another [4,5].

Bioavailability is related to other two concepts bioaccessibility and bioactivity. In this sense, bioaccessibility is described as the amount of any food constituent that is released from the food matrix, detectable in the gut, and that may be able to pass through the intestinal barrier [6]. This is very important because only the compounds that are released from the food matrix or absorbed in the small intestine are potentially bioavailable and bioactive [7].

Furthermore, it was recently proposed that once a compound is absorbed it is inevitably bioactive, therefore it was suggested that the concept of bioaccessibility

includes bioactivity [8]. Nonetheless, it is important to note that the fact that a compound is bioaccessible does not always imply it is bioactive.

It is important to mention that bioavailability is influenced by phenolic structure, food processing and matrix, host, among others; besides all this factors can interact with each other and influence phenolic compounds bioavailability, which makes harder to define the exact mechanisms of action of phenolic compounds . Nonetheless, in this work we will focus on glycosylation and food matrix.

Glycosylation

Phenolic compounds exist as free aglycones and glycoside forms, the last ones can be as O-glycosides or as C-glycosides, with a number of sugars, glucose is the most commonly encountered, followed by galactose, rhamnose, xylose and arabinose, while mannose, fructose, glucuronic and galacturonic acids are unusual [9 10].

Aglycones and polyphenols bound to glucose, galactose or xylose are absorbed in the small intestine after deglycosylation by β-glucosidase and lactase phlorizin hydrolase [11], these enzymes releasing the aglycone within the intestinal lumen for absorption by a diffusion mechanism. Phenolic compounds bound to rhamnose must reach the colon to be hydrolyzed by bacterial ramnosidases prior to its absorption [12].

Flavonoids bound to sugars as ß-glycosides are considered non-absorbable, only aglycones are able to pass through the gut wall. The major sites of flavonoid metabolism are the liver and the colonic flora. In the liver occurs O-methylation, sulphation and glucuronidation of hydroxyl groups improving flavonoid absortion; moreover, flavonoid glycosides are hydrolysed only by colon microorganisms, after this they can be absorbed [13].

Most of *in vivo* studies show gastric absorption of aglycones as quercetin and daidzein, while glycosides are poorly absorbed [12]. However, Hollman and Katan observed that quercetin glycosides from onions were absorbed far well than the pure

aglycone. Isoflavones aglycones are absorbed in the stomach, while their glycosides are absorbed in the intestine [11].

Within the glycosylated polyphenols, anthocyanins appear to be an exception, since the predominant forms in blood are their intact glycosides. Some authors have suggested the existence of a specific mechanism of anthocyanins absorption at the gastric level, which could involve transport via gastric bilitranslocase [12 14 15]

Food Matrix

The biological properties and bioavailability of some polyphenolic depends largely on their release from the food matrix and their subsequent interaction with target tissues. Today, the food matrix is considered as the factor most decisive in the bioavailability and absorption of dietary polyphenols [16].

Most cereal phenolics have covalent interactions with glycosides from the cell wall, forming ester linkages which are not hydrolysed by Phase I and II biotransformation enzymes, thus limiting their release into the colon to be metabolized by intestinal microbiota [17 18]. Such interactions depend on the specific porosity and surface properties of the cell wall that can measure between 4 and 10 mm diameter which restricts the penetration of molecules with high molecular weight polyphenols (>10 kDa) [19]. These bound phenolics are also denominated conjugated.

Free and some conjugated phenolic acids are thought to be readily available for absorption in the human small and large intestines [12 20], however, those covalently bound to indigestible polysaccharides can only be absorbed after being released from cell structures by digestive enzymes or microorganisms in intestinal lumen [20 21]. The bound phenolic acids have very low bioavailability because the bran matrix severely hinders their access to the necessary enzymes (such as ferulate esterases, xylanases) that contribute to their release in the human gastrointestinal tract [18 21 22].

In addition, during the mastication of plant foods, the cells are disrupted and polyphenols are released from the cell; this can cause phytochemicals interact with

components of dietary fiber as cellulose, hemicellulose and pectin, which affects bioavailability by increasing or decreasing it [23-25].

Furthermore, some phenolic acids such as chlorogenic and caffeic acids, can form interactions with proteins, however, these interactions proved to be slightly disrupted during an *in vitro* digestion process and does not affect its bioavailability and absorption [26].

Biotransformation Of Dietary Phenolic Compounds

Biotransformation of xenobiotic compounds is the process of converting lipophilic chemicals into hydrophilic chemicals; thus, making the readily absorbed compounds into readily excreted compounds. Exceptions to this process are acetylation and methylation that can decrease the water solubility of certain xenobiotics. Phenolic compounds are categorised into xenobiotic compounds because humans do not produce them. Therefore, they undergo through xenobiotic biotransformation reactions [27 28].

Xenobiotic biotransformation reactions are divided into four categories 1) hydrolysis, 2) reduction, 3) oxidation and 4) conjugation. Nonetheless, oxidation and conjugation reactions are the most important in dietary phenolic studies. These reactions can occur whether in the cytosol or cellular organelles such as microsomes or mitochondria, and the main tissues in which these processes occur are small intestine and liver [27].

The structure of most dietary phenolic compounds reaching the peripheral circulation and tissues is different from the structure of those present in foods, this due to the metabolism to which they are subjected after intake [11 29-31].

Some enzymes involved in these processes are phenol-sulfotransferases, β-glucosidase, lactase phlorizin oxidase and UDP-glucuronosyl transferases. The absorption and bioavailability of phenolic compounds depends largely on their metabolism in the small intestine. Moreover, only compounds that are not absorbed in the stomach and small intestine are degraded by the colonic microbiota [29].

Nevertheless, during mastication and gastric digestion, the structure and interactions of phenolic compounds with food matrix may be modified. This can diminish or improve their bioaccessibility in the small intestine [29 32].

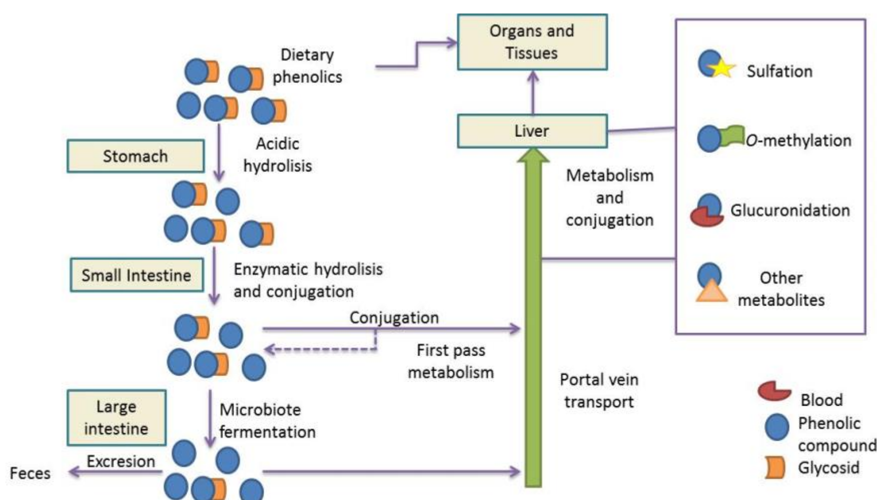

Figure 1. Schematic simplified representation of the metabolic pathway of plant phenolics in humans after ingestion. Adapted from [33]

The resultant metabolites of these conjugation reactions are transported through blood bound mainly to albumin and distributed to different tissues and organs. The fraction of phenol compounds not absorbed in the small intestine and that reaches the large intestine is metabolized by colonic bacteria. The action of colonic bacteria involves releasing aglycones and breakage of simple aromatic rings which may be absorbed after processing in benzoic acid derivatives and conjugation with glycine molecules, glucuronic acid or sulfate [12 34].

Conjugation of hydroxycinnamic acids *in vivo* has implications for the bioactivity of these compounds. Because the antioxidant capacity of hydroxycinnamic acids is given by the presence of free hydroxyl groups and these are the main sites of glucuronidation and sulfation [35].

Phase I Metabolism of Dietary Phenolic Compounds

The phase I biotransformation reactions are oxidation, reduction and hydrolysis. These reactions may increase, decrease or nullifying the biological activity of phenolic compounds [27].

Reactions of phase I aim at changing the structure of the xenobiotic molecules. This modification is achieved by the introduction of hydroxyl functional groups, amino, carboxyl, among others. The purpose of this process is to increase the polarity of the xenobiotic phenolic compound for easy excretion [27].

Hydrolysis

Hydrolytic reactions mainly target functional groups as carboxylic acid ester, amide, lactone and others. One of the major hydrolytic enzymes in mammals are carboxylesterases. Nevertheless, aldehyde dehydrogenases, carbonic anhydrases, carboxypeptidases, lipases and proteases have shown to have hydrolytic activity [27].

The enzyme lactase-phlorizin hydrolase (LPH) is found in humans. It is present mostly in the luminal side of the enterocytes of the small intestine. LPH hydrolyses lactose to glucose and galactose. It has also been reported that LPH can hydrolyse flavonoid-O-β-D-glycosides; this process causes a decreased polarity in the resulting aglycones, which can increase flavonoids cellular absorption [36 37]. Nevertheless, the activity of LPH can be inhibited by stearic factors and it may not hydrolyse glycosides like rhamnosides [29].

Oxidation

Oxidative reactions are the most important of the phase I biotransformations of phenolic compounds. These reactions are mainly mediated by the enzymatic oxidative system regulated by microsomal cytochrome P-450 (CYP450). Human CYP450 is an enzyme with a broad spectrum of substrates. CYP3A4, a subfamily enzyme of CYP450 is involved in the metabolism of xenobiotics in gut. It has been reported that CYP3A4

interacts with dietary phenolic compounds. This suggests that co-administration of drugs and phenolic compounds may stimulate some toxicity consequences [38].

The metabolic action of CYP450 on phenolic compounds depends largely on their functional groups, molecular weight, stereostructure, glycosylation, polymerization, and conjugation with other phenolic compounds [38 39]. Moreover, flavonoids rich in hydroxylic groups are less likely to be metabolized by CYP450; paradoxically, tea catechins (flavonoids rich in hydroxyl groups) are reported to inhibit CYP450 [40].

Nevertheless, phenolic compounds metabolism is still under-studied. But the potential health benefits of phenolics urges more studies on the subject.

Phase II-Metabolism of Dietary Phenolic Compounds

Phase II biotransformation reactions include the addition of various chemical radicals to xenobiotic compounds. The transferred radicals are derived from endogenous, polar and high availability molecules in the body. The ultimate purpose of this process is to increase the polarity of the xenobiotic molecules. This increased polarity facilitates the excretion of xenobiotics through urine [27 30].

The enzymes involved in phase II metabolism of dietary polyphenols are uridine-5'-diphosphate-glucuronosyl transferase, sulfotransferases and catechol-O-methyltransferase. The resultant molecules are conjugated with sulfate, glucuronide and/or methylation groups [27 30].

Phenolic-conjugated compounds differ from the parental molecule in size, polarity and ionic form. Therefore, the physiological behaviour of conjugated molecules is different from the native compound. Hence, there is an increasing need in knowing the possible contribution on health of these compounds. One way to achieve this is using phenolic-conjugated compounds in *in vitro* studies [30].

Glucuronidation

The process of glucuronidation is the main conjugation reaction in humans. Glucuronidation incorporates to xenobiotic compound the glucuronic acid molecule through the substrate UDP-glucuronic acid (UDPGA). The reaction may also utilize UDP-glucose, UDP-xylose and UDP-galactose as a substrate [27]. The enzyme responsible for catalysing glucuronidation process is UDP-glucuronosyl transferase. This enzyme is found in the microsomal fraction of tissues as liver, kidney, skin, brain and small intestine [41].

The site of glucuronidation is an electron rich nucleophilic heteroatom O, N or S. Therefore, the substrates for glucuronidation contain functional groups such as aliphatic alcohols and phenols (forming ethers O-glucuronide) [42]. Therefore, glucuronidation is one of the main conjugation reactions of phenolic compounds metabolism in humans [43].

Steffen et al. (2008) reported that glucuronide metabolites of (–)-epicatechin bind to a lesser degree to serum albumin than their aglycone counterpart, therefore, this can increase their absorption in enterocytes through β-glucuronidase or LPH activity. Moreover, aglycones lipophility is higher than flavonoids glycosides, therefore they can be more readily absorbed. Additionally, it would be interesting to study glucuronidation of phenolic acids and its effect on their bioaccessibility and bioactivity [44].

Acetylation and Methylation

The acylated flavonoids such as epicatechin and epicatechin gallate are absorbed without prior hydrolysis or deconjugation [11]. Studies have shown that approximately 50% of the amount of (–)-epicatechin that reaches the intestinal cells are absorbed with a percentage of metabolites (especially sulfate conjugates) eliminated by efflux into the intestinal lumen, and there exist a relatively modest elimination of (–)-epicatechin by

bile, also have been observed a potential absorption of (–)-epicatechin and elimination by efflux in another segment of the gut lumen [45].

Methylation differs from other conjugation reactions because it generally decreases hidrosolubility of phenolic compounds, also it masks functional groups that may be targeted by other conjugating enzymes [27].

As previously stated, flavonoids are mainly glucuronidated, nevertheless, methylated metabolites have also been detected, for example, perfusion studies with catechin or epicatechin have found O-methylated and O-methylated-glucuronide forms. This process is thought to be mediated by catechol-O-methyl-transferase (COMT) [46 47].

The O-methylation of flavonoids is a natural xenobiotic transformation by the O-methyl transferases, they are high selective enzymatic systems in plants, microbes and mammalians [48]. Methylation of phenolic compounds significantly increases their ability to be transported across biological membranes, making them more stable to metabolic changes, this can also increase their biological efficacy, particularly its antitumor activity. In this sense, O-methylated flavonoids exhibited a superior anticancer activity than the corresponding hydroxylated derivatives, being more resistant to the hepatic metabolism and showing a higher intestinal absorption [49]. Moreover, methylated flavonoids showed effects on transport proteins which play a central role in the defense of organism against toxic compounds (multidrug resistance proteins) [50]. It has been suggested that increasing the degree of methylation and decreasing the number of free hydroxyl groups that are available for conjugation with glucuronic acid and sulfate groups, stability and ability to transport across biological membranes is increased [51].

Conclusions

Several epidemiological studies relate a decreased rate of chronic diseases in population with high intake of fruits, vegetables and cereals. Phenolic compounds are a group of antioxidant phytochemicals that are present in those plant-based foods; a large number of studies relate health promoting effects of plant-based foods to phenolic compounds. Nevertheless, there is a lack of knowledge on the metabolism of these

compounds; these have led to a poor understanding of phenols mechanism of action. There is a need to go further into this kind of research in order to create better strategies to take advantage of phenols health promoting properties.

Conflict of interest

The authors declare no conflict of interest

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III. REVIEW: DIETARY PHENOLIC COMPOUNDS, HEALTH BENEFITS, AND BIOACCESSIBILITY

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Summary

Phenolic compounds are ubiquitous in plant-based foods. High dietary intake of fruits, vegetables and cereals are related to a decreased rate in chronic diseases. Phenolic compounds are thought to be responsible, at least in part, for those health effects. Nonetheless, the bioaccessibility of phenolic compounds is not often considered in these studies; thus, a precise mechanism of action of phenolic compounds is not known. In this review, we aim to present a comprehensive knowledge of the potential health promotion effects of polyphenols and the importance of considering the factors that affect their bioavailability on research projects.

Keywords: phenolic compounds, biotransformation, metabolism

Resumen

Los compuestos fenólicos son ubicuos en alimentos de origen vegetal. La alta ingesta de frutas, vegetales y cereales está relacionada con un bajo índice en padecimientos crónicos. Se cree que los compuestos fenólicos son, en parte, responsables de este efecto benéfico. Sin embargo, la bioaccesibilidad y biotransformación de los compuestos fenólicos generalmente no es considerada en este tipo de estudios. Por lo tanto, no se ha podido obtener un mecanismo de acción de los compuestos fenólicos. En este trabajo, presentamos una revisión de literatura del potencial benéfico de los compuestos fenólicos y cómo diversos factores pueden afectar su absorción y metabolismo.

Palabras clave: compuestos fenólicos, biotransformación, metabolismo

Introduction

In recent years, there has been an increased awareness of the effect of food on health, thus leading to a rise in the consumption of fruit, vegetables, and cereal-based food. Many studies have approached the bioactive properties of bioactive compounds such as phenolic compounds. Nonetheless, bioactive claims are made without taking into consideration the further modifications to which phenolic compounds are subjected once ingested. This study is a comprehensive review of the health claims and bioavailability of phenolic compounds.

Phenolic Compounds in Cereals, Fruits, and Vegetables

Phenolic compounds constitute a substantial and an important group of phenylpropanoids produced by plants as secondary metabolites. Plants synthesize them to function as a chemical defense against predators and to participate in reproduction as well as in plant-plant interference (1). Phenolic compounds have an aromatic ring and several hydroxyl groups attached to it. Phenolic compounds can be classified into different groups. They are grouped as a function of the number of phenolic rings that they contain and the radicals that bind these rings to another one (2).

Recently, phenolic compounds have received considerable attention because their dietary intake is related to lower incidence of chronic degenerative diseases, such as cancer, diabetes, Alzheimer’s disease and cardiovascular diseases. Cereals, fruits, and vegetables are rich sources of phenolic compounds. In fact, the health benefits of their dietary intake have been related, at least in part, to their phenolic compounds content.

Epidemiological studies have related dietary intake of phenolic-rich food with lower incidence in the appearance of several chronic diseases (3, 4). In this section of the review, we will discuss the epidemiological evidence that supports phenolics health benefits.

Phenolics in Cereals and Their Relationship With Health

Phenolic compounds are among the health-promoting phytochemicals present in cereals. Phenolic compounds are receiving much attention because of their antioxidant properties. Phenolic acids and flavonoids are the most common types of phenolic compounds found in whole grains. In cereals, phenolic compounds can be present in the free or bounded form; bound phenolics are mostly attached to arabinosyl chains of cell wall arabinoxylans (5, 6). Most of these bound phenolic compounds are located in the aleurone layer, but can also found in seed and embryos (7-10).

Irakli, Samanidou (11) reported that phenolic acids such as coumaric, ferulic, gallic, hydroxybenzoic, vanillic, syringic, and sinapic acid are found in both, free and bounded form in durum wheat, bread wheat, barley, oat, rye, rice, corn, and triticale. In cereals, the free phenolic acids constitute a small portion of the total phenolic content while bound phenolic acids are the most predominant. In this case, it was proven that the total of bound phenolic acids constitutes from 88 % (rye) to 99.5 % (corn) of the total phenolic acids. Regardless phenolic acids being the most predominant in cereals, flavonoids are also present in grains. 20 genotypes of small grains cereals, including bread wheat, durum wheat, rye, hull-less barley, and hull-less oat were analyzed for total phenolic and flavonoid content. The highest content of these phytochemicals was found in hull-less barley, followed by hull-less oat, rye, durum wheat, and bread wheat. Nevertheless, monomeric phenolic compounds like catechin and epicatechin were only

detected in hull-less barley genotypes (12). So it is concluded that the phenolic composition depends greatly on the type and variety of cereal.

It has been suggested that phenolic compounds play a significant role in the prevention of many chronic diseases due to their antioxidant, anti-inflammatory and anti-carcinogenic properties (7). In this sense, Hole, Grimmer (13) analyzed the anti-inflammatory action of ferulic, caffeic, ρ-coumaric and sinapic acids found in extracts of free and bound phenolic acids from oat, barley, and wheat flour by studying the modulation of NF-κB activity. NF-κB is a transcription factor involved in the regulation of pro-inflammatory genes that plays a critical role in the control of innate immunity processes and whose increased activation has been detected in several human cancers (14). The results of this study indicated that modulation of NF-κB activity exposed to cereal extracts containing phenolic acids is the result of phenolic acids synergic action. Moreover, a combination of ferulic, caffeic, ρ-coumaric and sinapic acid in low concentrations had a significant synergistic effect on NF-κB activity; while higher concentrations had better effect suppressing NF-κB activity (13). An important thing to remark is that in this study, the concentrations of extracts from cereal grains are similar to those found in the human diet, so their results increase the knowledge about the health-promoting effect of phenolic acids from cereal consumption.

Whole cereal grains are an excellent source of phenolic acids, and its consumption is associated with lower incidence of chronic diseases. Giacco, Costabile (15) reported that a diet based on whole-grain cereal products reduces postprandial insulin, and plasma triglyceride concentrations in individuals with metabolic syndrome, in 29 and 43 % respectively. The effects of whole-grain cereals on postprandial insulin and plasma triglyceride concentrations might explain the relationship between consumption of cereals and a reduced risk of type 2 diabetes and cardiovascular diseases. Nevertheless, the responsible compounds of these effects were not reported.

Phenolics in Fruits and Their Relationship With Health

Apples are one of the most popular fruits whose health benefits are attributed to phenolic compounds. The four polyphenol groups predominant in apples are flavan-3-ols, phenolic acids, dihydrochalcones and flavonols (16). Some phenolics such as chlorogenic acid, phloretin, epicatechin, quercetin and procyanidin B_2 have been identified as major antioxidants in apples (17). In this sense, some *in vitro* properties of apple polyphenols have been elucidated, among these: enhancement of glutathione S-transferases, reduced formation of H_2O_2, protection against oxidative-induced DNA damage, inhibition of intestinal glucose absorption (which could help against metabolic syndrome) (18-20).

Mango fruits contain several bioactive compounds, such as vitamins, carotenoids, terpenoids and phenolic compounds. Phenolic acids like gallic, protocatechuic, chlorogenic and vanillic acids are predominant in mango pulp (21). On this subject, Noratto, Bertoldi (22) reported the presence of hydrolysable tannins and mangiferin in mango pulp of different varieties. Additionally, mango peel is a rich source of these bioactive compounds. Furthermore, gallic, protocatechuic, syringic and ferulic acids were phenolic acids identified in the bound phenolic fraction of mango peel dietary fiber. Among the bound flavonoids identified, kaempferol and quercetin were predominant, but traces of rutin were also present (23). Amongst the *in vitro* health properties of mango polyphenols, it has been suggested that they can inhibit adipogenesis (24), also, anti-cancer properties of mango extracts have been proven, this anti-cancer bioactivity was mainly attributed to polyphenolics compounds in mango (22, 25, 26); also, *in vivo* studies have shown anti-cancer and antioxidant capacity of mango (27).

Citrus fruits are rich in various nutrients, such as vitamins A and C, folic acid and dietary fiber. Furthermore, these fruits are a source of bioactive compounds, being cinnamic acid derivatives, coumarins, and flavonoids the major groups of phenolic compounds (28). Citrus fruit has considerable amounts of flavonoids like, flavones, flavonols, and anthocyanins; however the main flavonoids are flavanones, which the most frequently found are hesperidin, naringin, narituring and eriocitrin (29, 30). Other phenolics often found in citrus are ρ-coumaric, ferulic, caffeic and sinapic acids (30).

The daily consumption of grapefruit and orange juice has shown to decrease diastolic blood pressure (31, 32)

It is always important to remember that phenolic composition and concentration are dependent on the variety and ripeness stage of the fruit, as of the part of the fruit that is being analyzed.

There is epidemiological and experimental evidence that consumption of fruits has a positive effect on health; this effect has been, in part, attributed to their content of phenolic compounds (33-36). In this regard, McCann, Gill (37) investigated the ability of a phenolic extract of apple waste (material left after juice extraction) to affect a range of colon cancer biomarkers, namely: DNA damage, colonocyte barrier function, cell cycle progression and invasion *in vitro* using HT29, HT115 and CaCo-2 cell lines as models. Flavan-3-ols, phloretin glycosides, quercetin glycosides, cyanidin glycoside and hydroxycinnamic acids were among the phenolic compounds present in apple phenolic extracts. Phenolic compounds from apples proved to help to decrease DNA damage in HT29 cells significantly, to enhance the colonic barrier function of Caco-2 cells and to reduce the invasive potential of HT115 cells. These authors concluded that apple consumption may serve to protect against colon cancer by protecting gut cells against DNA damage and abnormal extracellular behavior. Additionally, some studies relate apple consumption to lower plasma cholesterol and reduction of risk of cardiovascular disease (38).

Studies on yuzu (*Citrus junos* Siebold ex Tanaka) show that peel and pomace exerted anti-obesity effects in zebrafish with diet-induced obesity. Yuzu peel suppressed the rise in plasma triacylglycerol and liver lipid accumulation (39). Yuzu peel and pomace are rich in flavonoids, such as hesperidin, naringin, and eriocitrin that are recognized to have the ability to lower total blood cholesterol (40). In this sense, a recent study by Wu, Jiang (41), high-fat-diet-induced obese mice were fed from 50-200 mg/kg of blueberry anthocyanins, found that supplementation at high dose of anthocyanins decreased serum glucose, attenuated epididymal adipocytes, improved lipid profiles and down-regulated expression levels of inflammation-related genes TNFα, IL-6 PPARγ and FAS; their results suggest that anthocyanins could help to reduce obesity.

Phenolics in Vegetables and Their Relationship With Health

Phenolic acids and isocoumarins were the predominant phenolics in carrots (42, 43). Among the most common phenolic compounds found in vegetables are flavonoids, phenolic acids and isocoumarins. For example, most of the compounds detected in black carrot roots and black carrot juice are composed of ρ-coumaric, caffeic and ferulic acids; 5-caffeoylquinic acid was the predominant phenolic acid. Besides, some phenolic glycosides like dihydroxybenzoic acid hexoside and quercetin-3-*O*-galactoside were detected (43). Alasalvar, Grigor (44) examined the phenolic content of carrots of four different colours: orange, purple, yellow and white. The four colored carrots contained mainly hydroxycinnamic acid derivatives, namely 3'-caffeoylquinic acid, 5'-caffeoylquinic acid, 3'-ρ-coumaroylquinic acid, 3'-feruloyquinic acid, 3',4'-dicaffeoylquinic acid, 5'-feruloyquinic acid, 5'-ρ-coumaroylquinic acid, 4'-feruloylquinic acid, 3',5'-dicaffeoylquinic acid, 3',4'-diferuloylquinic acid and 3',5'-diferuloylquinic acid. Anthocyanin content, a type of flavonoids, has also been reported in carrots. The anthocyanins found in purple carrots roots are cyanidin-3-xylosyl (glucosyl)-galactoside, cyanidin-3-xylosylagalactoside, cyanidin-3-xylosyl-(sinapoylglucosyl)-galactoside, cyanidin-3-xylosyl (feruloylglucosyl)-galactoside, cyanidin-3-xylosyl (coumaroylglucosyl)-galactoside, pelargonidin-3-xylosyl (feruloylglucosyl)-galactoside and peonidin-3-xylosyl (feruloylglucosyl)-galactoside (45).

In this sense another vegetable that has been widely studied are tomatoes, which are a key component in the Mediterranean diet and its dietary intake is associated with lower risk of chronic degenerative diseases, such as cancer and cardiovascular diseases (46). Vallverdú-Queralt, Regueiro (47) conducted an extensive study to identify the number of phenolic compounds extracted from tomato samples. They identified a total of 38 phenolic compounds, among which gallic acid, protocatechuic acid, caffeic acid derivatives, ferulic acid derivatives, kaempferol, rutin, naringenin, phloridzin, and quercetin were present.

Lettuce might be relevant as a dietary source of phenolic compounds. Several phenolics were identified in five varieties of lettuce (iceberg, romaine, continental, red oak leaf and lollo rosso). The phenolic compounds identified were 5-*O*-caffeoylquinic acid, caffeoylmalic acid, dicaffeoyltartaric acid, and 3,5-dicaffeoylquinic acid, flavonoid-malonyl glycosides (quercetin-3-malonylglucoside-7-glucuronide, quercetin-3-malonylglucoside-7-glucoside, quercetin-3-malonylglucoside) and flavonoid glycosides (quercetin-3-glucuronide, quercetin-3-glucoside, quercetin-3-rutinoside, luteolin-7-glucuronide, and luteolin-7-glucoside, luteolin-7-rutinoside) groups. Caffeic acid derivatives were the main phenolic compounds in green varieties; while flavonols were present in red varieties in higher quantities; besides, anthocyanins were present only in red-leafed varieties (48).

Onions, spinach and pepper fruits are a rich source of flavonoids (5). A blended mix of juice and pulp of carrot, parsley, beet, kale, broccoli, cabbage, tomato, and spinach, as well as sugar beet fiber, garlic powder, oat bran fiber, and rice bran was analyzed in order to identify the phenolic composition of this vegetable mix. Among the variety of phenolic compounds found, flavonols were the most abundant. A total of 28 compounds were identified which belong to the dihydrochalcones, flavone, flavonols, hydroxycinnamic acids, lignans and glucosinolate groups (49).

Reactive oxygen species (ROS) is a collective term that describes O_2-derived free radicals, such as superoxide anion ($O_2\bullet^-$), hydroxyl (HO•), peroxyl ($RO_2\bullet^-$), and alkoxy (RO•) radicals; O_2-derived nonradical species such as hydrogen peroxide (H_2O_2) are also included. Reactive species of oxygen (ROS) are relevant mediators and influential factors in the development of colorectal cancer (50). Phenolic compounds with antioxidant potential are shown to play an important role in modulating the ROS level in the intestinal contents. Olejnik, Kowalska (45) studied the effect of digested purple carrot extract, rich in anthocyanins, on ROS generation and oxidative DNA damage in colon cells. Digested purple carrot extract exhibited intracellular ROS-inhibitory capacity, with 1 mg/mL showing the ROS clearance of 18.4 %. Digested purple carrot extract showed a 20.7 % reduction in oxidative DNA damage in colon mucosa. These findings indicate that purple carrot extract is capable of colonic cells protection against

the adverse effects of oxidative stress. For this reason, phenolics contained in purple carrot extract may have a protective capacity of colonic cells (45).

Treatment of rats with CCl_4 plus lettuce extract ameliorated the toxic effects of CCl_4. This plant contains flavonoids that scavenge the oxidative damage to different cells and organs. These results demonstrate that ethanol lettuce extract treatment increases the antioxidants defense mechanism against CCl_4-induced toxicity and provides evidence that it may have a therapeutic role in free radical mediated diseases (51).

Nevertheless, sole dietary consumption of plant-based foods allows us to utilize fully the phenolic compounds present in those foods. There are a lot of factors that affect the bioactivity of phenolic compounds present in plant foods. This subject will be further discussed.

Dietary Phenolic Compounds

Phenolic compounds are derived from the secondary metabolism of plants. Phenolics are chemical compounds that have at least one aromatic ring to which one or more hydroxyl groups are bonded to aromatic or aliphatic structures (52). There is a wide variety of phenolic compounds. Nonetheless, this study will focus only in 2 groups:

1) Flavonoids, composed of two aromatic rings linked through an oxygen heterocycle and depending on the degree of hydrogenation (Figure 2) and the replacement of the heterocycle they can be sub-classified as flavonols, flavones, isoflavones, anthocyanins, proanthocyanidins, flavanones, etc. Flavonoids are mostly found in the form of glycoside, the main sugars to which they are linked are glucose, rhamnose, galactose and xylose.
2) Non-flavonoids, like benzoic and cinnamic compounds, which are commonly called phenolic acids, these contain an aromatic ring that can be attached to different functional groups or esterified to organic acids (Figure 3). Some other phenolic compounds are stilbenes, tannins, lignins, and lignans. Properties, like color, flavour and astringency are caused by the presence of such compounds.

3)

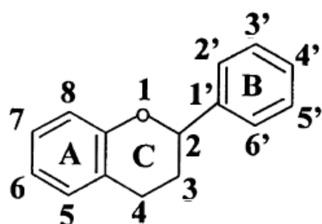
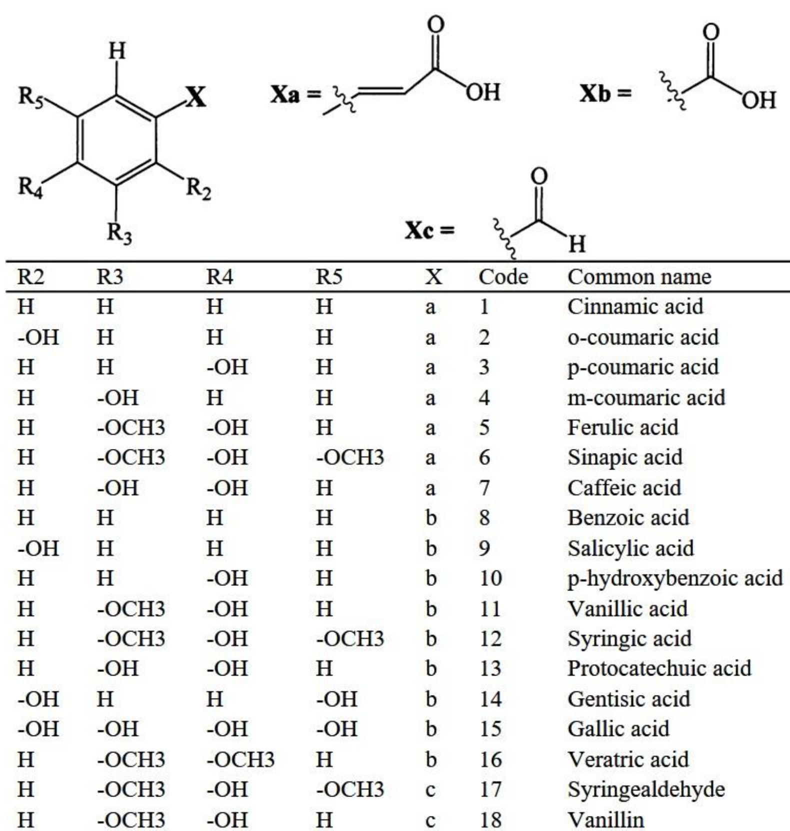


Figure 2. Graphic representation of flavonoids common structure (40)



R2	R3	R4	R5	X	Code	Common name
H	H	H	H	a	1	Cinnamic acid
-OH	H	H	H	a	2	o-coumaric acid
H	H	-OH	H	a	3	p-coumaric acid
H	-OH	H	H	a	4	m-coumaric acid
H	-OCH3	-OH	H	a	5	Ferulic acid
H	-OCH3	-OH	-OCH3	a	6	Sinapic acid
H	-OH	-OH	H	a	7	Caffeic acid
H	H	H	H	b	8	Benzoic acid
-OH	H	H	H	b	9	Salicylic acid
H	H	-OH	H	b	10	p-hydroxybenzoic acid
H	-OCH3	-OH	H	b	11	Vanillic acid
H	-OCH3	-OH	-OCH3	b	12	Syringic acid
H	-OH	-OH	H	b	13	Protocatechuic acid
-OH	H	H	-OH	b	14	Gentisic acid
-OH	-OH	-OH	-OH	b	15	Gallic acid
H	-OCH3	-OCH3	H	b	16	Veratric acid
H	-OCH3	-OH	-OCH3	c	17	Syringealdehyde
H	-OCH3	-OH	H	c	18	Vanillin

Figure 3. Graphic representation of the common structure of phenolic acids

In recent years, phenolic compounds have been of increasing interest to science and industry for their beneficial health effects, especially because of its antioxidants properties. Halliwell and Gutteridge (53) defined an antioxidant as “any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate”. An antioxidant should also

have the ability that after scavenging the radical, to form a new radical that is stable enough intramolecular hydrogen bonding on further oxidation (54). Free radicals, in turn, are molecules with an unpaired electron and seek electrons from other molecules to gain stability. Molecules as proteins, lipids or DNA, are known to function as a target for these free radicals, which may lead to a deteriorative process called oxidation (2, 55).

Antioxidant activity of phenolic compounds is attributed to their capacity to act as reducing agents to free radicals. Some common phenolic compounds found in nature are flavonoles, flavones, isoflavones, anthocyanins, flavonones, chatequines and proanthocyanidins (2, 35, 55). Additionally, the potential phenolic compounds has also been attributed, at least partially, to its anti-inflammatory properties (56).

The main factor on antioxidant activity of phenolic compounds is its number and position of hydroxyl groups. Flavonoids possess more hydroxyl groups thus present higher antioxidant activity.

Moreover, the solubility and stearic effects of each molecule may be affected by the structure the molecule; for example, the presence of glycosylated derivatives of other adducts, can increase or decrease the antioxidant activity of phenolic compounds. Flavonoid compounds are commonly present in plants as glycosides, but can be released by the action of enzymes to its corresponding aglycone. The antioxidant activity of phenolic acids is also based on the binding of these compounds to organic acids and sugars. The mechanisms by which these compounds act may vary depending on the concentration and types of compounds present in foods (52).

Factors Affecting The Bioavailability of Dietary Phenolic Compounds

A balanced diet provides many different phenolic compounds. Thus their bioavailability may vary, besides the diet changes in every country and every season. To consider phenolic compounds nutraceutical potential it is important to know how much of a phenolic is present in specific food or dietary supplement; also, it is necessary to know how much of it is bioavailable.

Bioavailability is defined as that fraction of an ingested nutrient or compound that reaches the systemic circulation and the specific sites where it can exert its biological action. Bioavailability depends on proper absorption, the release of a dosage form and presystemic elimination. Therefore, bioavailability also depends on the route of administration and dosage form used, but can vary from one individual to another, especially when factors that alter the absorption (57).

Bioavailability is related to other two concepts: bioaccessibility and bioactivity. In this sense, bioaccessibility is described as the amount of any food constituent that is released from the food matrix, detectable in the gut, and that may be able to pass through the intestinal barrier (21). This is crucial because only the compounds that released from the matrix or absorbed in the small intestine are potentially bioavailable and bioactive (Figure 4) (58).

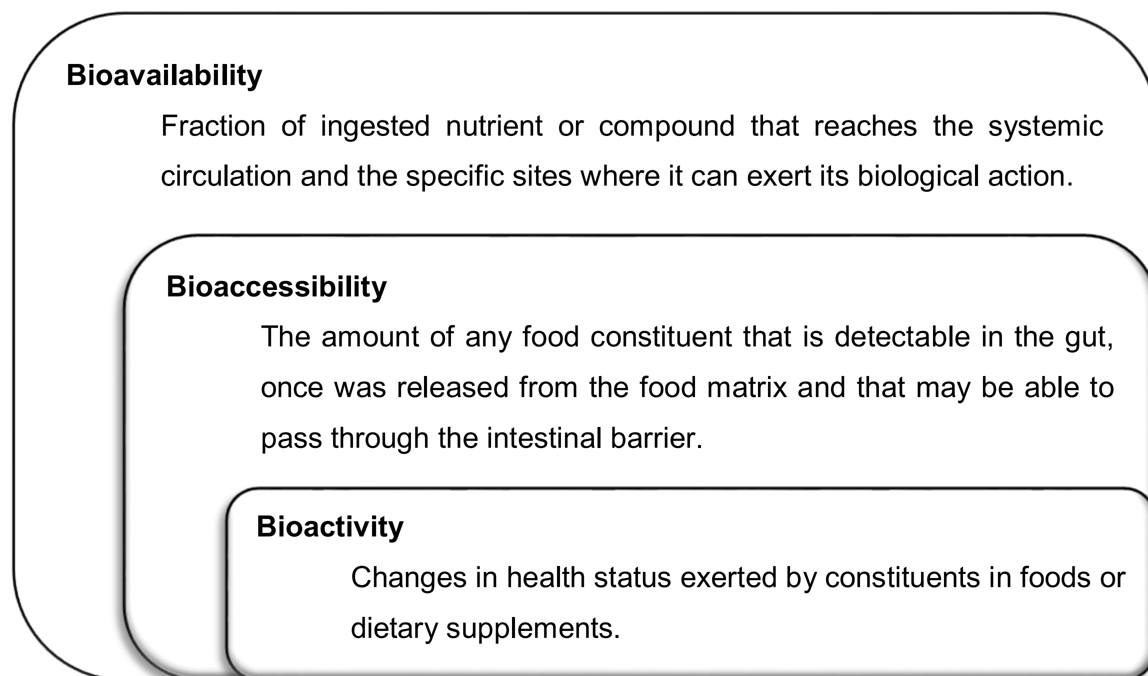


Figure 4. Differences between bioavailability, bioaccessibility and bioactivity concepts.

Furthermore, recently it was proposed that once a compound is absorbed it is inevitably bioactive, because of this was suggested that the concept of bioavailability

includes bioactivity (59). Nonetheless, it is important to note that the fact that a compound being bioavailable does not always imply its bioactive (Figure 4).

Phenolic bioavailability varies over a wide range from 0.3% estimated for anthocyanins to 43% in the case of isoflavones (55). In this sense, bioavailability is influenced by phenolic structure, food processing and matrix, host, among others; besides all these factors can interact with each other and influence phenolic compounds bioavailability (Table 1).

Table 1. Factors that can affect dietary phenolic compounds bioavailability

Factors related to phenolics	Chemical structure	Chemical structure; solubility; bond with sugars (glycosides), methyl groups, etc.; stereoconfiguration.	(52, 55, 60)
	Interaction with other compounds	Bonds with proteins (*i.e.*, albumin) or with polyphenols with similar mechanism of absorption.	(23, 76, 81, 82, 103)
Factors related to Food	Food processing	Thermal treatments; liophylization; cooking and methods of culinary preparation; storage.	(3, 83, 84)
	Food interaction	Food matrix; presence of effectors of absorption (positive or negative) (i.e., fat, fiber).	(74, 75)

Factors related to Host	Dietary intake	Differences between countries and seasons; quantity and frequency of exposure, single or multiple dose.	(33, 87, 88)
	Absortion and metabolism	Intestinal factors (i.e., enzyme activity; intestinal transit time; colonic microflora). Systemic factors (i.e., gender and age; disorders and/or pathologies; genetics; physiological condition)	(20, 88-92, 95)
Others	Distribution and Food Content	Exclusivity in some foods (i.e., soy isoflavones, flavanones in citrus, etc.).Ubiquity (i.e., quercetin).	(98, 100)
	External factors	Environmental factors (*i.e.*, different stress conditions, degree of ripeness).	(102)

Factors Related to Phenolic Chemical Structure

Solubility

The degree of solubility is given by the chemical structure of a molecule. It has been observed in both *in vivo* and *in vitro* studies, using phenolic compounds with different solubility, that there is variation in susceptibility to digestion, fermentation, and absorption from the gastrointestinal tract (52). These lead to suggest a classification of phenolic compounds that distinguishes between extractable and non-extractable phenols.

Extractable polyphenols have low-intermediate molecular mass, and they can be extracted using different solvents such as water, methanol, aqueous acetone, etc. Aamong the major extractable polyphenols, we can find some hydrolysable tannins and proanthocyanidins. In the other hand, nonextractable polyphenols are high molecular weight compounds that are mostly bound to dietary fiber or protein that remains insoluble in the usual solvent and requires an extra step of hydrolysis during extraction to make them soluble and bioavailable (60). Also, it has been observed that the specific absorption of various extractable phenolics, depends on their extractability with different solvents (61).

Glycosylation

Phenolic compounds exist as free aglycones and glycoside forms, the last ones can be as O-glycosides or as C-glycosides, with a number of sugars, glucose is the most commonly encountered, followed by galactose, rhamnose, xylose and arabinose, while mannose, fructose, glucuronic and galacturonic acids are unusual (62).

Aglycones and polyphenols bound to glucose, galactose or xylose are absorbed in the small intestine after deglycosylation by β-glucosidase and lactase phlorizin hydrolase (4), these enzymes releasing the aglycone into the intestinal lumen for absorption by a diffusion mechanism. Phenolic compounds bound to rhamnose must reach the colon to be hydrolysed by bacterial ramnosidases before its absorption (55).

Flavonoids bound to sugars as ß-glycosides are considered non-absorbable, only aglycones can pass through the gut wall. The major sites of flavonoid metabolism are the liver and the colonic flora. In the liver occurs O-methylation, sulphation, and glucuronidation of hydroxyl groups improving flavonoid absorption; moreover, flavonoid glycosides are hydrolysed only by colon microorganisms, after this they can be absorbed (63).

Most of *in vivo* studies show gastric absorption of aglycones as quercetin and daidzein while glycosides are poorly absorbed (55). However, Hollman and Katan (64)

observed that quercetin glycosides from onions were absorbed far well than the pure aglycone. Isoflavones aglycones are absorbed in the stomach while their glycosides are absorbed in the intestine (4)

Within the glycosylated polyphenols, anthocyanins appear to be an exception, since the predominant forms in blood are their intact glycosides. Some authors have suggested the existence of a particular mechanism of anthocyanins absorption at the gastric level, which could involve transport via gastric bilitranslocase (2, 55, 65).

Acetylation and Methylation

The acylated flavonoids such as epicatechin and epicatechin gallate are absorbed without prior hydrolysis or deconjugation (4, 66). Studies have shown that approximately 50% of the amount of (–)-epicatechin that reaches the intestinal cells is absorbed, and a percentage of metabolites (especially sulfate conjugates) eliminated by efflux into the intestinal lumen, and there exists a relatively modest elimination of (–)-epicatechin by bile; it also has been observed a potential absorption of (–)-epicatechin and elimination by efflux in another segment of the gut lumen (67).

The O-methylation of flavonoids is a natural xenobiotic transformation by the O-methyl transferases, they are high selective enzymatic systems in plants, microbes, and mammalians (68). Methylation of phenolic compounds significantly increases their ability to be transported across biological membranes, making them more stable to metabolic changes also increase biological efficacy, particularly its antitumor activity. In this sense, O-methylated flavonoids exhibited a superior anticancer activity than the corresponding hydroxylated derivatives, being more resistant to the hepatic metabolism and showing a higher intestinal absorption (69). Moreover, methylated flavonoids showed effects on transport proteins which play a central role in the defense of organism against toxic compounds (multidrug resistance proteins) (70). It has been suggested that increasing the degree of methylation and decreasing the number of free hydroxyl groups

that are available for conjugation with glucuronic acid and sulfate groups, stability and ability to transport across biological membranes is increased (71).

Polymerization

Bioavailability and metabolism of monomeric phenols have been extensively studied, but little is known about the bioavailability of polymeric phenolics such as tannins, and the results are still controversial. Tannins bioactivity mostly depends on their grade of polymerisation and solubility. For example, highly polymerized tannins exhibit low bioaccessibility in the small intestine and low fermentability by colonic microflora (72).

Factors Related to Food

Food Matrix

The biological properties and bioavailability of some phenolics depends largely on their release from the food matrix and their subsequent interaction with target tissues. Today, the food matrix is considered as the factor most decisive in the bioavailability and absorption of dietary polyphenols (73).

Most cereal phenolics have covalent interactions with glycosides from the cell wall, forming ester linkages which are not hydrolysed by Phase I and II biotransformation enzymes, this limiting their release into the colon to be metabolized by intestinal microbiota (74, 75). Such interactions depend on the specific porosity and surface properties of the cell wall that can measure between 4 and 10 mm diameter which restricts the penetration of molecules with high molecular weight polyphenols (>10 kDa) (76). These bound phenolics are denominated conjugated.

Free and some conjugated phenolic acids are thought to be readily available for absorption in the human small and large intestines; however, those covalently bound to indigestible polysaccharides can only be absorbed after being released from cell structures by digestive enzymes or microorganisms in intestinal lumen. The bound phenolic acids have very low bioavailability because the bran matrix severely hinders their access to the necessary enzymes (such as ferulate esterases, xylanases) that contribute to their release in the human gastrointestinal tract(55, 77).

Also, during the mastication of plant foods, the cells are disrupted, and polyphenols are released from the cell; this can cause phytochemicals interact with components of dietary fiber as cellulose, hemicellulose, and pectin, which affects bioavailability, increasing or decreasing (78-80).

Furthermore, some phenolic acids such as chlorogenic and caffeic acids, can form interactions with proteins, however, these interactions proved to be slightly disrupted during an *in vitro* digestion process and does not affect its bioavailability and absorption (81, 82).

Food Processing

Food processing can either increase or decrease the phenolic content, it depends on the process. For example, milling processes have shown to increase the solvent-extractable phenolic content, because this process increase the specific surface area of the particle and thus increase the accessibility of phenolic compounds to extraction solvents (77). Thermal processing techniques such as steaming, autoclaving, drying, roasting, and microwave heating are widely used in cereal processing for improving sensorial characteristics, stability and safety of the products, also, some of these techniques have shown the potential to increase the extractability of phenolic compounds in the materials, especially autoclaving (increase ≈ 50%) (83).

On the other hand, some processes tend to decrease the extractable phenolic content. For example, extrusion cooking causes decomposition of heat-labile phenolic compounds (3).

The storage is included as a food process that can or cannot cause-effect, increase or decrease the phenolic content. Broccoli and lettuce were stored at modified atmospheres (argon, helium and nitrogen atmosphere containing 2% oxygen) during 9 days, the authors observed that the content of total phenolics was reduced in relation to the control sample (stored in air) (84). Oppositely, frozen red raspberries were stored at 4 °C for 3 days, and then at 18 °C for 24 h, after this, the authors observed that anthocyanin levels were unaffected while elligitannins increased (85).

Factors Related to Host

Dietary Intake

Phenolics dietary intake it is affected by the food availability, every country and seasons offer a different wide range of possibilities; besides, the quantity and frequency of exposure to particular compounds is determinant. Additionally, the diet of each host is diverse, because of this every host responds in a different way to phenolics. There are reports demonstrating that subjects with low circulating levels of antioxidants respond promptly to a rich phenolic diet. However, once plasma levels reach a certain concentration, there is no significant increase (86). This could be a defense mechanism of the human body homeostasis, avoiding accumulation in tissues where antioxidants might be potentially dangerous (87).

Absorption and Metabolism

Renouf et al. (88) performed a dose–response study with ten healthy subjects, measuring plasma bioavailability of coffee phenolics and their metabolites; they observed significant interindividual variability in plasma appearance, and this variability was greater for the most abundant metabolites. Multiple factors could explain this variability, from genetic background to gut microbial composition. However, they affirm that to identify the cause(s) of this variability of the metabolic fate of coffee metabolites remains very difficult.

Mechanism of Absorption of Phenolic Acids

Nowadays, the precise mechanism of absorption of phenolic compounds is being studied. In this study, the mechanism in which ferulic acid is absorbed will be explained as a model of all phenolic acids.

A study by Konishi and Shimizu (89), on the transepithelial transport of ferulic acid, using Caco-2 cell monolayers, reported that ferulic acid transport was dependent on pH, in the apical-basolateral direction, and that the permeation rate of ferulic acid was concentration-dependent; they also reported that ferulic acid uses various substrates for monocarboxylic acid transporters (MCTs); and it is suggested that other phenolic acids could be recognized and transported by MCT by intestinal absorption. In contrast, a study published by Poquet, Clifford (90) used co-cultured Caco-2 and mucus-producing HT29-MTX cells to study ferulic acid uptake, they found that ferulic acid was permeated by passive diffusion suggesting a transcellular transport. On the other hand, phenolic acids could have different transport mechanisms since they possess different chemical structures and properties. This is the case of rosmarinic acid, a study by Konishi and Kobayashi (91), reported that rosmarinic acid could be absorbed via paracellular, and that it could be further metabolized and degraded into m-coumaric and

hydroxylated phenylpropionic acids by gut microflora, and then absorbed and distributed by the MCT. In a similar way, the transport mode of ρ-coumaric acid and gallic acid, under a Caco-2 cell monolayer was studied. The authors reported that while the transepithelial transport of ρ-coumaric acid is via the MCT, the permeation of gallic acid appears to be via paracellular (92).

Additionally, once absorbed some phenolic acids may undergo through phase II metabolism. As it is the case of caffeic and dihydrocaffeic acids, both possess the catechol group, this makes it a target for sulfation at the 3-OH group by human liver S9, intestinal S9 and SULT1A1; the latter has been suggested as the most active enzyme in the sulfation of caffeic and dihydrocaffeic acids. Furthermore, glucuronidation can also occur in caffeic acid, UGT1A1 and UGT1A9 are the active enzymes reported in this process. While UGT1A1 catalyses the formation of caffeic acid-4-O-glucuronide, UGT1A9 conjugated the 3-OH and 4-OH groups of caffeic acid (93).

Moreover, *in vivo* studies

As it can be observed, the absorption of phenolic acids is not well known nor its mechanisms. The information on this subject is yet quite limited, and more studies are needed to understand the exact mechanisms of the most important phenolic acids in regard of their beneficial health effect.

Mechanism of Absorption of Flavonoids

In 2015, Actis-Goretta, Dew (94) reported a study on the absorption of hesperetin-7-O-rutinoside (hesperidin) using *in vivo* models (humans); they report that hesperidin was hydrolysed by brush border enzymes without involvement of pancreatic, stomach, or other enzymes, additionally no hesperidin metabolites were detected in blood, only amount traces were excreted in urine. In this sense, epicatechin is suggested to be absorbed in the jejunum and that its conjugation is a major determinant of the metabolic fate of epicatechin in the body (67). A study using Caco-2 cell monolayers found that quercetin and naringenin are poorly absorbed by Caco-2 cells; quercetin was absorbed

by passive diffusion and a pH-dependent mechanism mediated by the organic anion transporting protein B, and that intestinal permeability was higher for naringenin than for quercetin. It is also mentioned that, naringenin transport is somewhat ATP-dependent (95). Moreover, genistein absorption was studied by Liu and Hu (96) using Caco-2 cell monolayers and rats; in Caco-2 cells they reported that aglycones of genistein are 5 times more permeable than their corresponding glycosides; besides in rats, aglycones of genistein underwent phase II transformations of glucuronidation and sulfation.

The wide variety of flavonoids, their different molecular size, chemical structure, and chemical properties can vary the way in which they are absorbed, transported, metabolized and excreted. For this reason, it is highly important that the mechanisms of absorption are studied for each phenolic compound of interest, without assuming any general behaviour on their absorption and further metabolism.

Other Factors

Distribution and Food Content.

Plant polyphenol composition is highly variable both qualitatively and quantitatively; while some phenolics are ubiquitous, as quercetin, others are restricted to specific families or species (i.e. isoflavones in legumes, flavanones in citrus). Some phenolics as anthocyanins are very common, they can be found in all parts of the plant, although they are accumulated mostly in flowers and fruits, but are also present in leaves, stems and storage organs. In general, the level of anthocyanins in fruits is much higher than in vegetables, among fruits the richest in anthocyanins are various berries and black currants (97).

It has been observed that genetic factors, environmental conditions, and growth or maturation stages are determinant influences in phenolic content, causing large variations even within single species (52).

For example, in grapes and apples, anthocyanins are found only in the red varieties (because of the color) and accumulate toward the end of ripening. Pinot noir grapes contain only anthocyanin 3-glucosides, whereas other grape cultivars also contain acylated anthocyanins (98). Most of the phenolics in apples are hydroxycinnamic acids, flavanols and dihydrochalcones, these compounds are very common in most of the fruits, but dihydrochalcones like phloridzin and phloretin are unique to apples and apple trees (17, 38, 99).

In the case of tannins, hydrolysable tannins are characterized by a restricted taxonomic distribution and are mainly associated with dicotyledonous plants; it has also been observed that most of the plants that can synthesize hydrolysable tannins are unable to synthesize condensed and vice versa (100).

External Factors

There several factors related to polyphenolics production in plants, among this there are external factors as environmental conditions, UV light exposure, insect and pathogens attack. Plants responses to light UV radiation include increased content of phenolic compounds, such as of hydrolysable tannins, flavonol, and anthocyanin compounds. Flavonols have been implicated in providing photoprotection against UV irradiation through a screening function. Furthermore, the total phenol content increased proportionately with the dose of UV radiation (101). These increase in phenolic content is bigger especially with UV-C light, exposure of C. nivalis cells to UV-A light (365nm) for 5 days resulted in a 5–12% increase in total phenolics, whereas exposure to UV-C light (254 nm) led to a 12–24% increase in phenolics after 7 days of exposure (102).

Among all phenolic compounds, tannins have been major associated to insect attack. It has been observed that insect damage and wounding can have strong stimulatory effects on tannin production in some plants, suggesting that tannin synthesis contributes to induced defense by deterrence and/or toxicity (100, 103).

Conclusions

Epidemiological studies relate a decreased rate of chronic diseases in individuals with higher intake of phenolic-rich foods. Nevertheless, little is known about the biological activities of phenolic compounds that have gone through the digestion process and its relation to the factors affecting its bioaccessibility. In this regard, it has been argued that the phenolic compounds once ingested, are oxidized during digestion and lose their biological properties. On the other hand, epidemiological evidence suggests that consumption of polyphenol-rich foods reduces the incidence of chronic diseases.

There have been efforts to study phenolic's metabolism; nonetheless, the main limiting factor in this kind of research is the lack of chromatographic standards of each metabolite produced. Obtaining these standards may be difficult considering the wide spectrum of phenolic compounds that exist in nature and the high number of metabolites that are produced during digestion and metabolism.

Finally, it is recommended as highly critical that each factor that affects polyphenols bioavailability must be considered to be properly used in the pharmacological industry. In this sense, it is important to obtain the pharmacokinetic of phenolic compounds, their interaction with other drugs, interaction with other food constituents and their effective doses. Additionally, to create further dietary recommendations, it is relevant to understand phenolic compounds relationship with food matrix and how it affects their bioaccessibility.

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IV. EFFECT OF *IN VITRO* DIGESTION ON THE TOTAL ANTIOXIDANT CAPACITY AND PHENOLIC CONTENT OF THREE SPECIES OF OREGANO (*Hedeoma patens*, *Lippia graveolens*, *Lippia palmeri*)

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ABSTRACT: Oregano phenolic compounds have been studied for their anti-inflammatory properties. Nonetheless, after ingestion, the gastrointestinal environment can affect their antioxidant stability and thus their bioactive properties. To evaluate the effect of *in vitro* gastrointestinal (GI) digestion on the phenolic compounds of three species of oregano (*Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri*), the total reducing capacity, total flavonoid content, and antioxidant capacity were evaluated before and after *in vitro* GI digestion. In addition, the phenolic compounds of the 3 oregano species were identified and quantified by UPLC-PDA before and after *in vitro* GI digestion. It was shown that the reducing capacity, flavonoid content and antioxidant capacity were affected by the GI digestion process. Moreover, the phenolic compounds identified were apigenin-7-glucoside, scutellarein, luteolin, luteolin-7-glucoside, phloridzin and chlorogenic acid, and their levels were affected by the *in vitro* GI process. Our results showed that the phenolic compounds from these three species of oregano are affected by the *in vitro* digestion process, and this effect is largely attributable to pH changes. These changes can modify the bioavailability and further anti-inflammatory activity of oregano phenolics, and thus, further research is needed.

Keywords: *in vitro* digestion, polyphenols, *Lippia graveolens*, *Lippia palmeri*, *Hedeoma patens*.

Practical Application: Oregano is a rich source of polyphenols that have shown bioactive properties like antiinflammatory potential. However, little is known of the gastrointestinal fate of oregano polyphenols which is imperative to fully understand its bioaccessibility. Our results are important to develop new administration strategies which could help protect the antioxidant and antiinflammatory potential and bioaccessibility of such compounds.

Introduction

Several plants and herbs have been traditionally used in folk medicine to treat illnesses related to chronic conditions. In this sense, some herbs have been used for their potential health-promoting properties (Fleisher and Sneer 1982). Among these herbs, oregano is one of the most widely used (Rubió and others 2013; Barros and others 2010). In Mexico, the most commonly commercialized species of oregano are *Hedeoma patens* (HP), *Lippia graveolens* (LG), and *Lippia palmeri* (LP). The beneficial properties of oregano are attributed to its bioactive compounds, of which phenolic compounds are an important constituent (Leyva-López and others 2016; Lin and others 2007).

Oregano is a good source of polyphenols with antioxidant and bioactive properties (Leyva-López and others 2016; Lin and others 2007). Phenolic compounds are a heterogeneous group of compounds that are present in the vast majority of plant foods. There is epidemiological and *in vitro* experimental evidence associating the consumption of foods rich in phenolic compounds with a low incidence of chronic diseases, which are the leading cause of death worldwide (Bennett and others 2012; Mohamed 2014; Urias-Lugo and others 2015). The main characteristics through which phenolic compounds have been reported to be bioactive are related to their antioxidant, immunomodulatory, chemopreventive, anti-platelet aggregation and anti-inflammatory properties (de la Lastra and Villegas 2007; Fraga and others 2010). In this regard, recent research has focused on the antioxidant and anti-inflammatory properties of the phenolic compounds of *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri* (Leyva-López and others 2016). Nonetheless, the antioxidant properties and polyphenolic profiles of these species of oregano have not been studied after an *in vitro* digestion process. Therefore, the aim of this study was to evaluate the stability of the antioxidant capacity and the changes of the phenolic content from three species of oregano (*Lippia graveolens*, *Lippia palmeri* and *Hedeoma patens*) after a simulated *in vitro* GI digestion.

Plant Material

Three species of oregano were used, *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri*. Plants of HP were collected in Surutato, Sinaloa, Mexico in August 2013 (coordinates: N 25° 51′ 6.2″ W 107° 34′ 56.6″). Plants of LG and LP were purchased from a local market in Durango in October 2013 and Baja California Sur in November 2013, respectively. The oregano leaves were dried and ground until a fine powder was obtained. The oregano powder was stored at -20 °C until use.

Reagents and Chemicals

All reagents used in this experiment were purchased from Sigma-Aldrich (St. Louis, MO). The reagents used for the preparation of the artificial oral digestive fluids were potassium chloride (KCl), potassium thiocyanate (KSCN), sodium phosphate dibasic (Na_2PO_4), monosodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), urea, α-amylase, uric acid, and mucin. The phenolic compound analytical standards used here for UPLC analysis were: quercetin, galangin, apigenin, apigenin-7-O-glucoside, scutellarein, luteolin, luteolin-7-O-glucoside, pinocembrin, phloretin, phloridzin dihydrate, taxifolin, eriodictyol, luteolin, ferulic acid, cinnamic acid, and benzoic acid. The following reagents were used in the *in vitro* bioactivity assays: methanol (80%), Folin-Ciocalteu reagent, DPPH reagent (1,1'-diphenyl-2-picrylhydrazyl), AAPH reagent (2',2'-azobis(2-amidinopropane) dihydrochloride), and Trolox (6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid).

Sample Preparation

Phenolic extracts from LG, LP and HP were extracted as follows: briefly, 1 g of dried oregano powder was incubated with 50 mL of 80% methanol for 12 h in the absence of light. After incubation, the samples were centrifuged at 10,000 rpm for 15 min; supernatant was collected, stored at – 20 °C, and used in further experiments. Extracts were prepared in triplicate (n = 3) for each oregano species.

Chemical Analysis by UPLC-PDA

Ultra-performance liquid chromatography (UPLC) was used to separate and determine individual phenolic compounds from oregano extracts and digested samples. Briefly, samples of 0.5 µL were injected into an ACQUITY UPLC H-Class system that employed a Waters (Milford, MA, USA) UPLC Pump (QSM) separation module equipped with an auto injector flow-through-needle (FTN) and a Waters PDA eλ photodiode array detector. Separation of phenolic acids was performed with an UPLC BEH C18 column (1.7 µm x 2.1 mm x 100 mm) at 50°C, with a gradient elution solution A (water-formic acid 0.1%), and solution B, composed of methanol, which was delivered at a flow rate of 0.3 $mL.min^{-1}$. The gradient elution procedure was as follows: 0 min, 90% (A); 3 min, 70% (A); 9 min, 60% (A); 11 min, 50% (A); 12 min, 0% (A), 13 min, 0% (A); 15 min, 90% (A); and 17 min, 90% (A). On the other hand, the separation of flavonoids was performed with a different set of conditions that included a UPLC BEH C18 column (1.7 µm x 2.1 mm x 100 mm) at 30 °C, with a gradient elution solution A composed of water-formic acid (0.05% formic acid), and solution B composed of acetonitrile, which was delivered at a flow rate of 0.3 $mL.min^{-1}$. The gradient elution procedure was as follows: 0 min, 93% (A); 5 min, 73% (A); 20 min, 60% (A); 20.1 min, 0% (A); 22 min, 0% (A), 22.1 min, 93% (A); 24 min, 93% (A). The UV spectra were recorded between 210 and 620 nm for peak characterization. Phenolic

compounds were quantified based on the peak area of the maximum absorption wavelength. Phenolic compound contents are expressed in mg.100•g^{-1} of oregano leaf. Calibration curves for each phenolic compound analyzed were elaborated with the chemical standards mentioned in the materials section. The identification of phenolic compounds by UPLC was conducted in duplicate (n = 2).

Total Reducing Capacity by Folin-Ciocalteu Assay

The Folin-Ciocalteu assay was used with some modifications as reported by (Swain and Hillis 1959). Briefly, the procedure consisted of mixing 15 µL of phenolic oregano extract, 240 µL of distilled water, and 15 µL of Folin-Ciocalteu reagent in a 96-well microplate. The mixture was incubated for 3 min, and then 30 µL of 4 N Na_2CO_3 was added and incubated at room temperature (25 °C) for 2 h in the dark. After incubation, the absorbance was measured at 725 nm using a 96-well using a Synergy HT spectrophotometer (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT), and the digestive mixtures were measured as blank. The calculations were performed using a gallic acid standard curve (from 0 to 0.4 mg•mL^{-1}), and the results are expressed as milligrams of gallic acid equivalents per gram of dried oregano powder (mg GAE•g^{-1}). Dilutions were prepared when needed.

Total Flavonoid Content

Total flavonoid content was determined by a colorimetric method reported elsewhere (Ghasemi and others 2009). Briefly, aliquots (200 µL) of samples were used, and 1112 µL of deionized water was added, followed by 60 µL of methanol and 40 µL of 10% $AlCl_3$; finally, 40 µL of 1 M $C_2H_3KO_2$ was added and incubated for 30 min. Absorbance was measured at 415 nm using a 96-well using a Synergy HT spectrophotometer, and

the digestive mixtures were measured as blank. A quercetin curve (from 0 to 0.4 mg•mL^{-1}) was used to calculate concentration, and the results were expressed as quercetin equivalents per gram of dried oregano powder (mg QE•g^{-1}). Each sample was measured in triplicate (n = 3). Dilutions were prepared when needed.

Antioxidant Capacity

Oxygen radical absorbance capacity (ORAC) assay. The oxygen radical absorbance capacity of samples was evaluated before and after *in vitro* GI digestion. The ORAC assay was conducted using fluorescein probe, AAPH (2,2'-azobis (2-amidino-propane) dihydrochloride) was used as a peroxyl radical generator, and Trolox was used as a standard, as previously described by (Huang and others 2002). The reaction mixture contained 25 µL of sample, 25 µL of 75 mM phosphate buffer (pH 7.4), 75 µL of 0.8 M AAPH, and 200 µL of 0.106 µM fluorescein. The 75 mM phosphate buffer was used as a blank. The samples, phosphate buffer and fluorescein were pre-incubated at 37°C for 15 min. AAPH was added to start the reaction, and the fluorescence was measured every 70 seconds for 70 min with a 485 nm excitation filter and a 580 nm emission filter using a Synergy HT spectrophotometer. The digestion solution was used as a blank for the digested samples. The values were calculated using a regression equation describing the relationship between the Trolox concentration and the net area under the fluorescein decay curve. The Trolox curve from 6.25 to 125 (µmol TE•g^{-1}) was used to calculate the results, which are expressed as µmol of Trolox equivalent per gram of oregano leaf (µmol TE•g^{-1}). Each sample was measured in triplicate (n = 3). Dilutions were prepared when needed.

DPPH scavenging capacity. DPPH radical scavenging assay was carried out according to Karadag and others (2009). Briefly, 20 μL of sample was placed in a 96-well microplate. Then, 280 μL of DPPH was added and left to incubate for 30 min in the absence of white light. Finally, absorbance was measured at 540 nm using a Synergy HT spectrophotometer, and the digestion mixture was used as a blank. The Trolox curve from 0.05 to 1 mmol TE•g^{-1} was used to calculate the results, which are expressed as mmol of Trolox equivalent per gram of oregano leaf (mmol TE•g^{-1}). Each sample was measured in triplicate (n = 3). Dilutions were prepared when needed.

Total equivalence antioxidant capacity assay (ABTS assay). The ABTS was dissolved in distilled water to a 7.4 mM concentration. The ABTS radical cation ($ABTS^{\cdot+}$) was produced by the reaction of the ABTS stock solution with 2.6 mM potassium persulfate at the ratio of 1:1 and by leaving the mixture to stand in the dark at room temperature for 12-16 h before use. The resulting blue-green ABTS radical solution was diluted in methanol to an absorbance of 0.700 ± 0.050 at 734 nm. Absorbance was measured using a Synergy HT spectrophotometer. The results were expressed as μM TE.g^{-1} of sample.

In vitro Gastrointestinal Digestion

An *in vitro* digestion was carried out using the method developed by (Versantvoort and others 2004). This *in vitro* digestion model consists of a three-step procedure that simulates the digestion in mouth, stomach and small intestine applying a physiologically based model of the human digestive tract, mimicking the chemical composition, digestive fluid pH, temperature (37 °C) and transit times. The composition of the synthetic digestive juices is detailed in Table 2. The simulated digestion process was performed using 50 mL tubes as follows: the digestion began by adding 2 mL of artificial saliva to 3 mL of phenolic extracts of oregano, and the mixture was gently stirred (55 rpm) for 5 min at 37°C. Afterward, the pH was increased to 5.4 with 1 M

NaOH. In the next step, the samples were supplemented with 4 mL of gastric juice, the pH was adjusted to 2, and the mixture was incubated at 37 °C (1 h, 55 rpm). Finally, 4 mL of intestinal juice was added, and the samples were incubated for 1 h at 37 °C (55 rpm); at the end of the intestinal digestion, the pH was adjusted to 7.4. At the end of the *in vitro* digestion process, the digestion tubes were centrifuged at 10,000 rpm for 10 min at 4°C, and the resulting supernatant was kept at -20 °C for further analysis. Samples without added oregano extracts were run in parallel as blanks to differentiate the effects of digestive fluids in the assays. Each sample was processed in triplicate (n = 3) for a total of 27 independent digestions. At the end of each digestive phase, the whole digested sample in the tube was centrifuged under the aforementioned conditions, and the supernatants were kept frozen until further use (-20 °C).

Percentage of Variation (% Var)

The percentage of variation (% var) expresses the results as the difference between the phenolic concentration before and after digestion at each digestive phase. In this sense, to evaluate the effect of digestion on the stability of phenolic compounds, the percentage of variation was calculated for total reducing capacity, total flavonoid content, antioxidant capacity (DPPH, ORAC) and for each individual phenolic compound detected by UPLC-PDA. The percentage of variation is measured according to:

% Variation (% Var) = (100 - ((Final value x 100) / Initial value))

Experimental Design

A randomized complete block design with one factor and four levels was used to analyze the data. The blocked factor was type of oregano, and the four levels were the phases of GI digestion (prior or undigested, oral, gastric and intestinal). All of the

treatments were performed in triplicate (n = 3). Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test (prior phase used as a comparison control) using the statistical package Minitab Express (Minitab Inc., State College, Pennsylvania). Statistical differences at the level $P < 0.05$ were considered to be significant.

Results and Discussion

Identification and Auantification of Phenolic Compounds

Previous studies have shown that the phenolic extracts from HP, LG, and LP have anti-inflammatory properties in that they inhibit the production of inflammation-related molecules, such as reactive oxygen and nitrogen species, as well as cytokines. This effect is attributed, in part, to the flavonoid content in the oregano species. Nonetheless, there are no studies regarding the antioxidant capacity and bioaccessibility of the oregano species HP, LG, and LP. In particular, no studies to date have provided information on the stability of the antioxidant and bioactive properties of the phenolic compounds from these species of oregano after the digestion process. It is noteworthy that to take advantage of and understand the potential beneficial effects of these herbs, it is imperative to evaluate the bioaccessibility of these compounds. One way to achieve these goals is by studying the effects of digestion on the antioxidant potential and content of the phenolic compounds from the HP, LG and LP oregano species, which quickly and cost-effectively provide relevant data on the bioaccessibility of these phenolic compounds.

Stability of Phenolic Compounds During *in vitro* Digestion

The individual content of phenolic compounds from the three oregano species submitted to *in vitro* digestion and analyzed by UPLC and percent changes during the GI digestion are presented in Table 3. Three main phenolic groups were identified in the oregano species studied here: a hydroxycinnamic acid derivative, a dihydrochalcone and four flavones. Overall, these compounds were identified as apigenin-7-*O*-glucoside (A7G), chlorogenic acid (CHA), luteolin (LUT), luteolin-7-*O*-glucoside (L7G), phloridzin (PHL) and scutellarein (SCUT). Chlorogenic acid is an hydroxycinnamic acid derivative formed from the esterification of caffeic and quinic acid. Phloridzin is a dihydrochalcone, which is part of a group of flavonoids with a linear saturated C3-chain connecting the two rings (Tomás-Barberán and Clifford 2000). Moreover, apigenin-7-glucoside, luteolin, luteolin-7-glucoside, and scutellarein are flavones, which contain a ketone group and have an unsaturated carbon-carbon bond at the C2-C3 position.

Flavones have been reported to be one of the main phenolic components of different oregano species (Lin and others 2007; Leyva-López and others 2016), which is consistent with our findings. Overall, flavones were the most abundant phenolic compounds in *Hedeoma patens* (1223.97 mg•100 g^{-1}), *Lippia graveolens* (1190.25 mg•100 g^{-1}), and *Lippia palmeri* (1228.99 mg•100 g^{-1}). In this regard, prior to *in vitro* gastrointestinal digestion, the most abundant individual phenolic compounds in HP, LG and LP were apigenin-7-*O*-glucoside, scutellarein and luteolin-7-*O*-glucoside. It is important to mention that apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside were found in the three oregano species (Table 2).

To some extent, the contents of all the individual phenolic compounds in *Hedeoma patens*, *Lippia graveolens*, and *Lippia palmeri* were affected during *in vitro* GI digestion. Following the oral phase of *in vitro* digestion, the variation of the content of each individual compound depended on the type of oregano. As indicated in Table 2, the oral digestion had a significant effect ($P<0.05$) on the phenolic content of *Lippia palmeri*, with a total loss of luteolin and an overall loss of 62% of its original phenolic

content. The total individual phenolic content of *Hedeoma patens* and *Lippia graveolens* showed a reduction of only 9.94% and 20.21%, respectively (Table 2).

The gastric phase of the *in vitro* digestion had a significant effect ($P<0.05$) on the loss of the phenolic compounds in *Lippia palmeri*, with a loss approximately 97%, followed by *Hedeoma patens* (-57.8%) and *Lippia graveolens* (-36.69%). After gastric digestion, the only detected compounds from *Lippia palmeri* were luteolin-7-*O*-glucoside and scutellarein. The phenolic compounds in *Hedeoma patens* and *Lippia graveolens* showed more stability, with an overall loss of only 57.85% and 36.69% of their original content. In this sense, it can be observed in Table 3 that the only individual phenolic compound with a decrease was chlorogenic acid, with a loss of only 16.21% of its initial content.

As shown in Table 2, at the end of the intestinal phase of the *in vitro* digestion process, no individual phenolic compound from the *Lippia palmeri* oregano was detected. On the other hand, *Lippia graveolens* and *Hedeoma patens* maintained 33.06% and 64.12% of their original content, respectively. Additionally, it is important to mention that the contents of apigenin-7-glucoside, chlorogenic acid and luteolin-7-O-glucoside increased at the end of the GI digestion in *Hedeoma patens*. Moreover, chlorogenic acid was the most stable phenolic compound of the three oregano species, maintaining 93.11% of its original content. It is important to mention that there is a lack of data regarding the effect of GI digestion on the phenolics of herbs and spices like the oregano species studied here; additionally, as far as we know, there are few reports on the chemical stability of flavones during GI digestion, limiting further comparisons.

Some studies have attributed the stability of chlorogenic acid during gastric GI digestion to the acidic pH of the compartment, which provides a more suitable environment for the chemical stability of this hydroxycinnamic acid derivative (Bermúdez-Soto and others 2007). Similarly, the stability of chlorogenic acid throughout the *in vitro* digestion process has been previously reported in food matrices such as edible flowers, fruit seeds and pomegranate pulp and juice (Chen and others 2015; Chen and others 2014; Mosele

and others 2015; Bermúdez-Soto and others 2007). Additionally, the stability of chlorogenic acid from apple was analyzed by He and others (2016), who reported that chlorogenic acid is somewhat stable throughout the GI process and shows an increase at the end of gastric and pancreatic digestion, which is consistent with our results. Moreover, Cilla and others (2009) analyzed the stability of phenolic compounds in fruit beverages (apricot-grape-orange) during simulated GI digestion and reported that the most affected compounds were flavones and flavan-3-ols; this result is in accordance with our data, which show that *Lippia graveolens* and *Lippia palmeri*, the samples rich in flavones, were the most affected by the GI physiological conditions. Contrary to our results, some authors have reported higher stability for flavonoids and tannins than for the phenolic acids, this finding may be attributed to the different matrixes studied in their work (Gullon and others 2015; Mosele and others 2015; Velderrain-Rodríguez and others 2014). It has been suggested that the chemical structure and distribution of the –OH radicals on the rings of the phenolic molecules are related to their stability under different pH conditions, as occurs during gastrointestinal digestion. Herein, conjugated non-phenolic acids such as trans-cinnamic acid, as well as ferulic acid, are stable at a high pH. It is important to mention that the loss of individual phenolics after the GI process has been attributed, in addition to the pH changes, to the possible interactions between polyphenols and other components of the digestive juices, such as enzymes and electrolytes, which needs to be studied further (Friedman and Jürgens 2000; Lamothe and others 2014). Additionally, the structural transformation that occurs as a result of the conjugation of polyphenols with proteins might render them undetectable in the individual chromatographic analysis by UPLC-PDA. In addition, these modifications can affect the bioaccessibility and bioactivity of the phenolic compounds in HP, LG and LP. More research is needed on this subject to fully understand the nature of these interactions and their effect on the possible protective effect of the antioxidant capacity and bioactive properties of the phenolic compounds. To the best of our knowledge, there are few reports on the effects of salivary fluids on the antioxidant and chemical stability of phenolic compounds during simulated gastrointestinal digestion. In this regard, most publications have focused on the interactions of wine tannins with salivary proteins. Ginsburg and others (2012) reported that the oxidant scavenging activity of cinnamon

polyphenolic extracts was increased after incubation with salivary fluids; the presence of phenolic compounds in the oral cavity has been associated with the prevention of the adverse effects of free radicals and reactive oxygen species. Additionally, it has been argued that the interactions of phenolic compounds and salivary proteins could protect phenolic compounds from oxidation and potentially maintain a higher redox status in the oral cavity, thus increasing protection against oxidative stress (Quintero-Flórez and others 2015).

Total Reducing Capacity and Total Flavonoid Content

The total reducing capacity is expressed as mg GAE•g^{-1} of dried oregano leaf. As shown in Table 4, of the three oregano species studied here, *Lippia graveolens* had the highest total reducing capacity ($P<0.05$) in undigested samples (51.26 ± 2.36 mg GAE•g^{-1} of oregano), followed by *Hedeoma patens* (40.74 ± 2.08 mg GAE•g^{-1}). On the other hand, *Lippia palmeri* had the lowest total reducing capacity (22.87 ± 0.26 mg GAE•g^{-1}). At all stages of *in vitro* digestion, the changes in the total reducing capacity of HP, LG and LP were significant ($P<0.05$). At the end of the oral phase of digestion, the TRC decreased significantly ($P<0.05$) for the three oregano species, to 0.34-, 0.60- and 0.85-fold for LP, HP and LG, respectively. Thus, LP had the most drastic decrease in the TRC of the three species. Interestingly, during the gastric phase of digestion, the TRC increased significantly in the three oregano species ($P<0.05$) between 1.97-3.20-fold; similarly some authors have reported an increase in the TRC and the content of individual phenolics at this stage and that this increase is mainly attributable to the acidic environment, where polyphenols are more stable (Bermúdez-Soto and others 2007; Chen and others 2015; Chen and others 2013; Wootton-Beard and others 2011). In contrast, at the end of the intestinal step of *in vitro* digestion the TRC decreased significantly ($P<0.05$) in the three oreganos studied; it is worth noting that the TRC for LP at this stage was null, while the TRC for LG and LP showed 0.44- and 0.32-fold changes, respectively, with values of 37.84 and 18.58 mg GAE•g^{-1}, respectively.

The total flavonoid content is expressed as mg•QE.g^{-1} of dried oregano leaf. As shown in Table 3, the total flavonoid content of the three oregano species was similar ($P<0.05$), with the highest TFC for the LG oregano (11.80 ± 0.12 mg•QE.g^{-1}), followed by HP and LP oreganos (10.48 ± 0.03 and 10.44 ± 0.03 mg•QE.g^{-1}, respectively). After the oral phase of *in vitro* digestion, the total flavonoid content increased significantly ($P<0.05$) by approximately 1.5-fold for the three oregano species. Interestingly, this significant ($P<0.05$) increase in the TFC was observed throughout the gastric and intestinal phases of the *in vitro* digestive process for the three oregano species.

The Folin-Ciocalteu assay is typically used to determine the total phenolic compounds in food samples and is based on the electron transfer reactions between the Folin-Ciocalteu reagent and the phenolic compounds in the sample. However, the Folin-Ciocalteu reagent is not specific to phenolic compounds, and it is known to interact with other compounds in the sample by, for instance, reducing sugars (glucose, fructose, galactose, etc.) and interacting with ascorbic acid. Therefore, it is more appropriate to consider the Folin-Ciocalteu assay as a total reducing capacity assay than to assume that the result is completely attributable to the phenolic compounds in the sample (Sánchez-Rangel and others 2013). Interestingly, our results show that the total reducing capacity, as measured by the Folin-Ciocalteu assay and the total flavonoid content, was affected by the *in vitro* digestion process, except in the LP species, which had a null TRC at the end of the intestinal phase. Interestingly, at the gastric step of *in vitro* digestion, the TRC and TFC increased significantly ($P<0.05$) for the three oregano types.

Antioxidant Capacity

The antioxidant capacity (AOXC) of plant-based foods is thought to be linked in part to their content of phenolic compounds. Nonetheless, during GI digestion, the chemical antioxidant capacity of these compounds can be modified, hence the need to measure the antioxidant capacity of the oregano species throughout the *in vitro* digestive process.

Additionally, the antioxidant capacity of a given compound can be assessed using several chemical mechanisms, making it necessary to measure antioxidant capacity via different methods. Therefore, in this study antioxidant capacity was measured using three different methodologies, i.e., DPPH, ABTS and ORAC methods, where the first two measure the capacity of a molecule to donate electrons, while the latter measures the capacity of a molecule to transfer hydrogen atoms (protons). As a consequence, these methods only estimate the antioxidant capacity that is associated with its conditions and reagents (Schaich and others 2015; Gullon and others 2015).

The $DPPH^{\cdot}$ scavenging capacity was quantified in terms of mM TE•g^{-1} of dried oregano leaf in each sample. As presented in Table 3, the initial DPPH values (mM TE•g^{-1} of dried oregano leaf) of the extracts from the three oregano species (HP, LG, and LP) varied from 225 to 500 mM TE•g^{-1}. Among them, the highest initial DPPH scavenging capacity ($P<0.05$) was shown for the LG species with 500.54 ± 9.63 mM TE•g^{-1}, followed by the HP species with 377.44 ± 13.53 mM TE•g^{-1}, and the LP species with 225.42 ± 1.01 mM•TE g^{-1}. At the end of oral *in vitro* digestion, the DPPH scavenging capacity increased significantly ($P< 0.05$) for the three oregano species between 5 and 9-fold in relation to undigested samples, with the highest increase observed in the LP species. Following the gastric phase of *in vitro* digestion, the DPPH scavenging capacity of the three oregano species decreased significantly ($P< 0.05$) by approximately 0.5-fold. At the end of the intestinal phase of the *in vitro* digestion, the $DPPH^{\cdot}$ scavenging capacity of HP, LG and LP decreased significantly ($P<0.05$), an approximately 0.9-fold change, and the higher values were found in the following order: LG>HP>LP ($P<0.05$).
The $ABTS^{\cdot+}$ scavenging capacity was quantified in terms of mM TE•g^{-1} of dried oregano leaf in each sample. There was no significant variation in the antioxidant capacity of the three oregano species before digestion ($P<0.05$), as determined by the $ABTS^{\cdot+}$ scavenging capacity (Table 4). Following the oral phase of *in vitro* digestion, the $ABTS^{\cdot+}$ scavenging capacity increased significantly ($P<0.05$) for the three oregano species by approximately 3-fold and in the following order: HP>LG>LP. Interestingly, the antioxidant capacity, as measured by the DPPH and ORAC assays, also increased after this digestive phase in almost all oregano species; the exception was the ORAC

scavenging activity in LG. Similarly, after the gastric phase of *in vitro* digestion, the $ABTS^{\cdot+}$ antioxidant capacity increased significantly ($P<0.05$); HP and LG had the highest antioxidant capacity of the three oregano species ($P<0.05$), with 1.87- and 2.03-fold changes for HP and LG, respectively, and a 1.7-fold increase for the LP species. At the end of intestinal *in vitro* digestion, there was significant ($P<0.05$) variation in the $ABTS^{\cdot+}$ scavenging capacity of the three oregano species; nonetheless, the LP oregano had a lower antioxidant capacity (819.40 ± 76.16 mM $TE \bullet g^{-1}$) than HP and LG (1319.92 ± 23.94 and 1445.70 ± 54.19 mM $TE \bullet g^{-1}$, respectively) ($P<0.05$). Despite the observed decrease in ABTS values during the intestinal phase, the antioxidant capacities shown for HP, LG and LP was higher than those of the non-digested samples of each oregano, which shows that the antioxidant capacity can be maintained through the digestion process.

The ORAC antioxidant capacity was measured in terms of μM $TE \bullet g^{-1}$ of dried oregano leaf in each sample. The ORAC antioxidant capacity prior to digestion was highest ($P<0.05$) for the LG (812.31 ± 35.46 μM $TE \bullet g^{-1}$) and HP oregano (753.95 ± 57.31 μM $TE \bullet g^{-1}$), followed by LP species (342.44 ± 11.03 μM $TE \bullet g^{-1}$). After the oral phase of *in vitro* digestion, the ORAC antioxidant capacity increased significantly ($P<0.05$) for the HP oregano. Interestingly, after the gastric phase, the ORAC antioxidant capacity increased significantly ($P<0.05$) in the LG and LP samples, with fold changes of 1.18 and 1.37, respectively, while the HP oregano decreased ($P<0.05$), showing a 0.29-fold change. On the other hand, at the end of the intestinal phase, the ORAC antioxidant capacity of the HP and LG oreganos did not significantly change, while the ORAC values for LP ($P<0.05$) oregano decreased, showing 0.63-fold change.

The DPPH and ABTS scavenging capacity was maintained and even increased in the three oregano species during *in vitro* digestion with regard to non-digested samples. Similarly, the ORAC antioxidant capacity was maintained in the digestates of the three oregano species. Overall, various authors have partially attributed this finding to the pH changes of the gastrointestinal environment, which cause the polyphenols to enhance their ability to donate protons (H^{+}) from their hydroxyl moieties on their aromatic rings.

These changes create different proportions of unionized phenolics, which might be reflected by their reactivity towards the reagents used in each assay (Friedman and Jürgens 2000). Moreover, the stability of the antioxidant capacity in HP, LG and LP might suggests that they have antioxidant potential. This result means that some phenolic compounds from these oregano species are somewhat resistant to pH changes; this finding is similar to that reported by several authors in polyphenols from different foodstuffs after similar *in vitro* digestion processes (Wootton-Beard and others 2011; Chen and others 2016; Chen and others 2015; Chen and others 2014; Chen and others 2013; Bermúdez-Soto and others 2007).

It is important to mention that while we observed a significantly reduced content of chromatographically detected phenolic compounds after digestion, the antioxidant capacity and total flavonoid content of the three oregano species diminished to a lesser extent. This resistance has been attributed to the possible formation of flavonoid derivatives, which could not be detected under the chromatographic conditions employed here. It is also worth noting that further studies need to be conducted because the formation of derivatives of the phenolic compounds detected can affect their bioaccessibility and further bioactivity (Campos-Vega and others 2015; Pinacho and others 2015).

Conclusions

The antioxidant stability of the phenolic compounds from the three oregano species (HP, LG, and LP) in terms of total reducing capacity, total flavonoid content, and antioxidant capacity by the DPPH, ABTS, and ORAC methods depended on the oregano species and was affected by the *in vitro* digestion process. Additionally, it was found that the species *Hedeoma patens* and *Lippia graveolens* had the most abundant phenolic content at the end of the simulated GI digestion. Our work shows that the phenolic compounds of oregano are affected during each GI phase; in this regard, it is imperative to promote

new studies on the properties of digested phenolic compounds in order to better understand their bioaccessibility and bioavailability. Additionally, it would be of interest to study the effects of the phenolic compounds from HP, LG and LP on the colon microbiota; although this was not the purpose of the present work, it is nonetheless considered as part of the metabolic pathway of polyphenols before reaching systemic circulation. Finally, since phenolic compounds from the oregano species have shown anti-inflammatory bioactivity, further absorption assays of oregano phenolic compounds are necessary.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Author Contributions

Erick Paul Gutiérrez-Grijalva: PhD student, responsible for conducting the research and the writing of the manuscript draft. Dr. Miguel Angel Angulo-Escalante: Responsible for the recollection of the plant materials (oregano species) used in the research. Supported research planning and revised the manuscript. Dra. Josefina León-Félix: Supported research planning and revised the manuscript. Dr. José Basilio Heredia: Obtained the funding for the project, provided guidance throughout the design and experimental phases of the project, as well as through the writing process.

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TABLES

Table 2. Composition of artificial digestive juices

Artificial saliva	Gastric juice	Intestinal juice
Inorganic components		
10 mL KCl 89.6 g.L^{-1}	15.7 mL NaCl 175.3 g.L^{-1}	40 mL NaCl 175.3 g.L^{-1}
10 mL KSCN 20 g.L^{-1}	3 mL NaH_2PO_4 88.8 g.L^{-1}	40 mL $NaHCO_3$ 84.7 g.L^{-1}
10 mL NaH_2PO_4 88.8 g.L^{-1}	9.2 mL KCl 89.6 g.L^{-1}	10 mL KH_2PO_4 8 g.L^{-1}
10 mL Na_2PO_4 57 g.L^{-1}	18 mL $CaCl_2$ 22.2 g.L^{-1} x $2H_2O$	6.3 mL KCl 89.6 g.L^{-1}
1.7 mL NaCl 175.3 g.L^{-1}	10 mL NH_4Cl 30.6 g.L^{-1}	10 mL $MgCl_2$ 5 g.L^{-1}
	8.3 mL HCl 37% g.g^{-1}	180 µL HCl 37% g.g^{-1}
Organic components		
8 mL urea 25 g.L^{-1}	10 mL glucose 65 g.L^{-1}	4 mL urea 25 g.L^{-1}
	10 mL glucuronic acid 2 g.L^{-1}	
	3.4 mL urea 25 g.L^{-1}	
Enzymes and other compounds added to mixture		
145 mg α-amylase	1 g BSA	9 mL $CaCl_2$ 22.2 g.L^{-1} x $2H_2O$
15 mg uric acid	1 g pepsin	1 g BSA
50 mg mucin	3 g mucin	3 g pancreatin
		0.5 g lipase
pH 6.5 ± 0.1	pH 1.0 ± 0.1	pH 7.8 ± 0.2

Table 3. Content (mg.100g^{-1} of dried oregano leaf) and percentage of variation of main phenolic compounds of three oregano species: *Lippia graveolens* (LG), *Lippia palmeri* (LP) and *Hedeoma patens* (HP) before and after the oral, gastric and intestinal *in vitro* digestion.

		Content of individual phenolic compounds (mg.100g^{-1})				**% Variation**		
		U	**O**	**G**	**I**	**O**	**G**	**I**
Hedeoma patens	**A7G**	673.02 ± 31.45a	646.92 ± 14.47a	151.33 ± 43.19b	361.06 ± 38.64c	-3.88	-77.52	-46.35
	CHA	545.15 ± 6.47a	448.23 ± 1.58b	456.78 ± 8.49b	507.58 ± 4.92c	-17.78	-16.21	-6.89
	L7G	550.95 ± 45.90a	498.04 ± 8.02a	137.56 ± 39.14b	265.64 ± 6.73c	-9.60	-75.03	-51.78
	Total	1769.12	1593.19	745.66	1134.29			
Lippia graveolens	**A7G**	47.30 ± 4.39a	32.32 ± 0.91a	7.98 ± 9.27b	0.00b	-31.67	-83.13	-100.00
	L7G	344.19 ± 10.65a	275.08 ± 0.03b	199.01 ± 6.41c	132.77 ± 23.76d	-20.08	-42.18	-61.42
	PHL	737.68 ± 25.80a	616.49 ± 0.28b	542.21 ± 34.90c	204.70 ± 9.79d	-16.43	-26.50	-72.25
	SCUT	798.76 ± 29.15a	614.35 ± 13.83b	471.46 ± 46.54c	299.97 ± 5.45d	-23.09	-40.98	-62.45
	Total	1927.93	1538.24	1220.65	637.44			
Lippia palmeri	**A7G**	291.90 ± 9.66a	147.16 ± 4.12b	0.00c	0.00c	-49.59	-100.00	-100.00
	LUT	141.47 ± 31.40a	0.00b	0.00b	0.00b	-100.00	-100.00	-100.00
	L7G	441.40 ± 4.19a	213.27 ± 14.56b	25.90 ± 9.21c	0.00c	-51.68	-94.13	-100.00
	SCUT	354.22 ± 23.29a	105.87 ± 2.48b	7.83 ± 2.61c	0.00 ± 0c	-70.11	-97.79	-100.00
	Total	1228.99	466.30	33.73	0.00			

[a] Means that do not share a letter are significantly different ($P < 0.05$)

%Variation in relation to undigested samples

U: Undigested, O: After oral digestion, G: After gastric digestion, I: After intestinal digestion, A7G: Apigenin-7-*O*-glucoside, CHA: Chlorogenic acid, LUT: Luteolin, L7G: Luteolin-7-*O*-glucoside, PHL: Phloridzin, SCUT: Scutellarein.

Table 4. Total reducing capacity (TRC, mg GAE.g^{-1}), total flavonoid content (TFC, mg QE.g^{-1}), DPPH scavenging capacity (DPPH Value, mmol TE.g^{-1}), ORAC antioxidant capacity (ORAC Value, µmol TE.g^{-1}), ABTS scavenging capacity (ABTS value, µmol TE.g^{-1}) and percentage of variation of three species of oregano before and after gastrointestinal *in vitro* digestion.

Digestive phase	***Hedeoma patens***		***Lippia graveolens***		***Lippia palmeri***	
	TRC (mg GAE.g^{-1}†)	**% Var**	**TRC (mg GAE.g^{-1}†)**	**% Var**	**TRC(mg GAE.g^{-1}†)**	**% Var**
U	40.74 ± 2.08a B		51.26 ± 2.36a A		22.87 ± 0.26a C	
O	24.46 ± 3.40b B	-39.95	43.70 ± 5.16b A	-14.75	7.81 ± 0.87b C	-65.86
G	58.81 ± 2.91c B	44.35	86.27 ± 2.00c A	68.30	24.97 ± 1.73c C	9.14
I	18.58 ± 2.83d	-54.39	37.84 ± 4.72d	-26.18	0 ± 0d	-100
	TFC (mg QE.g^{-1}†)	**% Var**	**TFC (mg QE.g^{-1}†)**	**% Var**	**TFC (mg QE.g^{-1}†)**	**% Var**
U	10.48 ± 0.03a B	-	11.80 ± 0.12a A	-	10.44 ± 0.03a B	-
O	16.19 ± 0.068b B	54.44	16.75 ± 0.13b A	41.97	16.16 ± 0.0b B	54.81
G	29.25 ± 0.05c B	179.13	29.47 ± 0.04c A	149.84	29.22 ± 0.02c B	179.84
I	42.50 ± 0.02d B	305.49	42.66 ± 0.01d A	261.49	42.48 ± 0.01d A	306.90
	DPPH Value (mM TE.g^{-1}†)	**% Var**	**DPPH Value (mM TE.g^{-1}†)**	**% Var**	**DPPH Value (mM TE.g^{-1}†)**	**% Var**
U	377.44 ± 13.53a B	-	500.54 ± 9.63a A	-	225.42 ± 1.01a C	-
O	2372.42 ± 17.90b B	528.56	2619.29 ± 17.18b A	423.30	2078.49 ± 30.78b C	822.05
G	1176.27 ± 5.25c B	211.65	1234.99 ± 18.79c A	146.73	1100.12 ± 3.31c C	388.03
I	1046.07 ± 2.94d B	177.15	1068.86 ± 5.16d A	113.54	989.29 ± 6.69d C	338.86
	ORAC Value (µmol TE.g^{-1}†)	**% Var**	**ORAC Value (µmol TE.g^{-1}†)**	**% Var**	**ORAC Value (µmol TE.g^{-1}†)**	**% Var**
U	753.95 ± 57.31a A	-	812.31 ± 35.46a A	-	342.44 ± 11.03a B	-
O	1698.92 ± 218.13b A	125.34	896.19 ± 9.90ab B	10.33	312.73 ± 20.96a C	-8.68
G	485.23 ± 14.22a B	-35.64	1055.45 ± 100.95b A	29.93	428.64 ± 8.83b B	25.17
I	682.91 ± 34.11a A	-9.42	761.11 ± 62.93b A	-6.30	268.98 ± 2.45c B	-21.45

Table 4. Cont.

Digestive phase	***Hedeoma patens***		***Lippia graveolens***		***Lippia palmeri***	
	ABTS Value (µmol TE.g^{-1}†)	**% Var**	**ABTS Value (µmol TE.g^{-1}†)**	**% Var**	**ABTS Value (µmol TE.g^{-1}†)**	**% Var**
U	351.55 ± 2.19a A	-	350.07 ± 0.45a A	-	348.85 ± 1.02a A	-
O	1196.96 ± 5.22b A	240.48	1191.98 ± 2.60b A	240.49	1110.67 ± 6.76b B	218.38
G	2236.87 ± 103.18c B	536.29	2424.69 ± 1.93c A	592.62	1889.89± 16.48c C	441.74
I	1319.92 ± 23.94b A	275.46	1445.70 ± 54.19d A	312.97	819.40 ± 76.16d B	134.88

U: Undigested, O: after oral digestion, G: after gastric digestion, I: after intestinal digestion

[a] Means that do not share a letter are significantly different by Tukey test. Comparison between digestion phases ($P< 0.05$)

[A] Means that do not share a letter are significantly different by Tukey test. Comparison between oregano species ($P< 0.05$)

%Var in relation to undigested samples

† Per gram of dried oregano leaf

V. FLAVONOIDS AND PHENOLIC ACIDS FROM OREGANO: OCCURRENCE, BIOLOGICAL ACTIVITY AND HEALTH BENEFITS

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Abstract

Several herb species classified as oregano have been widely used in folk medicine to alleviate inflammation-related diseases, respiratory and digestive disorders, headaches, rheumatism, diabetes and others. These potential health benefits are partially attributed to the phytochemical compounds in oregano such as flavonoids (FL) and phenolic acids (PA). Flavonoids and phenolic acids are among the most abundant and most studied phytochemicals in oregano species. Epidemiological, *in vitro* and *in vivo* experiments have related long-term consumption of dietary FL and PA with a decreased risk of incidence of chronic diseases. The aim of this manuscript is to summarize the latest studies on the identification and distribution of flavonoids and phenolic compounds from oregano species and their potential antioxidant, anti-inflammatory and anti-cancer health benefits.

Keywords: oregano; flavonoids; phenolic acids; antioxidant; flavones; flavonols; hydroxycinnamic acids; hydroxybenzoic acids; phytochemicals

1. Introduction

It has been stated that oregano is the name used to refer to a numerous variety of plants that share a particular flavor and odor. At least 60 species and 17 genera belonging to diverse botanical families are known as oregano. However, the most relevant families are Verbenaceae and Lamiaceae [1]. Interestingly Franz and Novak [2] discusses this issue and reports a table listing species and family information of the oregano plants. Moreover, in this study we have considered as oregano the species in that list. Oregano has been traditionally used in folk medicine to alleviate conditions such as asthma, bronchitis, coughs, diarrhea, indigestion, stomachache, menstrual

disorders, general infections, inflammation-related illnesses and diabetes [3]. The benefits of oregano on human health have been attributed to their phytochemical content [3,4]. Phytochemicals are a heterogeneous class of compounds derived from the secondary metabolism of plants, thus most of them do not appear to participate in essential metabolic roles. Research indicates that the main physiological function of phytochemicals is to serve as a plant defense mechanism against plant pathogens, pests, herbivores, UV-light and oxidative stress [5,6]. And their content in plants depends on factors such as cultivar, geographical localization, weather, daylight, temperature, soil conditions, water stress, harvesting time and others [7]. The most important phytochemicals found in oregano are grouped depending on their hydrophilic and hydrophobic properties into two categories: essential oils and phenolic compounds. Although research has often focused on the study of essential oils [8], they represent only one of the main groups of phytochemicals found in oregano. Thus, the study of hydrophilic compounds such as phenolic compounds is frequently ignored.

Flavonoids (FL) and phenolic acids (PA) are the main types of phenolic compounds present in oregano [9]. Oregano FL and PA have been studied due to their therapeutic potential, which has been partly attributed to their antioxidant properties [4,10]. Flavonoids and phenolic acids are molecules characterized by having at least one aromatic ring with one or more hydroxyl groups attached. FL and PA are compounds with a wide range of structures and can be classified based on the number and arrangement of their carbon atoms in: flavonoids (flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, isoflavones and others) and non-flavonoids (phenolic acids, hydroxycinnamic acids, hydroxybenzoic acids, stilbenes and others) and they are commonly found conjugated to sugars and organic acids [11–13]. All flavonoids are derived from the aromatic amino acids, phenylalanine and tyrosine and are C15 compounds arranged in three rings (C6-C3-C6) which are designated as A, B and C (Figure 5) [14]. Their structure varies due to the degree and pattern of hydroxylation, prenylation, alkalization or glycosylation reactions that modify the primary molecule [13]. These modifications can alter the water solubility of flavonoids, which can directly affect their bioavailability [15]. Flavones, which are the most abundant flavonoids present in oregano species, are characterized by the presence of a ketone group between

C-2 and C-3 and the attachment of the B ring to C-2. Among the most widely distributed flavones in nature are apigenin, luteolin and their derivatives [14]. For further information regarding the physiological function and health effects of flavones we suggest the studies by Jiang et al. [16] and Singh et al. [17]. On the other hand, hydroxycinnamic acids are part of the non-flavonoid phenolics and the main subgroup of phenolic acids distributed in the different oregano species [18]. They are formed with an aromatic ring and a three-carbon chain (C6-C3) (Figure 5) [11]. There are four basic structures: coumaric acid, caffeic acid, ferulic acid and sinapic acid. In nature, they are usually associated with other compounds such as chlorogenic acid, which is the link between caffeic acid and quinic acid [19].

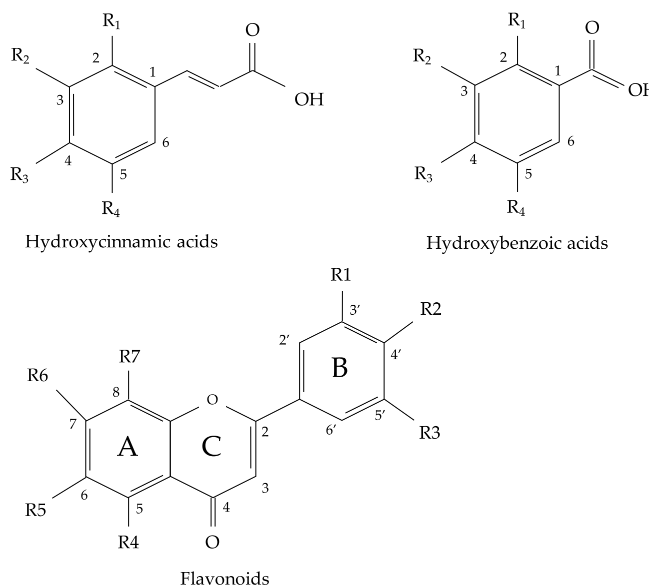

Figure 5. General structure of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids.

2. Flavonoids and Phenolic Acids Composition of Oregano Species

With the growing evidence on the biological activity from flavonoids and phenolic acids of oregano species, the identification of these compounds in oregano is important. Reports from different oregano species have shown that flavones are among the most

abundant sub-group of FL followed by flavonols (Figure 6), flavanones and flavanols. Among the most common PA in oregano are: hydroxycinnamic acid and hydroxybenzoic acid derivatives and other phenolics [9]. The major individual FL and PA that have been identified in oregano species are rosmarinic acid, apigenin, luteolin, quercetin, scutellarein, and their derivatives [4,9].

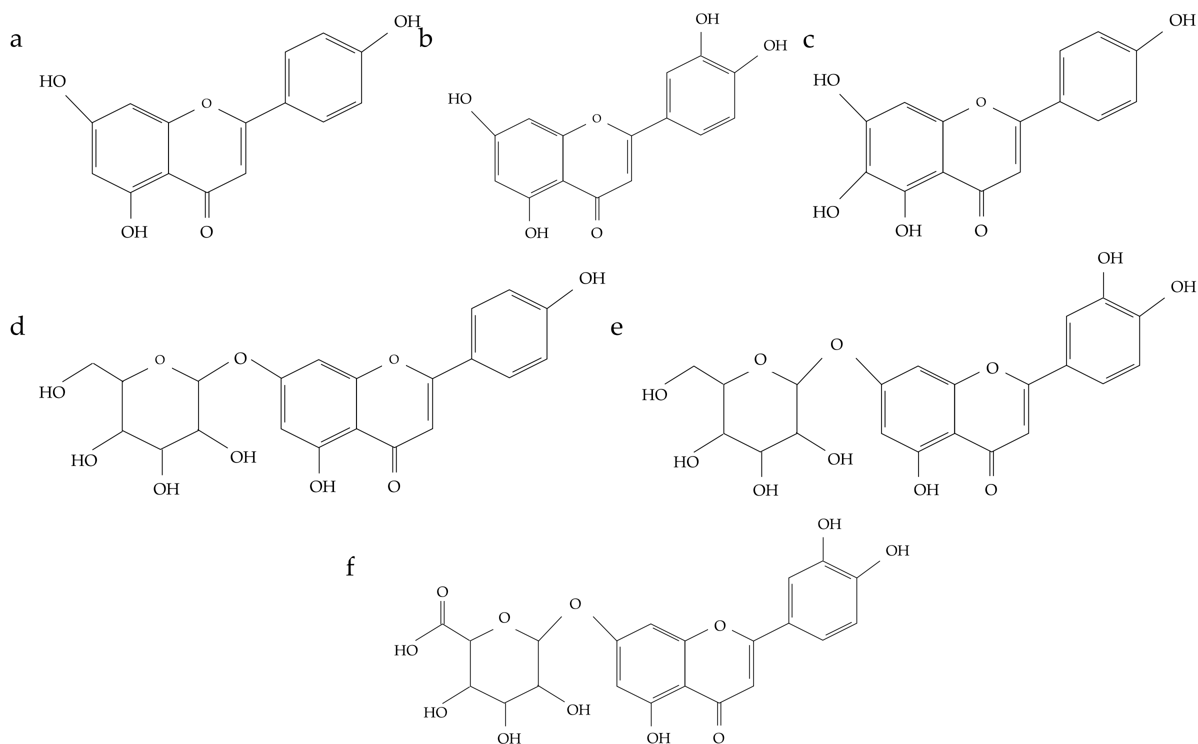

Figure 6. Chemical structure of common flavones commonly found in oregano species. a) apigenin, b) luteolin, c) scutellarein, d) apigenin-7-O-glucoside, e) luteolin-7-O-glucoside, f) luteolin-7-O-glucuronide. Structures from Phenol-Explorer Database, Version 3.6 [20].

The content and distribution of FL and PA in oregano can vary depending on the cultivar, geographical and environmental factors aforementioned. It has been indicated that the flavonoid and phenolic acids profile of each oregano can be used to differentiate between oregano chemotypes within the same species. For instance, Timóteo et al. [21] reported that the flavonoids tricin-7-O-diglucuronide, chrysoeriol-7-O-diglucuronide and luteolin-7-O-glucuronide can be used to discriminate between three Brazilian chemotypes of *Lippia alba*. Similarly, Barbosa et al. [22] reported that two flavonoids,

namely 5,5″-dihydroxy-6,4′,6″,3″,4″′-pentamethoxy-[C7-O-C7″]-biflavone and 4′,4,5,5″-tetrahycroxy-6,6″,3″′-trimethoxy-[C7-O-C7″]-biflavone, can be used to identify the limonene-carvone chemotype of *Lippia alba*. Likewise, the levels of rosmarinic acid have been reported to vary between the chemotypes within the species of *O. vulgare* ssp. *hirtum*, *O. vulgare* and *O. syriacum* [23]. Additionally, the FL and PA content of oregano species can vary depending on the vegetative stage of the plant. For instance, Baâtour et al. [24] suggested that during the late vegetative states of O. majorana L, there is a higher content of trans-2 hydroxinnamic, gallic and quercetin-3-galactoside, which makes it the best stage for harvesting *O. majorana* plants.

It can also be observed that oregano genotypes from the same species but from different places of origin can vary in their FL and PA composition, as is the case with the species *Lippia graveolens*, *Lippia alba*, *O. majorana*, *O. vulgare* and other oregano species. To illustrate, a recent study by Leyva-López et al. [4] reported 5 flavonoids in *Lippia graveolens* collected in Mexico, which are mainly flavones, namely quercetin-O-hexoside, scutellarein-7-O-hexoside, phloridzin, a trihydroxy-methoxyflavone derivative and 6-O-methylscutellarein. Whilst *L. graveolens* collected from Colombia reported by Stashenko et al. [25] had a different flavonoid profile with only 4 flavonoids, namely apigenin, luteolin, naringenin and quercetin. In contrast, *O. vulgare* collected from two different countries, China and Greece, showed a highly similar profile in their FL and PA content [26,27].

A summary of the major flavonoids and phenolic acids found in oregano species can be found in Table 5.

Table 5. Principal components of flavonoids and phenolic compounds of different oregano species

Oregano Species	Origin	Extraction solvent	Flavonoids and phenolic acids constituents	Reference
Hedeoma patens	Mexico	Methanol/acetone/water (50:40:10)	3-*O*-caffeoylquinic acid, luteolin-7-*O*-glucuronide, scutellarein-7-*O*-hexoside, salvianolic acid A	[4]
Lippia alba (Mill.) N. E. Brown	Brazil	Methanol 62.5%	Apigenin-7-*O*-diglucuronide, chrysoeriol-7-*O*-diglucuronide, luteolin-7-*O*-glucuronide, tricin-7-*O*-diglucuronide, tricin-7-*O*-glucuronide	[25]
Lippia alba (Mill.) N. E. Brown	France	Methanol/hydromethanol	Luteolin-7-diglucuronide, verbascoside, chlorogenic acid	[28]
Lippia alba (Mill.) N.E. Brown	Brazil	Chloroform	5,5″-dihydroxy-6,4′,6″,3‴,4‴- pentamethoxy-[C7–O–C7″]-biflavone, 4′,4,5,5″-tetrahydroxy-6,6″,3‴-trimethoxy-[C7 –O–C7″]- biflavone	[22]
Lippia citriodora	Germany	Water	Verbasoside, luteolin-7-diglucuronide, apigenin-7-diglucuronide,	[29]
Lippia graveolens	Mexico	Methanol/acetone/water (50:40:10)	Quercetin *O*-hexoside, scutellarein 7-*O*-hexoside, phloridzin, trihydroxy-methoxyflavone derivative, 6-*O*-methylscutellarein	[4]
Lippia graveolens	NR[3]	Methanol 70%	6-Hydroxyluteolin 7-*O*-hexoside, pentaflavanone-B hexoside-1, pentaflavanone-B hexoside-2, scutellarein 7-*O*-hexoside, luteolin 7-*O*-glucoside, taxifolin, 6-hydroxyluteolin 7-*O*-rhamnoside, 6-hydroxyluteolin, 3-hydroxyphloretin 6’-*O*-hexoside, apigenin 7-*O*-glucoside, phloridzin, scutellarein, eriodictyol, luteolin, quercetin, naringenin, 6-methylscutellarein, 6,7-dimethylscutellarein, sakuranetin, pinocembrin, galangin, methylgalangin	[9]

[1] Species not mentioned, [2] Not reported

Table 5. Cont.

Oregano Species	Origin	Extraction solvent	Flavonoids and phenolic acids constituents	Reference
Lippia graveolens	USA	Methanol 100%	Eriodictyol, naringenin, hispidulin, cirsimaritin	[30]
Lippia micromera	Colombia	Methanol/hydromethanol	Naringenin, apigenin	[28]
Lippia origanoides	Colombia	Methanol 62.5%	Quercetin, naringenina, luteolin, pinocembrin	[25]
Lippia palmeri	Mexico	Methanol/acetone/wáter (50:40:10)	Quercetin-*O*-hexoside, luteolin 7-*O*-glucuronide-3′-*O*-glucoside, scutellarein 7-*O*-hexoside, trihydroxy-methoxyflavone derivative	[4]
Majorana hortensis Moench.	Poland	Water	Caffeic acid, lithospermic acid, rosmarinic acid	[31]
O. acutidens	Turkey	Water	Gallic acid, caffeic acid, 4-hydroxybenzaldehyde, *p*-coumaric acid, rosmarinic acid	[32]
O. dictamus	Greece	Methanol 62.5%/BHT	Vanillic acid, protocatechuic acid, syringic acid, gallic acid, cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, rosmarinic acid, chrysin, epicatechin, naringenin, catechin, genistein, quercetin	[33]
O. dictamus	Greece	Water	Chlorogenic acid, rutin, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoisde, rosmarinic acid, luteolin	[34]
O. indercedens	Greece	Methanol	Caffeic acid, rosmarinic acid	[35]
O. majoram L.	Germany	Methanol 60%/Formic acid 1%	Apigenin-6,8-di-C-glucoside, luteolin-7'-*O*-glucuronide, rosmarinic acid, apigenin-glucuronide, lithospermic acid A isomer a, salvianolic acid B, apigenin	[36]

[1] Species not mentioned, [2] Not reported

Table 5. Cont.

Oregano Species	Origin	Extraction solvent	Flavonoids and phenolic acids constituents	Reference
O. Majoram L.	Tunisia	Methanol	Dihydroxybenzoic acid hexose, syringic acid, vanillic acid, dihydroxybenzoic acid, *p*-coumaric acid, chlorogenic acid, salvianolic acid I, caffeoyl-arbutin, rosmarinic acid, gallocatechin isomer 1, gallocatechin isomer 2, 3-*O*-methyl-catechin, luteolin-6,8-C-dihexose, apigenin-6,8-di-C-hexoside, isoorientin, orientin, isovitexin, kaempferol-*O*-sumbubioside, kaempferol-*O*-glucuronide, luteolin-*O*-glycoside, diosmin, apigenin-*O*-glucuronide, acacetin rutinoside, luteolin, apigenin, taxifolin, taxifolin methyl ether isomer 1, taxifolin methyl ether isomer 2, dihydrokaempferide, hesperidin, eriodictyol, sakuranetin, O-methy-quercetin, dimethyl myriceitn, quercetin dimethyl ether, jaceidin isomer 1, jaceidin isomer 2,	[37]
	Greece	Water	Vanillic acid, protocatechuic acid, syringic acid, gallic acid, cinnamic acid, *o*-coumaric acid, *p*-coumaric acid, ferulic acid, caffeic acid, sinapic acid, rosmarinic acid, chrysin, epicatechin, naringenin, catechin, kaempferol, quercetin,	[34]
	Poland	Methanol	Protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, chlorogenic acid, syringic acid	[38]
	Turkey	Methanol 80%	Caffeic acid glucoside, epigallocatechin, arbutin, luteolin ruitinoside, luteolin glucuronide, rosmarinic acid, dihydroquercetin, dihydroluteolin, apigenin, quercetin, quercetin arabinoside, luteolin-7-*O*-glucoside gallocatechin derivative	[39]
	Germany	Methanol 50%	Luteolin-6,8-di-C-glucoside, apigenin-6,8-di-C-glucoside, luteolin-glucuronide, rosmarinic acid, apigenin-glucuronide, lithospermic acid, apigenin	[40]

[1] Species not mentioned, [2] Not reported

Table 5. Cont.

Oregano Species	Origin	Extraction solvent	Flavonoids and phenolic acids constituents	Reference
O. majoram	USA	Methanol 80%	Eriodictyol 6,8-di-C-glucoside, eriodictyol 7-*O*-glucoside, apigenin 6,8-di-C-glucoside, luteolin 7,7'-di-*O*-glucuronide, luteolin 7-*O*-glucuronide-3'-*O*-glucoside, apigenin 7-*O*-diglucuronide, luteolin 7-*O*-glucuronide, luteolin 7-*O*-glucuronide, luteolin 7-*O*-glucoside, apigenin 7-*O*-glucoside, apigenin 7-*O*-glucuronide, rosmarinic acid	[41]
	Turkey	Water	Gallic acid, caffeic acid, ρ-coumaric acid, rosmarinic acid, chicoric acid, apigenin-7-glucoside, quercetin, kaempferol	[32]
	Italy	Cascade extraction with ethyl acetate and ethanol	Eriodictyol 7-*O*-rutinoside, aromadendrin, eriodictyol, naringenin, luteolin, sorbifolin, cirsiliol, apigenin, cirsilineol, cirsimaritin, xanthomicrol, caffeic acid, rosmarinic acid	[42]
	Greece	Cascade extraction with hexane and ethyl acetate	Salvianolic acid H, salvianolic acid B, rosmarinic acid, salvianolic acid C, eriodictyol, naringenin	[43]
	Finland	Methanol 40%	Calleryanin 3,4-dihydroxybenzoate, gastrodin 3,4-dihydroxybenzoate, calleryanin 3-hydroxy,4-methoxybenzoate	[44]
	Portugal	Methanol 80%	Gallic acid, 3,4-dihydroxybenzoic acid, (+)-catechin, caffeic acid, (-)-epicatechin, rosmarinic acid	[10]
Oregano[1]	Turkey	Methanol 80%	Gallic acid, syringic acid, vanillic acid, protocatechuic acid, chlorogenic acid, p-coumaric acid, quercetin-3-*O*-hexoside, luteolin-7-*O*-glucoside, ferulic acid, phloridzin, dicaffeoylquinic acid, apigenin-7-*O*-rutinoside, rutin, apigenin-7-*O*-glucoside, rosmarinic acid, luteolin-3-*O*-glucuronide, luteolin-7-*O*-rutinoside, quercetin, apigenin, 4'-methoxyapigenin, 6,7-dimethoxyscutellarein, luteolin	[45]
Poliomintha longiflora	NR[2]	Phosphate buffer	Vanillic acid, caffeic acid, luteolin, rosmarinic acid, hispidulin	[46]

[1] Species not mentioned, [2]Not reported

3. Physiological Functions of Flavonoids and Phenolic Acids from Oregano Species.

Flavonoids and phenolic acids are accumulated in plant tissues (such as leaves, flowers, stems and roots) as a response to biotic and abiotic stress like pathogen and insect attack, UV radiation and wounding [13,47]. Phenolic compounds help plants survive and adapt to environmental disturbances through various physiological functions. Phenolic acids such as hydroxycinnamic acid derivatives serve as precursor molecules for the stilbenes, chalcones, flavonoids, lignans and anthocyanins [48]. These compounds are present in most tissues as conjugates (esters of carboxylic acids or sterols, amides of amino acids or amines, glycosides of mono or disaccharides) or insoluble-bound (attached to the structural components of the plant cell wall); and are rarely found in free form (monomers or dimers) [18,49].

On the other hand, flavonoids have been reported to play a variety of roles in plants, such as UV protection, pigmentation, growth regulation, stimulation of nitrogen-fixing nodules and disease resistance [11,50]. Flavonoids have the ability to absorb UV-wavelength; this ability depends on the molecular structure and the nature of substitution on different rings of their molecule. For instance, dihydroxy B ring substituted flavonoids have a better antioxidant capacity, while their monohydroxy B ring substituted analogue have greater capacity to absorb UV wavelengths [50]. The flavones orientin and luteolin are found in high levels in plants exposed to high levels of solar UV-B radiation [51]. Moreover, flavonoids are produced in the cytosol of cells and are present in high concentrations in the epidermis of leaves [52]. Besides, flavonoids may regulate auxin movement and catabolism. For instance, they modulate different phenotypes and morphoanatomical features of plants, because of their ability to create auxin gradients [50]. Furthermore, flavonoids play a role in the protection of plants against plant feeding insects and herbivores; since their presence can alter the palatability of plants and reduce their nutritive value, decrease digestibility or even act as toxins [53]. Additionally, quercetin 3-O-rutinoside (rutin) has been reported to enhance membrane rigidity and protect the membrane from oxidative damage caused by lipid peroxidation [50].

For a more detailed description of the chemistry and biological functions of flavonoids in plants, please refer to the review in the literature Samanta et al. [52].

4. Health Benefits of Flavonoids and Phenolic Compounds from Oregano Species

Epidemiological, *in vitro*, and *in vivo* evidence has associated regular dietary intake of FL and PA with lower incidence of chronic diseases [54,55]. Interestingly, the flavonoids and phenolic acids that have been identified in oregano species have exhibited antioxidant, anti-inflammatory, and anti-cancer properties [10,56,57]. Interestingly, some *in vitro*, *in silico* and *in vivo* studies have proposed a structure-activity relationship of flavonoids and phenolic acids with their biological properties [58-60]. For instance, Ambriz-Pérez, et al. [58] summarized the following structure-anti-inflammatory activity relationship of flavonoids:

- A planar ring system in the flavonoid molecule
- Unsaturation in the C ring as ketonic carbonyl at C4 and/or C2-C3 double bond
- Hydroxyl groups in B ring and at C5 and C7 of A ring are necessary
- The number and position of hydroxyl groups as the catechol group at ring B
- The flavones and flavonoles having a hydroxyl group at 4' position of B ring
- The methylation of the hydroxyl groups at 3, 5 or 4' positions improves activity
- The methylation of the 3-hydroxyl group reduces cytotoxicity
- Flavones exhibited higher activities than isoflavones, flavonoles, and flavanones
- The 5, 6, 7-trihydroxyflavone core structure improves activity
- Aglycones are more bioactive than glycosides

Furthermore, in accordance with the aforementioned, some sub-classes of flavonoids that are present in oregano species, have been related with higher activity towards certain diseases. For example, flavones have been linked with many pharmacological properties such as neuroprotective, anti-inflammatory, antioxidant, anti-asthmatic, anti-ulcer, decreased risk of cardiovascular diseases, anti-diabetic, anti-cancer, etc [17,61-63]. Flavonols, for instance, have been associated with decreased risk of cardiovascular

diseases [64]. Hydroxycinnamic acid derivatives have been linked with anti-diabetic, antioxidant, and anti-cancer properties [65,66]. In the following sub-sections we will briefly discuss studies regarding the antioxidant, anti-inflammatory, and anti-cancer properties of oregano.

4.1. Antioxidant Properties of FL and PA from Oregano.

Flavonoids and phenolic acids from oregano have been reported with antioxidant properties [4]. These compounds can be extracted using different polar solvents like water, methanol and ethanol to obtain antioxidant-rich extracts. The antioxidant capacity of a sample is usually measured by different methods typically classified in two groups: hydrogen atom transfer (HAT) and electron transfer (ET). For instance, the oxygen radical absorbance assay (ORAC) is among the most common HAT assay used. On the other hand, the most commonly used ET methods are: the inhibition of the 1,1′-diphenyl-2-picrylhydrazyl radical (DPPH), the Trolox equivalent antioxidant capacity (TEAC) method/ABTS radical cation decolorization assay (also known as ABTS assay), the ferric reducing-antioxidant power assay (FRAP), the cupric ion reducing antioxidant capacity method (CUPRAC) and total phenolic content assay. Interestingly, DPPH and ABTS can also act like HAT [67,68]. Table 2 summarizes studies regarding antioxidant capacity in oregano species.

It has been reported that the antioxidant capacity of extracts from different oregano species is somewhat dependent on the solvents used during their extraction, which has been correlated with the FL and PA yielding during the process. A different study showed that the methanolic extract of the stem has strong antioxidant capacity against the DPPH radical (96% at 200 ppm), superoxide anion radical scavenging (61% at 250 ppm) and TAC (634 μM AAE/g of extract); which is attributed to their phenolic content such as rosmarinic acid, caffeic acid, rutin, gallic acid, quercetin and p-coumaric acid as reported by HPLC analysis [69]. Moreover, Singh et al. [70] studied various leaf extracts from *Eryngium foetidum*, interestingly the aqueous extract had the highest phenolic

content (256 mg gallic acid equivalents(GAE)/100 g fresh weight); furthermore, the methanolic extract was characterized by the presence of gallic, protocatechuic, syringic, p-coumaric, ferulic and sinapic acids.

The antioxidant capacity of oregano can also be affected by the cooking process. For instance, the effect of decoction and infusion on aqueous extracts of the leaves from *Lippia alba* has been studied by Timóteo et al. [21], they analyzed three chemotypes of *Lippia alba* and reported the presence of flavonoids of the class of flavones, especially mono and di-glucuronides of apigenin, luteolin and tricin. The authors noted that decoction samples with higher total phenolics content exhibited higher antioxidant activities (DPPH) than infusions, indicating that longer brewing time is better in the extraction of these compounds. Moreover, it was suggested that the presence of luteolin and luteolin-7-O-glucuronide in some chemotypes could improve the antioxidant capacity of *Lippia alba*. Aqueous extracts from *Lippia graveolens* (also known as Mexican oregano) have been studied for their antioxidant properties. For example, the oil free methanolic extract of *L. graveolens* showed a total phenolic content ranged from 211 to 270 mg GAE/g dried extract, where rosmarinic acid and naringenin were identified; meanwhile, in accordance with the TPC, the inhibition of the DPPH radical (IC50 = 152–207 μg/mL) was positively correlated to higher TPC [71]. However, another methanolic extract did not show the presence of rosmarinic acid but the flavonoids eriodictyol, naringenin, hispidulin and cirsimaritin were identified and, similarly to other studies, there was a positive correlation between the TPC and the antioxidant capacity by ORAC assay, around 5–9 mmol Trolox equivalents (TE)/mg of dry weight extract (DWE) [30].

Similarly, Lagouri and Alexandri [72] found a positive correlation between the TPC and antioxidant capacities by DPPH and FRAP of the leaves sequentially extracted with hexane, acetone and methanol, along with their infusion; they showed that the methanolic extract had higher DPPH value and the infusion higher TPC and FRAP value. Moreover, Kaliora et al. [34] observed a positive correlation between antiradical activity and quercetin protocatechuic acid, gallic acid, epicatechin, catechin, kaempferol and chlorogenic acid, found on the infusion of leaves and flowers.

Regarding *Origanum glandulosum* (syn. *Origanum compactum*), Béjaoui et al. [73] measured the antioxidant capacity of the methanolic extract, presenting an IC50 value of 0.6 mg/mL for DPPH, attributed principally to the presence of caffeic acid, which has been reported as a potent antioxidant [74]. Another widely studied oregano species regarding antioxidant capacity is *Origanum majorana*, characterized by the high content of rosmarinic acid. The antioxidant capacity of the microwave assisted methanolic extract from the aerial parts of O. majorana was analyzed by the CUPRAC, DPPH and TPC methods, showing high values attributed to the elevated content of rosmarinic acid, along with apigenin, caffeic acid and rutin [75]. Similarly, Elansary and Mahmoud [76] reported that rosmarinic acid and caffeic acid were the main compounds on the infusion and methanolic extract of the leaves from *Origanum majorana*, were the last one showed better antioxidant capacity by DPPH, β-carotene bleaching and TPC. Other flavonoids that have been found in methanolic extracts of *O. majorana* with correlation with TPC and ORAC values are eriodictyol and naringenin [30]. Similarly, rosmarinic acid was found to be the phenolic acid that provides the strongest antioxidant activity by FRAP and DPPH on hydromethanolic extract from Origanum majorana [39]. On the other hand, Vallverdú-Queralt et al. [77] did not find the presence of rosmarinic acid in the hydroethanolic extract of *Origanum majorana*, nevertheless protocatechuic acid, syringic acid and caffeic were the main compounds and the TPC exhibited a positive correlation with the DPPH and ABTS assays. Kogiannou et al. [26] reported relatively low TPC (38 mg GAE/200 mL) on the infusion of the leaves and flowers from *Origanum microphyllum*, with caffeic acid, syringic acid and naringenin as the main phenolics. Moreover, the antioxidant capacity was attributed to this content of phenolics, especially to caffeic acid, by FRAP assay.

Greek oregano (*Origanum vulgare*) is the most recognized herb as oregano around the world, generally reporting elevated content of rosmarinic acid. Yan et al. [78] analyzed the hydro-methanolic extract of the leaves from O. vulgare by TPC and ORAC, with results of 79–147 mg GAE/g DW and 1.59–3.39 mmol TE/g DW, respectively; but no correlation was found between the rosmarinic acid content and the antioxidant capacity measured by ORAC, which may indicate that other compounds are acting as antioxidant agents. In contrast, Gonçalves et al. [10] partially attributed the high

antioxidant capacity (DPPH, ABTS and FRAP) of the same kind of extract to the large quantity of rosmarinic acid (23.53 mg/g of dry extract) and the presence of other active compounds like (−)-epicatechin. A study by Koldaş et al. [32] in *O. vulgare* associated the presence rosmarinic, chicoric and caffeic acid with the antioxidant capacity of samples. Furthermore, eriodictyol and naringenin were also found in the methanolic extract of O. vulgare leaves, which exhibited high TPC (430 μg of GAE/mg of DWE) and a positive correlation with the ORAC value (~11 mmol TE/mg DWE) [30]. Likewise, the flavonoids luteolin-7-O-glucoside and apigenin-7-O-glucoside were obtained using accelerated solvent extraction with methanol, presenting a significant positive correlation between the TPC and the FRAP value [79]. Moreover, Balkan et al. [80] found a correlation between the TPC and DPPH scavenging activity with the presence of eriodictyol, apigenin and caffeic acid in the aqueous extract of *O. vulgare*.

For *Thymbra capitata*, the ethyl acetate extracts of aerial parts exhibited higher TPC, probably related to the higher content of taxifolin di-O-glucoside; whereas, the ethanolic extract was more efficient on the superoxide scavenging activity assay, the effect attributed to the higher content of rosmarinic acid in the extract [81].

As it can be seen, the polarity of the solvents used to extract FL and PA of interest from oregano can affect the yield and profile of compounds obtained, thus affecting their antioxidant capacity.

Table 6. A summary of the antioxidant capacity of flavonoids and phenolic acids of different oregano species

Oregano Species	Extract	Compounds	Plant Part	Antioxidant Assay	Reference
Coleus aromaticus	Methanol	Rosmarinic, caffeic, *p*-coumaric and gallic acids; quercetin and rutin	Stem	DPPH, superoxide, TAC	[69]
Eryngium foetidum	Aqueous, methanol	Gallic, protocatechuic, syringic, *p*-coumaric, ferulic and sinapic acids	Leaves	TPC, DPPH	[70]
Lippia alba	Aqueous	Apigenin-7-*O*-diglucuronide, chrysoeriol-7-*O*-diglucuronide, tricin-7-*O*-diglucuronide, luteolin-7-*O*-glucuronide	Leaves	TPC, DPPH	[21]
Lippia graveolens	Methanol	Rosmarinic acid, naringenin	Aerial parts	TPC, DPPH	[71]
	Methanol	Eriodictyol, naringenin, hispidulin, cirsimaritin	Leaves, commercial herbs	TPC, ORAC	[30]
Origanum dictamnus	Sequentially with hexane, acetone and methanol; Aqueous	Methanolic: gallic, caffeic, ferulic and rosmarinic acids. Aqueous: gallic, caffeic, protocatechuic and rosmarinic acids	Leaves	TPC, DPPH, FRAP	[72]
	Aqueous	Rosmarinic, caffeic and vanillic acids, epicatechin, catechin, genistein	Leaves and flowers	TPC, DPPH, FRAP	[34]
Origanum glandulosum	Methanol, previously deffated with n-hexane	Caffeic acid, luteolin glucoside	Not specified	DPPH, FRAP	[73]
Origanum majorana	Methanol microwave-assisted	Rosmarinic and caffeic acid, apigenin, rutin	Aerial parts	TPC, DPPH, CUPRAC	[75]
	Aqueous, methanol	Rosmarinic and caffeic acids	Leaves	TPC, DPPH, β-carotene bleaching	[76]
	Methanol	Rosmarinic acid, eriodictyol, naringenin, hispidulin, cirsimaritin	Leaves, commercial herbs	TPC, ORAC	[30]
	Methanol	Rosmarinic acid, epigallocatechin, quercetin, apigenin	Not specified	DPPH, FRAP	[39]

Table 6. Cont.

Oregano Species	Extract	Compounds	Plant Part	Antioxidant Assay	Reference
Origanum majorana	Ethanol	Chlorogenic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, rosmarinic and syringic acids, quercetin	Not specified	TPC, DPPH, ABTS	[77]
Origanum microphyllum	Aqueous	*p*-Hydroxybenzoic, protocatechuic, syringic and caffeic acids, naringenin	Leaves and flowers	TPC, FRAP	[26]
Origanum vulgare	Methanol	Rosmarinic acid	Leaves	TPC, ORAC	[78]
	Methanol	Rosmarinic acid and (−)-epicatechin	Leaves	DPPH; ABTS, FRAP	[10]
	Water, methanol, ethyl acetate, hexane	Rosmarinic, caffeic, chicoric and *p*-coumaric acids	Leaves	TPC, DPPH, TAC, RP, superoxide	[32]
	Methanol	Rosmarinic acid, eriodictyol, naringenin	Leaves	TPC, ORAC	[30]
Origanum vulgare	Methanol	Rosmarinic and caffeic acids, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside	Not specified	TPC, FRAP	[79]
	Aqueous	Eriodictyol, apigenin, caffeic acid, kaempferol	Not specified	TPC, DPPH	[80]
Thymbra capitata	Ethyl acetate, ethanol	Ethyl acetate: taxifolin di-*O*-glucoside, thymusin. Ethanol: taxifolin di-*O*-glucoside, rosmarinic acid	Aerial parts	TPC, superoxide	[81]

4.2. Anti-inflammatory Properties of FL and PA from Oregano.

Inflammation is a response of the organism to detect and destroy harmful agents [82]. During inflammation, the synthesis of pro-inflammatory mediators is activated. Some of these mediators are nitric oxide (NO), reactive oxygen species (ROS), cytokines and prostaglandins (PGs), as well as enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenases (COXs) [83]. When the inflammatory response is not well regulated an overproduction of these mediators is triggered causing pathological processes associated to diseases namely, arthritis, atherosclerosis and cancer, among others [84,85]. Therefore, the inhibition of the pro-inflammatory mediators mentioned is an important objective for the treatment of inflammation-related diseases.

It has been suggested that phenolic compounds from oregano, such as flavonoids and phenolic acids, might exert anti-inflammatory properties (Table 3) [86–88]. In this regard, Mueller et al. [89] evaluated the anti-inflammatory activity of *Origanum onites* and *O. majorana* hydrophilic extracts on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Pre-treatment of the cells with *O. onites* extracts at 500 μg/mL and 200 μg/mL reduced 19% and 49% the secretion of the pro-inflammatory cytokine IL-6, respectively, while the expression of iNOS was completely inhibited. Similarly, when macrophages were pre-treated with the extracts from *O. majorana* at 500 μg/mL and 200 μg/mL the levels of IL-6 were reduced 20% and 17%, respectively, while the iNOS expression was diminished 66%. Even though Mueller et al. [89] did not identify the compounds in the *O. onites* and *O. majorana* extracts, they mention that diosmetin, apigenin, luteolin and rosmarinic acid are the compounds to most likely be present in the hydrophilic extracts, so these molecules might be responsible for the activity of oregano extracts. On the other hand, aqueous infusion of *O. vulgare* was evaluated by Kogiannou et al. [26] in order to know their effect on IL-8 secretion, a pro-inflammatory and cancer promoting cytokine, in the cancerous cell lines HT-29 and PC3. When cells were treated with the *O. vulgare* infusion powder (0.2 μg powder/μL of medium) and stimulated with TNF-α, the IL-8 concentration on the medium was significantly reduced in both HT-29 and PC3 cells. The phenolic acid and flavonoid profile of the infusion from *O. vulgare*

was also determined. The authors attributed to caffeic acid present in the infusion part of the anti-IL-8 activity of oregano.

Recently, it has been demonstrated that extracts from the oregano species *Lippia graveolens*, *L. palmeri* and *Hedeoma patens*, containing quercetin, luteolin and scutellarein derivatives and salvianolic and neochlorogenic acids, showed anti-inflammatory activity by lowering ROS and NO production in LPS-induced inflammation in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells [4].

For a better understanding on how phenolic compounds exert their anti-inflammatory activity we recommend to review the papers by Ambriz-Pérez et al. [58] and Leyva-López et al. [90].

Table 7. A summary of the anti-inflammatory properties of flavonoids and phenolic acids of different oregano species

Oregano Species	Compounds	Effect	Reference
Eryngium foetidum	Kaempferol, chlorogenic acid, caffeic acid	Reduction of NO and ROS production and inhibition of the protein levels and gene expression of IL-6, TNF-α, iNOS and COX-2 in LPS-stimulated RAW 264.7 murine macrophages	[88]
Hedeoma patens	Neochlorogenic acid, Luteolin-7-*O*-glucuronide, scutellarein-7-*O*-hexoside, salvianolic acid A	Reduction of the levels of NO and ROS produced in murine macrophage cells	[4]
Lippia graveolens	Quercetin-*O*-hexoside, scutellarein-7-*O*-hexoside, phloridzin, trihydroxy-methoxyflavone derivative, 6-*O*-methylscutellarein	Inhibition of the NO and ROS production in LPS-stimulated murine macrophage cells	[4]
Lippia palmeri	Quercetin-*O*-hexoside, luteolin-7-*O*-glucuronide-3′-*O*-glucoside, scutellarein-7-*O*-hexoside, trihydroxy-methoxyflavone derivative, 6-*O*-methylscutellarein	Decrease of the levels of NO and ROS produced in murine macrophage cells	[4]
Origanum vulgare	Caffeic acid	Diminution of IL-8 secretion in HT-29 and PC3 cells	[26]

4.3. Anti-cancer Properties of FL and PA from Oregano.

Cancer is described as “a group of diseases characterized by unregulated cell growth and the invasion and spread of cells from the site of origin, or primary site, to other sites in the body” [91]. Several factors are involved in the onset of cancer such as age, alcohol, cancer-causing substances, diet, hormones, obesity, radiation, tobacco, etc.; and they may play a direct or indirect role in the development and progressions of different types of cancers. Carcinogenesis includes five known steps: initiation, promotion, progression, invasion and metastasis. The National Cancer Institute [92] states that *in vitro* and *in vivo* studies have shown that the increased presence of antioxidants prevents free radical damage that has been associated with cancer development. Plant foods are the most significance source of natural antioxidants; from which, flavonoids and phenolic acids have attracted the most attention as potential therapeutic agents against cancer. Shukla and Gupta [93] summarized that the potential anticancer properties of FL and PA as demonstrated by laboratory studies are due to different mechanisms of action, including antioxidation, induction of detoxification enzymes and inhibition of bioactivation enzymes, estrogenic and anti-estrogenic activity, antiproliferation, cell cycle arrest and apoptosis, promotion of differentiation, regulation of host immune function and inhibition of angiogenesis and metastasis. Several of the wide variety of FL and PA that have been identified in species classified as oregano have been reported with anti-cancer properties (Table 4). Thus, research has focused on the use of oregano FL and PA as potential anti-cancer therapy.

In this subject, the ethanolic extracts from *Origanum compactum* showed cytotoxic activity against human breast cancer cells (MCF7) and this effect was partially attributed to its FL content [94]. Calderón et al. [95] used the methanolic extracts of *Lippia cardiostegia* had cytotoxic activity at values from GI50 of 5.5, 5.2 and 7.5 (µg/mL) over breast (MCF-7), lung (H-460) and central nervous system (SF-268) human cancer cell lines. Methanolic extract from *Origanum compactum* also exhibited cytotoxic activity against MCF-7 cells, with IC50 values from 382 µg/mL to 374 µg/mL [96]. Hesperetin isolated from *Origanum majorana* has shown better antiproliferative activity than 5-

fluoroacil against Rattus norvegicus brain glioma (C6) and HeLa cells [97]. The FL and PA of hydroalcoholic extracts from *Origanum vulgare* L. subsp. viridulum showed antiproliferative activity, with 44% of inhibition of cell proliferation against human breast cancer cells (MFC-7), around 64% of inhibition against hepatic cancer cells (HepG2) and around 40% of inhibition of colorectal cancer (LoVo) cells. It is important to mention that *Origanum vulgare* extracts seemed to exhibit a selective antiproliferative activity against HepG2 [98].

Savini et al. [99] evaluated the ethanolic extracts from *Origanum vulgare* on cell proliferation and cell death in colon adenocarcinoma (Caco-2) cells; finding that at a concentration of 300 μg/mL oregano extract cell viability of decreased approximately 30% after 24 h; however, at a concentration of 500 μg/mL death time-dependently occurred. They also suggested that the mix of phenolic compounds found in *O. vulgare* extracts is more effective than individual phenolics, indicating a synergistic effect between compounds. Moreover, Nile et al. [100] suggested that the high cytotoxicity of *Origanum vulgare* extracts against breast cancer cells (MFC-7) is related to its high phenolic content

Likewise, García-Pérez et al. [57] evaluated different extracts from *Poliomintha glabrescens* for cytotoxic activity in colon cancer cells HT-29. The evaluated samples showed inhibition of the proliferation of HT-29 cell line, which was partially attributed to the luteolin and apigenin content in *Poliomintha glabrescens*.

According to all of these studies, the phenolic extracts and isolated flavonoids and phenolic acids of species classified as oregano are a promising source for the development of new drugs in the treatment of cancer, nevertheless more research is necessary to understand in detail the mechanisms of action of these compounds as well as their effects on *in vivo* models and the possible side effects that could occur their administration.

Table 8. A summary of the anti-cancer properties of flavonoids and phenolic acids of different oregano species

Oregano Species	Anti-cancer Activity	Effect	Reference
Origanum dictmnus	Cytotoxic	Activity against human bronchial epidermoid carcinoma (NSCLC-N6) and murine leukemia (P388) cells line.	[101]
Origanum compactum	Cytotoxic	Activity against human breast cancer cells (MCF-7)	[94]
	Antiproliferative	Inhibit human breast cancer (MCF-7) cell proliferation.	[96]
Origanum syriacum	Antiproliferative	Reduction in the proliferation of human breast cancer cells (MFC-7).	[102]
Origanum vulgare	Antiproliferative	Reduction in the proliferation of human breast cancer (MFC-7), colorectal cancer (LoVo), cervical epithelial carcinoma (HeLa) cells and selective antiproliferative activity on hepatic cancer cells (HepG2).	[32,98,102]
	Cytotoxic	Showed signs of cells death on cervical epithelial carcinoma (HeLa) cell line and cytotoxicity for breast cancer and colon adenocarcinoma (Caco-2) cells.	[98–100,103]
Lippia cardiostegia Benth	Cytotoxic	Activity against breast (MCF-7), lung (H-460) and central nervous system (SF-268) human cancer cell lines.	[95]
Origanum marjorana	Antiproliferative	Inhibit *Rattus norvegicus* brain glioma (C6) and cervical epithelial carcinoma (HeLa) cell proliferation.	[97]
	Cytotoxic	Activity against fibrosarcoma (HT-1080) cell line.	[98]
Origanum acutidens	Antiproliferative	Inhibit cervical epithelial carcinoma (HeLa) cell proliferation.	[32]
Poliomintha glabrescens Gray	Cytotoxic	Activity against human colon cancer cells (HT-29).	[57]

5. Enhancement of Flavonoid and Phenolic Acids Content in Oregano Species

As previously shown, oregano species are an important source of flavonoids and phenolic acids, which have been of special interest because of their potential use as antioxidants, anti-inflammatory and anti-cancer agents [4]. As a consequence, the

enhancement of FL and PA in oregano species is desired. Consequently, research has shown that flavonoids and phenolic acids in plants can be enhanced by several methods; however, research regarding enhancement of FL and PA content in oregano is scarce [104–108]; nonetheless there are few studies on enhancement of FL and PA from *Lamiaceae* herbs, like *Ocimum basilicum*, that can provide information for further research in plants known as oregano. Among the most studied are the agronomic approaches and through the use of plant elicitors [109]. The main agricultural method focuses on the manipulation of FL and PA content by the manipulation of nutrient (such as application of nitrogen fertilizers) and altering irrigation strategies. On the other hand, plant elicitors are compounds that can induce plant defense reactions that could result in the production of defense secondary metabolites such as FL and PA [108].

5.1. Nutrient Manipulation.

Few studies have focused on the effect of nutrient manipulation on the enhancement of flavonoid and phenolic acid content in oregano species. Briefly, boron toxicity was shown to increase phenolic content and anthocyanins in *Ocimum basilicum*, which is suggested to be a plant strategy to mitigate the negative effects of boron within the cells [110]. A couple of studies have also researched the effects of nitrogen manipulation on the phenolic content and antioxidant capacity in *Ocimum basilicum* and have shown that the levels of phenolics such as rosmarinic and caffeic acid can be enhanced as a result of nitrogen treatment [111,112]. Moreover, the total content of rosmarinic acid of *Origanum vulgare* can be increased when cultivated under manipulation of proline content [104]. However, caution is needed since these strategies can hinder plant growth.

5.2. Light Quality.

FL and PA are secondary metabolites that are metabolized as a response to UV stress in order to avoid oxidative stress on plants. In this regard, some studies have addressed this issue by studying the effect of light quality on FL and PA content. However, research regarding the effect of light stress on FL and PA content in oregano is scarce. For instance, Shiga et al. [113] showed that the rosmarinic acid content and antioxidant capacity of *Ocimum basilicum* L. were increased as a result of white light irradiation. Another study in Ocimum basilicum L. by Ghasemzadeh et al. [114] reported that UV-B radiation (3.60 W/m^2) improved the total phenolic and flavonoid content as well as the content of gallic acid, cinnamic acid, ferulic acid, quercetin, catechin, kaempferol, rutin and luteolin. UV treatment can also increase the content of rosmarinic acid in *Origanum vulgare* [106]. Additionally, UV-B treatment increased the activity of the enzyme chalcone synthase (EC: 2.3.1.74), which is a key enzyme in the phenylpropanoid metabolism, catalyzing the conversion of p-coumaroyl-CoA and malonyl-CoA to naringenin chalcone, which is a precursor of flavonoids [115].

5.1. Plant Elicitors.

5.1.1. Chitosan

Chitosan is a biopolymer produced the deacetylation of chitin. And has been extensively studied as a plant elicitor in several crops [116]. Some reports have shown the use of chitosan as a FL and PA enhancer in oregano species. For instance, the content of rosmarinic acid in *Ocimum basilicum* L. increased after chitosan treatment [117]. Similarly, Yin et al. [108] applied chitosan oligosaccharides in *Origanum vulgar*e ssp. *hirtum*, which resulted in increased content of lithospermic acid B and apigenin-6,8-diglucoside. Moreover, a combination of application of chitosan on *Ocimum basilicum* and reduced irrigation increased the total phenolic content and antioxidant activity [118].

Overall, these studies concluded that chitosan concentration had an impact on LF and PA stimulation.

5.1.1. Plant Growth Regulators

Growth and development in plants is regulated by endogenous phytohormones that play key roles on their metabolism. Additionally, these molecules can be used as potent elicitors. Among the most commonly used growth regulators are methyl jasmonate, jasmonic acid, spermine and arachidonic acid. In this regard, Koca and Karaman [119] showed that the application of a combination of methyl jasmonate and spermine enhanced the rosmarinic acid content in *Ocimum basilicum* L. Similarly, a study by Złotek et al. [120] in purple *Ocimum basilicum* showed an increased content of benzoic acid and rosmarinic acid after application of arachidonic and jasmonic acid. Malekpoor et al. [121] also showed an increased total phenolic content and antioxidant activity in *Ocimum basilicum* L. after treatment with jasmonic acid. Moreover, Kim et al. [122] showed that application of methyl jasmonate in *Ocimum basilicum* L. enhanced the rosmarinic and caffeic acid as well as the antioxidant activity of the samples. Additionally, acetyl salicylic acid in combination with fish protein hydrolyzate has been shown to stimulate the phenylpropanoid metabolism, which results in higher antioxidant activity [107].

Interestingly, even though enhancement FL and PA content is desired in oregano species due to their proposed human health effects, high concentrations of these compounds have shown a reduced plant growth and reproduction [109]. It is important to mention that even though the aim of this study was not to summarize flavonoid and phenolic acids enhancement methods, we consider of interest to recommend the studies by Malerba and Cerana [116], García-Mier et al. [109], Pichyangkura and Chadchawan [123] and Trivellini et al. [124].

6. Conclusion

In conclusion, due to the wide variety of herbs regarded as oregano, there is a complex mixture of flavonoids and phenolic acids that can be found throughout them, from which flavones are the main constituents. These compounds have been of particular interest for their potential bioactive properties and promising role as alternative treatment in several illnesses. Here we have summarized the most recent studies focusing on the characterization studies of oregano FL and PA as well as their antioxidant, anti-inflammatory and anti-cancer properties. Interestingly, most of the studies are based on *in vitro* approaches, limiting its extrapolation to human health. Hence, further *in vivo* research is needed to understand the bioavailability, pharmacokinetics and mechanism of action of oregano FL and PA as agents in human health improvement.

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Abbreviations

The following abbreviations are used in this manuscript:

FL	Flavonoids
PA	Phenolic acids
BHT	Butylated hydroxytoluene
HAT	Hydrogen atom transfer
ET	Electron transfer
ORAC	Oxygen radical absorbance assay
DPPH	1,1'-diphenyl-2-picrylhydrazyl radical
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
FRAP	Ferric reducing-antioxidant power assay
CUPRAC	Cupric ion reducing antioxidant capacity
TAC	Total antioxidant capacity
TPC	Total phenolic content
DW	Dried weight
DWE	Dried weight extract
NO	Nitric oxide
ROS	Reactive oxygen species
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
TNF-α	Tumor necrosis factor alpha
COX-2	Cyclooxygenase-2
LPS	Lipopolysaccharide
iNOS	Nitric oxide synthase
COXs	Cyclooxygenases

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VI. CELLULAR ANTIOXIDANT ACTIVITY AND ANTI-OBESITY PROPERTIES OF OREGANO POLYPHENOLS UNDER SIMULATED GASTROINTESTINAL DIGESTION

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ABSTRACT

Ethnopharmacological relevance: Different oregano species have been traditionally used as infusions in folk medicine to alleviate inflammation-related diseases. However, information regarding bioaccessibility of oregano polyphenols is limited.

Aim of the study: To evaluate the cellular antioxidant activity, anti-obesity properties and polyphenolic changes of three oregano species, namely, *Hedeoma patens* (HP), *Lippia graveolens* (LG) and *Lippia palmeri* (LP), subjected to simulated gastrointestinal digestion.

Materials and methods: We obtained hydromethanolic extracts from dried oregano leaves of HP, LG and LP. The hydromethanolic extracts were evaporated to dryness, resuspended in water and submitted to an *in vitro* gastrointestinal digestion process. Finally, we evaluated the polyphenol changes during *in vitro* digestion using HPLC-DAD and LC-TOF-MS. Moreover, we measured α-glucosidase, α-amylase and lipase inhibitory activity, and the cellular antioxidant activity (Caco-2 cells) of undigested and digested oregano polyphenolic extracts.

Results: The *in vitro* digestion process significantly affected measured antioxidant and bioactive properties. Oregano polyphenolic extracts and digests from HP, LG, and LP exhibited cellular antioxidant capacity.

Conclusions: Our results suggest that HP, LG and LP polyphenols exhibit potential for use as hypoglycemic, hypolipidemic, and antioxidant agents.

Keywords: bioaccessibility, *Lippia graveolens*, *Lippia palmeri*, *Hedeoma patens*, oregano, polyphenols, metabolic syndrome, antioxidant, cellular antioxidant activity, glucosidase, amylase, lipase.

1. Introduction

Several herb species are recognized as oregano in the literature and are generally comprised in the genera of Lamiaceae and Verbenaceae (Franz and Novak, 2010). One of the most common herb commercialized as oregano is *Lippia graveolens* (LG), also known as Mexican oregano; however, *Hedeoma patens* and *Lippia palmeri* are also distributed as oregano (Barros et al., 2010; Rivero-Cruz et al., 2011; Rubió et al., 2013). Herbs known as oregano have been traditionally used in folk medicine to treat respiratory and digestive disorders (vomiting, diarrhea), headaches, rheumatism and inflammation-related ailments (Charles, 2013; Laferriere et al., 1991). Moreover, different oregano species have been studied due to their anti-inflammatory, hypoglycemic, hypolipidemic and antioxidant properties, which have been attributed to the presence of phytochemicals, such as polyphenols (Gonçalves et al., 2017; Gutiérrez-Grijalva et al., 2017; Leyva-López et al., 2016). Polyphenols are a heterogeneous group of phytochemicals that are synthesized in plants as products of secondary metabolism. Polyphenols are of particular interest because of their potential health benefits in delaying the onset of non-communicable and chronic diseases, such as inflammation, metabolic syndrome and diabetes (Amiot et al., 2016; Pascual et al., 2001). In this regard, metabolic syndrome (MetS) is described as a cluster of heart attack risk factors, such as diabetes, increased fasting plasma glucose, abdominal obesity, high cholesterol and high blood pressure (Ahima, 2016; Alberti et al., 2006). Moreover, type-2 diabetes mellitus, commonly known as diabetes, is a chronic condition characterized by hyperglycemia and glucose intolerance. Currently, diabetes affects approximately 425 million people worldwide with a mean spent amount per person from 1,000 to over 4,000 billion US dollars (International Diabetes Federation, 2017). The most common clinical assessment in the management of MetS and diabetes involves the prescription of inhibitors of enzymes, such as acarbose for α-glucosidase and α-amylase, and orlistat for pancreatic lipase. However, the continuous intake of these drugs can cause adverse effects, such as gastrointestinal discomfort, flatulence, abdominal cramps, diarrhea, and

oily spotting (Filippatos et al., 2008; Leroux-Stewart et al., 2015). Thus, natural alternatives with fewer or no secondary effects are being currently studied. Regarding this subject, dietary polyphenols are efficient inhibitors of metabolic syndrome-related enzymes, such as α-glucosidase, α-amylase, and pancreatic lipase. This function has been attributed to their capacity to bind to proteins through hydrogen bonding (Ambigaipalan et al., 2016; Amiot et al., 2016; Mohamed, 2014). Nonetheless, it is frequently neglected that after ingestion, polyphenols can undergo chemical transformations due to the physiological conditions in the gastrointestinal tract, thus affecting their bioactive properties (Gutiérrez-Grijalva et al., 2016; Velderrain-Rodríguez et al., 2014). Moreover, we previously reported the antioxidant activity of oregano polyphenols during an *in vitro* digestion process using different *in vitro* chemical assays (Gutiérrez-Grijalva et al., 2017). Nonetheless, chemical assays are often questionable for predicting the antioxidant capacity of a sample due to non-physical conditions (Wan et al., 2015). Henceforth, the aim of this research was to study the hypoglycemic, hypolipidemic and antioxidant activities of oregano polyphenols through evaluation of their α-glucosidase, α-amylase and pancreatic lipase inhibitory activities as well as cellular antioxidant activity under simulated gastrointestinal digestion conditions. Moreover, the polyphenolic changes during GID were assessed by LC-TOF-MS.

2. Materials and Methods

2.1. Plant Material.

Three species of oregano were studied here, namely, *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri*. *Hedeoma patens* plants were collected in Surutato, Sinaloa, Mexico in August 2013 (coordinates: N 25° 51′ 6.2″ W 107° 34′ 56.6″). *Lippia graveolens* plants were collected in Santa Gertrudis, Durango in October 2013

(coordinates: N 23º 32’ 43.8” W 104º 22’ 20.8”). *Lippia palmeri* plants were collected in Todos Santos, Baja California Sur in November 2013 (coordinates: N 23º 27’ 26.1” W 110º 14’ 0.77”). The oregano leaves were dried (40 ºC, 24 h) and ground until a fine powder was obtained. The oregano powder was stored at – 20 °C until use. The voucher specimens were deposited at the Herbarium at the School of Biology from the Sinaloa State University (Sinaloa, México). Species identification was realized at the Herbarium from the School of Agriculture and Sinaloa State University.

2.2. Reagents and Chemicals.

α-Glucosidase from Saccharomyces cerevisiae (19.3 $U.mg^{-1}$ solid, Sigma G5003-100UN), α-amylase from porcine pancreas (150,000 $U.g^{-1}$ solid, Megazyme E-PANAA), ρ-nitrophenyl-α-glucopyranoside (Sigma N1377), ρ-nitrophenyl-palmitate (Sigma N2752), apigenin, luteolin, quercetin, rosmarinic acid, 2,2’-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), 2’,7’-dichlorofluorescin diacetate (DCFH-DA), water, formic acid, acetonitrile HPLC grade, and all chemicals used in the formulation of the simulated gastrointestinal juices were purchased from Sigma-Aldrich (St. Louis, MO). Lipase from porcine pancreas (100-500 $U.mg^{-1}$ solid) was purchased from Megazyme International (Wicklow, Ireland). Dulbecco’s Modified Eagle Medium (DMEM-F12) was obtained from Thermo Fischer Scientific (Waltham, MA). Fetal bovine serum (FBS), phosphate-buffered saline pH 7.4 (PBS), trypsin-EDTA 0.25%, penicillin (10000 $Unit.mL^{-1}$), and streptomycin (10000 $\mu g.mL^{-1}$) were acquired from GIBCO (Grand Island, NY). CellTiter 96 ® Aqueous One Solution Cell Proliferation Assay was obtained from Promega Corporation (Madison, WI).

2.3. Sample Preparation.

Phenolic compounds from HP, LG, and LP were extracted as follows. Briefly, 0.5 g of dried oregano leaf powder was incubated with 25 mL of 80% methanol for 12 h in constant shaking (100 rpm) in the absence of white light. After incubation, the samples were centrifuged at 10,000 rpm for 30 min. The supernatant was collected and stored at -20 °C. Extracts were prepared in triplicate (n = 3) for each oregano species. For enzyme inhibitory assays and *in vitro* digestion processes, methanolic extracts were evaporated to dryness (at 40 ºC) using a Savant Speed Vac Concentrator attached to a Labconco CentriVap Cold Trap (Labcon Co., Kansas, MO, USA) and later resuspended in distilled water.

2.4. *In vitro* Gastrointestinal Digestion.

An *in vitro* digestion process was performed as reported by Gutiérrez-Grijalva et al. (2017). The process consisted of a 3-step procedure that simulated digestion in mouth, stomach and small intestine, applying a physiologically based model of the human digestive tract that mimics the chemical composition and pH of digestive fluids at 37 ºC. At the end of the *in vitro* digestion process, the samples were centrifuged at 10,000 rpm (Thermo Scientific Sorvall Legend XTR) for 15 min at 4 °C, and the resulting supernatant was collected. Supernatant was freeze-dried obtaining the polyphenol extract. Afterwards, the PEE was extracted with ethyl acetate, and the organic phase dried and resuspended in 80% methanol for further analysis.

2.5. HPLC-TOF Analysis.

Changes in polyphenol profile of oregano extracts were performed based on the method and conditions reported by Leyva-López et al. (2016). Tentative identification of flavonoids was made by comparison of their UV spectra and characteristic ions m/z with

those previously reported in oregano species. Extracts of samples in methanol were used to quantify flavonoids and related compounds. Briefly, 20 µl of extracts was separated by HPLC (Agilent 1100 Santa Clara, CA) using a Zorbax Eclipse XDB-C18, 4.6 mm ID x 150 mm (5 µm) reverse column. Gradient elution was conducted with (A) water and 0.1% formic acid and (B) acetonitrile at a flow rate of 0.5 $mL.min^{-1}$. The following gradient was used: 0→5 min, 20% B; 5→20 min, 30% B; 20→40 min, 60% B; 40→45 min, 55% B. Mass spectrometric analyses were performed on Agilent Technologies LC/MSD TOF (Santa Clara, CA) with Analyst QS 1.1 software (Applied Biosystems, Carlsbad, CA). Mass spectra were collected using an electrospray source in negative mode (ESI-) under the following conditions: m/z range, 150-1500; nitrogen gas; gas temperature, 300 °C; drying gas low rate, 13 $L.min^{-1}$; nebulizer pressure, 40 psig; capillary voltage, 4000 V; and fragmentor voltage, 40 V.

2.6. Quantification of Phenolic Acids and Flavonoids.

Chromatograms were acquired at 280 and 320 nm according to the chromatographic conditions described in Section 2.5 using an Agilent 1100 HPLC-DAD and integrated by HP-Agilent Software (Chemstation for LC, Agilent Technologies, 1990-2003). Compounds were quantified as equivalents of apigenin, luteolin, or rosmarinic acid.

2.7. Bioaccessibility of Oregano Polyphenols.

In vitro bioaccessibility as measured through an *in vitro* gastrointestinal digestion model was calculated following the equation:

% Bioaccessibility = (Final concentration / Initial concentration) x 100

Here, the final concentration is the phenolic content at the end of the gastric and intestinal fractions, and initial concentration is the phenolic content in the undigested samples.

2.8. Total Reducing Capacity by Folin-Ciocalteu Assay.

The Folin-Ciocalteu assay was used as reported by Swain and Hillis (1959) with few modifications. Briefly, the procedure consisted of mixing 10 µL of phenolic oregano dried extract at a final concentration of 400 µg with 230 µL of distilled water and 10 µL of Folin-Ciocalteu reagent in a 96-well microplate. The mixture was incubated for 3 min. Then, 25 µL of 4 N Na2CO3 was added and incubated at room temperature (25 °C) for 2 h in the dark. After incubation, the absorbance was measured at 725 nm using a 96-well using a Synergy HT spectrophotometer (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). The calculations were performed using a gallic acid standard curve (from 0 to 0.4 $mg.mL^{-1}$), and the results are expressed as milligrams of gallic acid equivalents (mg GAE). Each sample was measured in triplicate (n = 3). Dilutions were prepared when needed.

2.9. Total Flavonoid Content.

Total flavonoid content was determined by a colorimetric method reported in Gutiérrez-Grijalva et al. (2017). Briefly, 10 µL of phenolic oregano dried extract at a final concentration of 400 µg. Then, 250 µL of deionized water was added followed by 10 µL of 10% AlCl3. Finally, 10 µL of 1 M $C_2H_3KO_2$ was added and incubated for 30 min. Absorbance was measured at 415 nm using a 96-well using a Synergy HT spectrophotometer. The calculations were performed using a quercetin curve (from 0 to 0.4 $mg.mL^{-1}$), and the results are expressed as milligrams equivalents of quercetin (mg

QE). Each sample was measured in triplicate (n = 3). Dilutions were prepared when needed.

2.10. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity.

DPPH radical scavenging assay was performed according to Karadag et al. (2009). Briefly, 20 µL of phenolic oregano dried extract at a final concentration of 400 µg was placed in a 96-well microplate. Then, 280 µL of DPPH was added, and the solution was incubated for 30 min in the absence of white light. Finally, absorbance was measured at 540 nm using a Synergy HT spectrophotometer. The calculations were performed using a Trolox curve (from 0.05 to 1 mmol TE). The results were expressed as a percentage of the DPPH radical inhibition and as mmol of Trolox equivalent (mM TE). Each sample was measured in triplicate (n = 3).

2.11. Cell Culture.

Human colorectal adenocarcinoma cells (Caco-2) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Caco-2 cells were grown in DMEM-F12 supplemented with 5% fetal bovine serum in a humidified atmosphere containing 5% CO_2.

2.12. Cell-Based Antioxidant Assay.

To evaluate cellular antioxidant activity (CAA) of oregano polyphenol-rich extracts before and after GID, the optimized method by López-Barrios et al. (2016) was used

with Caco-2 cells. Briefly, 24 h before the assay, Caco-2 cells were seeded in a black-walled, clear-bottom 96-well microplate (Costar, Corning Inc., Corning, NY) at a density of 5x105.mL^{-1}. Afterwards, cells were treated with 100 µL of HP, LG, and LP extracts at different concentrations (25, 50, 100, and 200 µg dried extract.mL^{-1}) containing DCFH-DA (60 µM). Then, cells were incubated for 20 min at 37 ºC. Afterwards, treatment solutions were removed, and the cells were washed thrice with PBS. Finally, 100 µL of 500 µM AAPH solution was added to each well, except for blank and negative control wells. Fluorescence emitted at 538 nm upon excitation at 485 nm was measured every 2 min for 90 min at 37 ºC using a microplate reader (Synergy HT, Bio-Tek, Winooski, VT). CAA values were calculated using the following equation:

$$\text{CAA Unit} = 1 - (\int SA / \int CA)$$

where $\int SA$ is the integrated area under the sample fluorescence versus time curve, and $\int CA$ is the integrated area from the control curve.

2.13. α-Glucosidase Inhibition.

α-Glucosidase inhibitory activity was determined according to an assay modified from the Worthington Enzyme Manual as reported by Cuevas-Juárez et al. (2014). Briefly, in 96-microwell plates, 50 µL of dried oregano extracts from HP, LG and LP and their digested samples at a concentration of 400 µg.mL^{-1} were incubated for 10 min at 37 °C with 100 µL of α-glucosidase from *Saccharomyces cerevisiae* (0.6 U.mL^{-1}) in phosphate buffer (0.1 M, pH 6.9). Then, 50 µL of 3 mM ρ-nitrophenyl-α-glucopyranoside in phosphate buffer pH 6.9 (ρNPG) was added, and the mixture was incubated again for 12 min at 37 °C. Enzyme activity was determined by measuring the release of ρ-nitrophenol from the ρNPG substrate. Absorbance at 405 nm was measured with a 96-well microplate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). In addition, 50 µL of 1 mM acarbose was used as a control.

2.14. α-Amylase Inhibition Assay.

The α-amylase inhibition assay was determined according to an assay modified from the Worthington Enzyme Manual (Worthington, 1993). Briefly, the assay contained 50 μL of substrate (starch at 1% in PBS) and enzyme (13 $U.mL^{-1}$) prepared in PBS, 50 μL PBS and 50 μL of inhibitor (oregano extract and digesta, or acarbose). PBS was used as a blank. Stock soluble starch solution was prepared in water by heating at 90 ºC on a hot plate for 15 min. Pancreatic α-amylase stock solution (13 $U.mL^{-1}$) was prepared in PBS. The enzyme solution and the assay mixture were pre-incubated at 37 ºC in a water bath for 10 min, and the reaction was started by adding the enzyme to the assay solution. The reaction was performed at 37 ºC for 10 min with 50 μL of pancreatic α-amylase at 13 $U.mL^{-1}$, 50 μL of substrate at 1 $mg.mL^{-1}$ and 50 μL of oregano extract (400 $μg.mL^{-1}$). Afterwards, 1 mL of dinitrosalicylic acid reagent was added, and the solution was heated at 85 ºC for 15 min. Then, the solution was transferred to ice to cool down to room temperature. Finally, 250 μL from each sample was placed in a 96-well plate, and the absorbance was recorded at 540 nm using a microplate reader.

2.15. Lipase Inhibition Assay.

The lipase inhibitory activity was determined using a spectroscopy method by Winkler and Stuckmann (1979) with minor modifications. In the assay, ρ-nitrophenyl palmitate (ρNPP) was used as substrate, which is hydrolyzed by lipase to ρ-nitrophenol (ρNP), a colored agent that can be monitored at 410 nm. Briefly, 20 μL of oregano extract at a concentration of 400 $μg.mL^{-1}$ and 20 ρL of the porcine lipase enzyme solution (1 $mg.mL^{-1}$) in sodium phosphate buffer (0.1 M, pH 6.9) were incubated for 10 min at 37 ºC. Then, 1800 μL of 0.1 M sodium phosphate buffer containing sodium cholate (1.15 $mg.mL^{-1}$) and arabic gum (0.55 $mg.mL^{-1}$) and 20 L of ρNPP in isopropanol (0.01 M)

were added and incubated for 10 min at 37 ºC. The released ρNP was monitored at 410 nm for 10 min at 37 ºC using a microplate reader.

2.16. Experimental Design.

Data were presented as the mean ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test using the statistical package JMP 9.0 (SAS Institute Inc., Cary, NC). Statistical differences at the level of $P < 0.05$ were considered significant.

3. Results

3.1. Identification of Polyphenols from *Hedeoma patens*, *Lippia graveolens*, and *Lippia palmeri* Under Simulated GID.

Polyphenols from different oregano species are related to antioxidant and anti-inflammatory properties (Gonçalves et al., 2017; Leyva-López et al., 2016). Moreover, we previously reported that the content of oregano polyphenols is negatively affected by GID as assessed by UPLC-PDA (Gutiérrez-Grijalva et al., 2017). However, UPLC-PDA analysis is limited by the availability of commercial standards. On the other hand, LC-TOF-MS is a more efficient method to study changes in the polyphenol composition of HP, LG and LP during gastrointestinal processes. Table 9 summarizes the compounds detected by LC-TOF. Phenolic compounds were identified by comparing the $[M-H]^-$ ion, fragment ions and the maximum absorption wavelengths with those reported in the literature for other species known as oregano.

LC-TOF-MS analysis allowed the identification of 13 phenolic compounds: 6 phenolic acid derivatives and 9 flavonoid derivatives. The identified phenolic acid derivatives were 1,6-di-O-syringoyl-β-D-glucopyranose with an $[M-H]^-$ ion at m/z 539, neochlorogenic acid with an [M-H]- ion at m/z 353, lithospermic acid A $[M-H]^-$ ion at m/z 537, rosmarinic acid with an $[M-H]^-$ ion at m/z 359, salvianolic acid A with an $[M-H]^-$ ion at m/z 493; and a dihydroxy methylcoumarin derivative with an $[M-H]^-$ ion at m/z 177. On the other hand, the identified flavonoids were quercetin O-hexoside with an $[M-H]^-$ ion at m/z 463; scutellarein 7-hexoside with an $[M-H]^-$ ion at m/z 447; luteolin-7-glucoside-3'-glucuronide with an $[M-H]^-$ ion at m/z 623; pentahydroxy dihydrochalcone derivative with an $[M-H]^-$ ion at m/z 331; luteolin 3',4',7-trimethyl ether with an $[M-H]^-$ ion at m/z 327; apigenin with an $[M-H]^-$ ion at m/z 269; and 6-O-methylscutellarein with an $[M-H]^-$ ion at m/z 299.

Interestingly, the simulated GID led to changes in the polyphenolic profile of HP, LG and LP. In the intestinal fraction of the GID from HP, peak 6 was not detected, and peak 1 appeared. In the case of the intestinal fraction from LG, peaks 4, 6, 7, and 11 were not detected, whereas peaks 1, 2, and 10 appeared. In the intestinal fraction from LP, peaks 4 and 5 were not detected, and peak 10 appeared.

Table 9. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data and tentative identification of polyphenols in methanolic and digested samples of *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri* by LC-DAD-ESI-MS/MS.

					HP			LG			LP			
Peak	RT (min)	Λ max (nm)	$[M-H]^-$	Tentative identification	U	G	I	U	G	I	U	G	I	Reference
1	9.052	220, 277	539	1,6-di-O-syringoyl-β-D-glucopyranose			x			x	x	x	x	DNP
2	9.911	217, 327	353	Neochlorogenic acid	x	x	x			x				Leyva-López et al. (2016)
3	12.138	280, 344	463	Quercetin O-hexoside				x	x	x	x	x	x	Leyva-López et al. (2016)
4	12.99	315	537	Lithospermic acid A				x			x			Martins et al 2014
5	13.266	287, 332	447	Scutellarein 7-Hexoside	x	x	x				x			Leyva-López et al. (2016)
6	14.163	266, 342	623	Luteolin-3'-glucuronide-7-glucoside	x	x	x	x	x	x	x	x	x	Leyva-López et al. (2016)
7	15.293	284	331	Pentahydroxy dihydrochalcone derivative				x						DNP
8	17.74	290, 330	359	Rosmarinic acid	x	x	x							Standard, Martins et al 2014
9	24.501	287, 323	493	Salvianolic acid A	x	x	x							Leyva-López et al. (2016); Greenham et al. (2003)

					HP			LG			LP			
Peak	RT (min)	Λ max (nm)	$[M-H]^-$	Tentative identification	U	G	I	U	G	I	U	G	I	Reference
11	28.698	288	327	Luteolin 3',4',7-trimethyl ether				x	x					DNP
12	29.3	266, 336	269	Apigenin	x	x	x	x	x	x	x	x	x	Standard
13	30.745	272, 342	299	6-O-Methylscutellarein				x	x	x	x	x	x	Leyva-López et al. (2016); Greenham et al. (2003)

* HP: Hedeoma patens, LG: Lippia graveolens, LP: Lippia palmeri, U: Undigested, G: After gastric digestion, I: After intestinal digestion

3.2. Quantification and Bioaccessibility of Polyphenols.

Based on the available commercial standards, we quantified the derivatives of apigenin, luteolin and rosmarinic acid as μg equivalents.mg^{-1} dried extract in non-digested and digested HP, LG and LP samples. The quantification of polyphenols from HP, LG and LP is presented in Table 10, and HPLC chromatograms are presented in Figures 7-9.

In accordance with a previous study from our group (Gutiérrez-Grijalva et al., 2017), simulated gastrointestinal digestion had a significant effect ($P < 0.05$) on the concentration of individual phenolics from HP, LG and LP. In the gastric fraction, most phenolics were not detectable in samples. Before the GID process, the main phenolic constituent in the evaluated oregano species was salvianolic acid A in HP (562.635 ± 2.086 μg RAE.mg^{-1} dried extract), and luteolin-7-glucoside-3'-glucuronide was the main compound in LG and LP (187.76 ± 4.48 and 78.67 ± 8.39 μg LE.mg^{-1} dried extract, respectively).

In the gastric fraction, the most bioaccessible compounds were salvianolic acid (25.31%) in HP and luteolin-7-glucoside-3'-glucuronide in LG (19.56%) and LP (39.19%). As previously mentioned, GID had a significant effect on the polyphenol content in the three oregano species; thus, the bioaccessibility of its components was lower. For instance, salvianolic acid A was no longer bioaccessible in the intestinal fraction in HP. Interestingly, in the intestinal fraction, luteolin-7-glucoside-3'-glucuronide remained the only bioaccessible compound of the three oregano species.

Table 10. Quantification of polyphenols in non-digested and digested samples of *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri*.

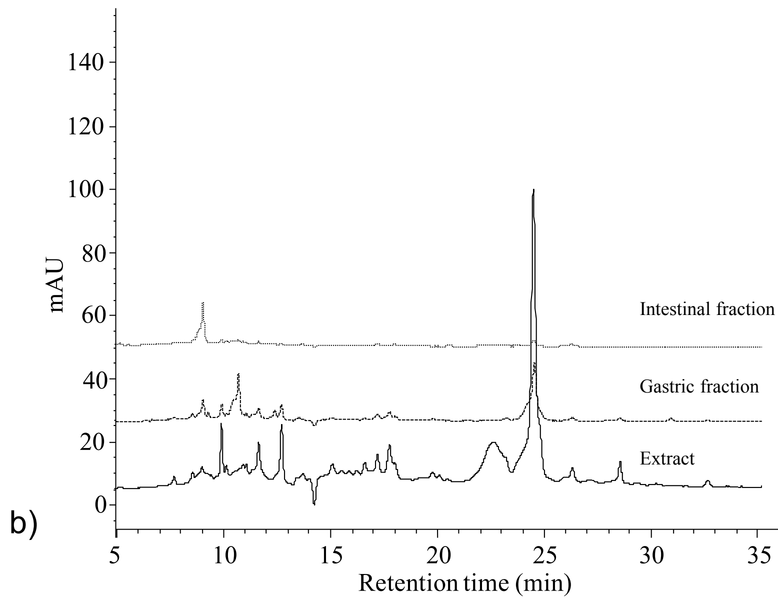
		Content (µg/mg dried extract)			Bioaccessibility (%)	
Oregano species	Compound	ND	Gast	Int	Gast	Int
HP	Luteolin-7-glucoside-3'-glucuronide*	424.91 ± 5.2 a	27.32 ± 2.45 b	13.7 ± 1.49 b	6.43	3.22
	Scutellarein-7-hexoside+	374.07 ± 77.11 a	0	0	0	0
	Apigenin+	19.76 ± 5.303 a	0	0	0	0
	Salvianolic acid A*+	562.64 ± 2.09 a	49.93 ± 3.03 b	0 c	25.31	0
	Rosmarinic acid*+	197.25 ± 50.62 a	12.36 ± 0.22 b	0 b	6.26	0
LG	Luteolin-7-glucoside-3'-glucuronide*	187.76 ± 4.48 a	36.73 ± 3.25 b	15.85 ± 1.48 c	19.56	8.44
	Luteolin 3',4',7-trimethyl ether*	11.46 ± 2.03 a	0 b	0 b	0	0
	Apigenin+	17.79 ± 2.59 a	0 b	0 b	0	0
	6-O-Methylscutellarein+	18.35 ± 3.44 a	0 b	0 b	0	0
LP	Luteolin-7-glucoside-3'-glucuronide*	78.67 ± 8.39 a	30.84 ± 1.90 b	14.4 ± 2.2 b	39.19	18.3
	6-O-Methylscutellarein+	19.28 ± 1.59 a	0 b	0 b	0	0

[a,b,c] Means that do not share a letter are significantly different ($P < 0.05$)

* ug Luteolin equivalents

+ ug Apigenin equivalents

*+ug Rosmarinic acid equivalents



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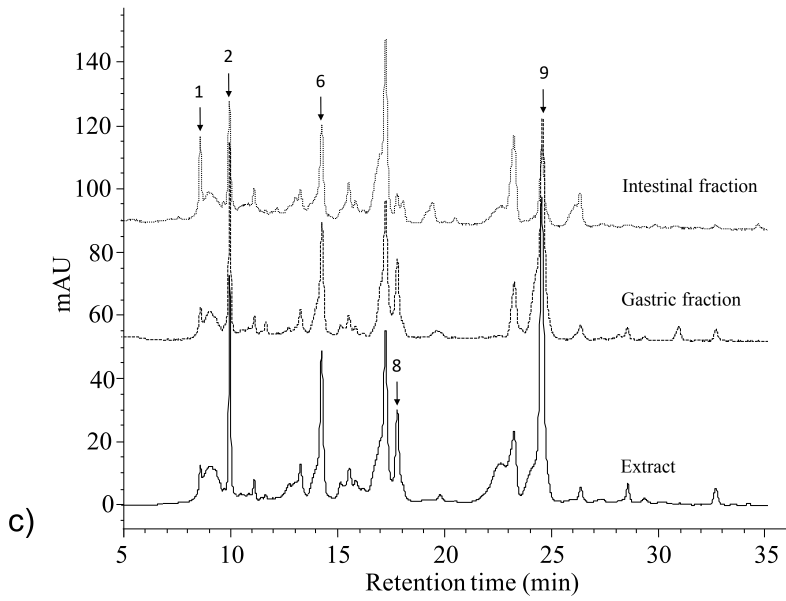

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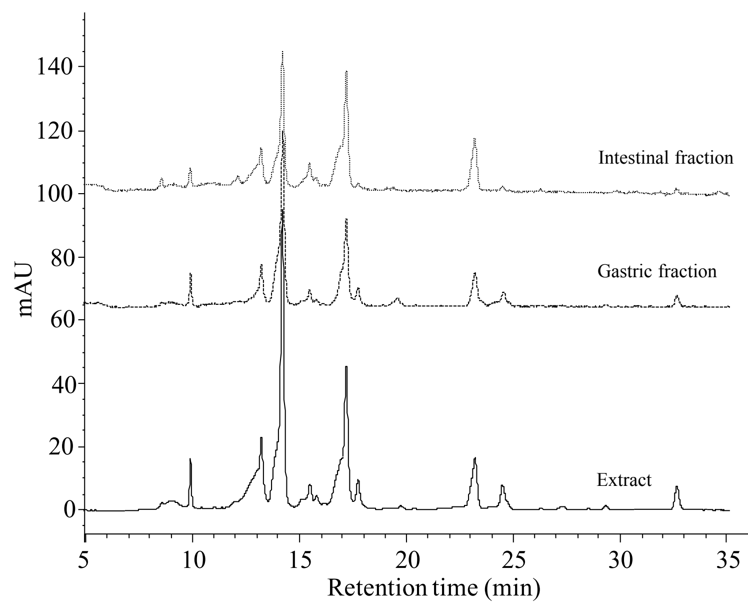

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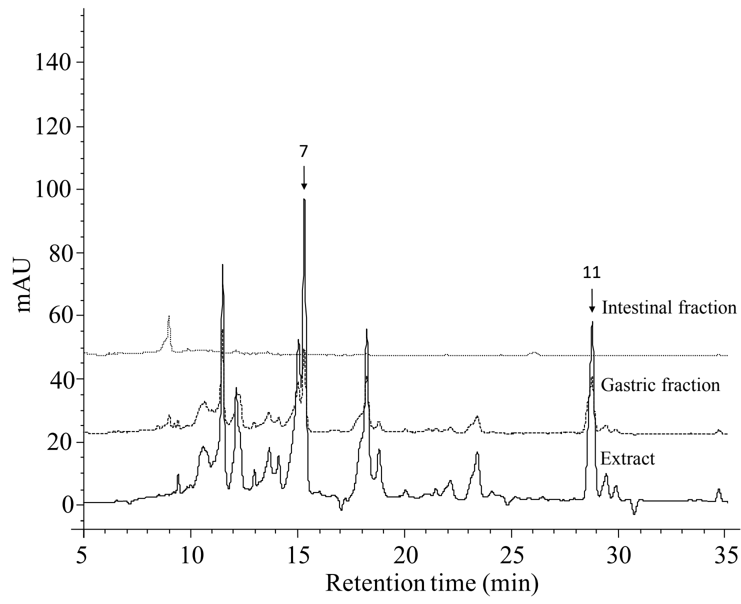
6 Figure 7. Base peak HPLC chromatograms at a) 280 nm, b) 320 nm and c) 365 nm with
7 the identification of polyphenol compounds detected in *Hedeoma patens* before and
8 after *in vitro* digestion. Peaks are labelled as described in Table 9 with components
9 appearing in undigested and digested samples.



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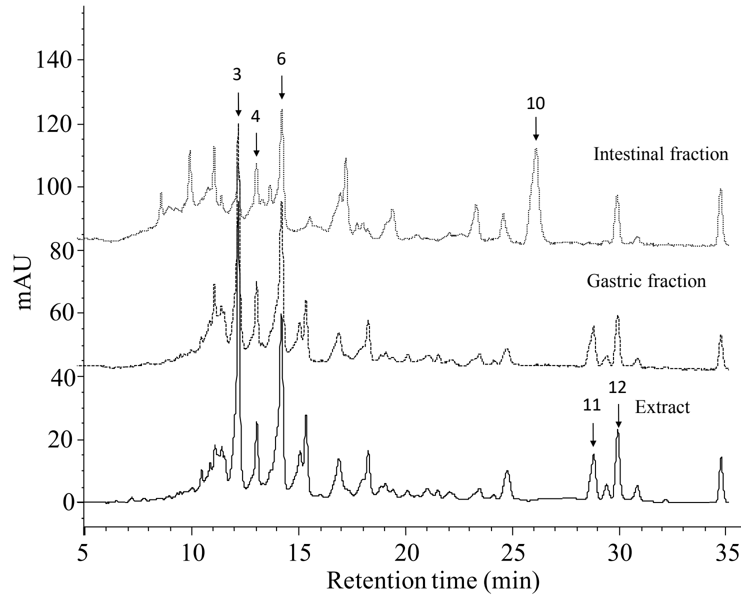

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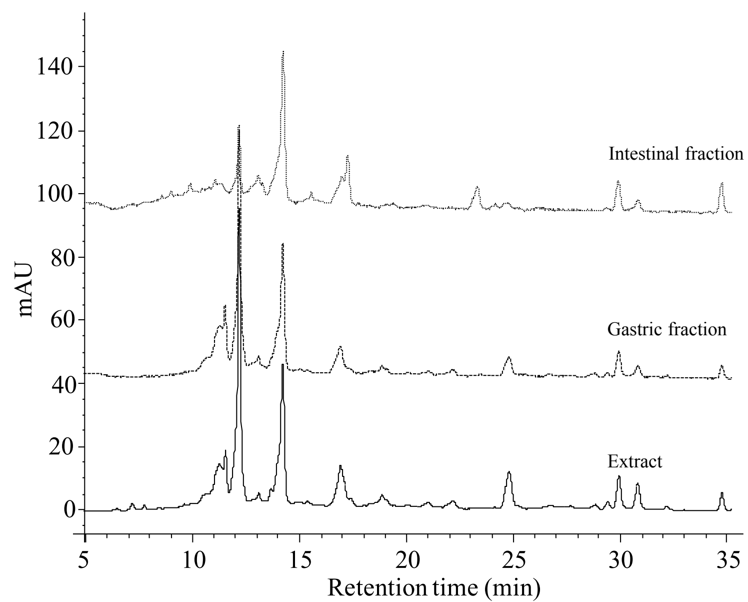

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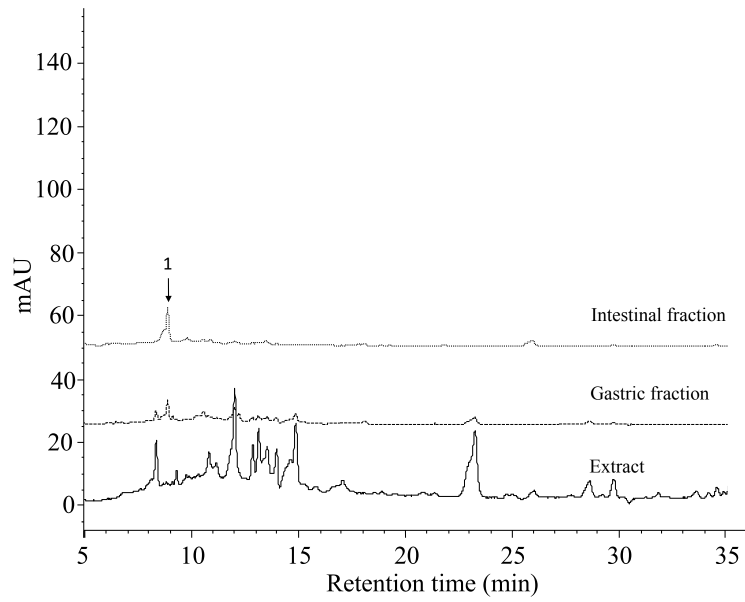
15 Figure 8. Base peak HPLC chromatograms at a) 280 nm, b) 320 nm and c) 365 nm with
16 the identification of polyphenol compounds detected in *Lippia graveolens* before and
17 after *in vitro* digestion. Peaks are labelled as described in Table 9 with components
18 appearing in undigested and digested samples.

19



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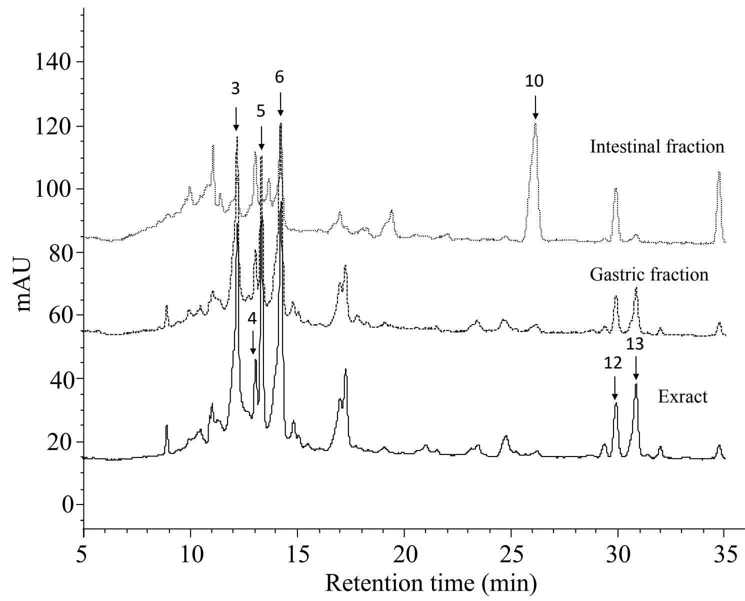

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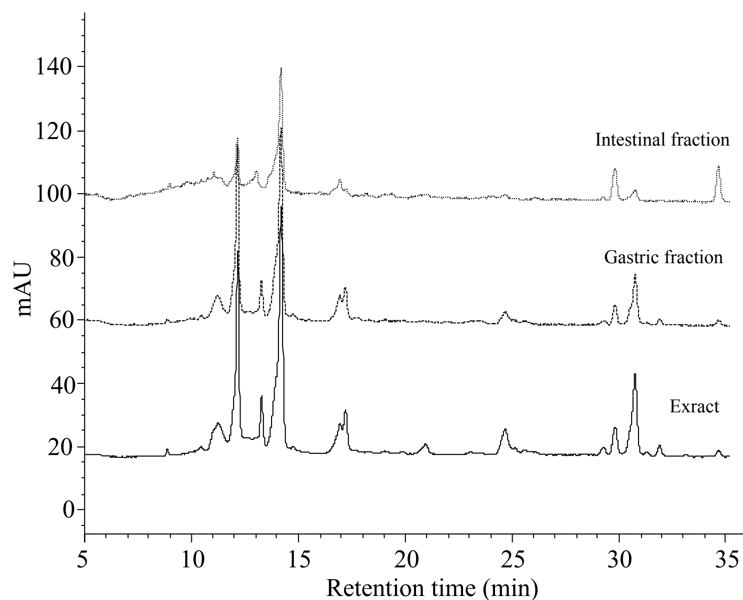

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24 Figure 9. Base peak HPLC chromatograms at a) 280 nm, b) 320 nm and c) 365 nm with
25 the identification of polyphenol compounds detected in *Lippia palmeri* before and after
26 *in vitro* digestion. Peaks are labelled as described in Table 9 with components
27 appearing in undigested and digested samples.

28

3.3 Total Reducing Capacity and Total Flavonoid Content.

The Folin-Ciocalteu assay is typically used as an inexpensive and rapid method to measure the content of total phenolics in a sample. However, the method is not specific to phenolics, and it is more appropriate to consider the technique as a method to evaluate the total reducing capacity of a sample (Sánchez-Rangel et al., 2013). The total reducing capacity and total flavonoid content of oregano polyphenols is presented in Table 11. The highest total reducing capacity is shown for the non-digested samples of LG > HP > LP at a concentration of 400 μg of dried extract ($P < 0.05$). Similarly, the highest total flavonoid content is presented for the non-digested samples of LG > HP = LP ($P < 0.05$). Simulated gastrointestinal digestion had a significant effect on the TRC and TFC of the samples ($P < 0.05$).

3.4. Inhibition of the DPPH Radical.

The antioxidant capacity of oregano polyphenols as evaluated by the inhibition of the DPPH radical is presented in Table 3. In accordance with the TRF and TFC results, the inhibition of the DPPH free radical as the percentage of inhibition and as mM TE.400 μg^{-1} dried extract was the highest for HP, LG and LP without statistical significance ($P < 0.05$). GID had a significant effect ($P < 0.05$) on the antioxidant capacity of the samples.

Table 11. Total flavonoid content, total reducing capacity, and antioxidant capacity of dried polyphenolic extracts of *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri* at a concentration of 400 μg dried extract from oregano leaves.

Oregano	Phase	TFC (mg QE)	TRC (mg GAE)	% Inhibition of DPPH	DPPH (mM TE)
Hedeoma patens	Undigested	0.4814 ± 0.0783 a f	0.6499 ± 0.048 a f	90.98 ± 1.46 a f	2.2724 ± 0.018 a f
	Gastric	0.0314 ± 0.0011 b	0.0772 ± 0.002 b	33.04 ± 2.83 b	1.5612 ± 0.035 b
	Intestinal	0.025 ± 0.0038 b	0.0708 ± 0.006 b	16.18 ± 2.87 c	1.3543 ± 0.035 c
Lippia graveolens	Undigested	1.0285 ± 0.0527 a f	0.9419 ± 0.091 a f	90.45 ± 0.12 a f	2.2658 ± 0.002 a f
	Gastric	0.0495 ± 0.0030 b	0.1045 ± 0.009 b	26.40 ± 3.32 b	1.4798 ± 0.041 b
	Intestinal	0.0199 ± 0.0021 b	0.0730 ± 0.002 b	14.95 ± 3.31 c	1.3392 ± 0.041 c
Lippia palmeri	Undigested	0.6469 ± 0.0 a f	0.4930 ± 0.005 a f	90.27 ± 0.47 a f	2.2636 ± 0.006 a f
	Gastric	0.0295 ± 0.0032 b	0.0532 ± 0.009 b	16.67 ± 1.76 b	1.3604 ± 0.022 c
	Intestinal	0.0351 ± 0.0059 b	0.2543 ± 0.033 c	27.10 ± 3.51 c	1.4884 ± 0.043 b

[a,b,c] Means that do not share a letter are significantly different by Tukey´s test ($P < 0.05$)

[f,g,h] Means that do not share a letter are significantly different by Dunnett´s test (Control: undigested sample; $P < 0.05$)

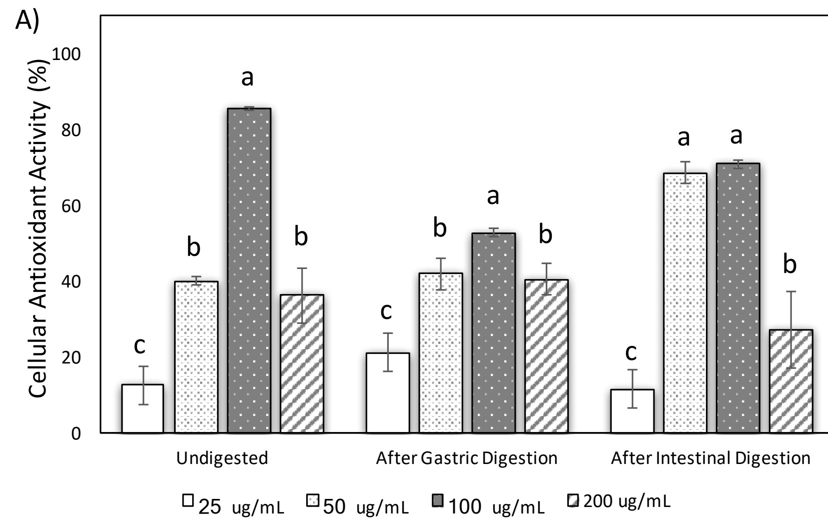
3.5. Cellullar Antioxidant Activity

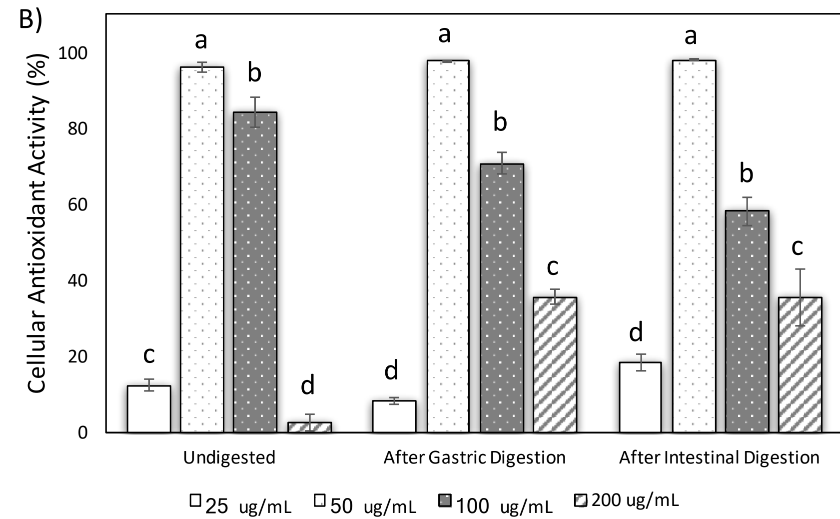
As reported by Wan et al. (2015), the antioxidant activity of a sample as assessed by the CAA assay is determined by measuring the prevention of the oxidation of 2’,7’-dichlorofluorescin diacetate to 2’,7’-dichlorofluorescin through quenching of the peroxyl radical ($RO_2^•$), which is reflected in a reduction in cellular fluorescence.

The cellular antioxidant activity of oregano polyphenols is presented in Figure 17. Our results demonstrated that the cellular antioxidant activity is concentration-dependent ($P < 0.05$) and significantly ($P < 0.05$) affected by the type of oregano species and the gastrointestinal digestion. The highest cellular antioxidant activity ($P < 0.05$) in Hedeoma patents was observed at 100 μg.mL^{-1} in the undigested fraction with 85.51% of CAA units. On the other hand, the highest CAA for *Lippia graveolens* was reported for the intestinal, gastric, and undigested fractions at 50 μg.mL^{-1} with CAA units of 97.94%, 97.72%, and 96.16%, respectively. *Lippia palmeri* samples exhibited the highest CAA values in the undigested, gastric, and intestinal fractions at 50 μg.mL^{-1} with CAA units of 97.96%, 97.56%, and 97.47%, respectively. Furthermore, when all treatments were compared, the highest CAA ($P < 0.05$) was observed at the concentration of 50 μg.mL^{-1} of dried extract of the non-digested LP fraction (97.96%), the LG intestinal fraction (97.94%), the LG and LP gastric fractions (97.72% and 97.56%, respectively), and the LP intestinal fraction (97.47%). It is important to mention that prior to the CAA assay, a cell-viability assay demonstrated > 80% cell viability in all oregano species (HP, LG, LP) at 100 μg.mL^{-1} (data not reported).

To evaluate the possible role of the main polyphenolic constituents on the CAA of our samples, we tested the CAA of the major available individual phenolics, namely, apigenin, luteolin, quercetin and rosmarinic acid, at different concentrations (25, 50, 100 and 200 μg.mL^{-1}). We observed that the CAA of the individual polyphenols was concentration-dependent ($P < 0.05$). Specifically, quercetin 200 μg.mL^{-1} (CAA 92.21%) > quercetin 100 μg.mL^{-1} (CAA 90.35%) > luteolin 200 μg.mL^{-1} (CAA 85.50%) >

rosmarinic acid 50 μg.mL^{-1} (CAA 81.45%) > luteolin 100 μg.mL^{-1} (CAA 81.04%) > quercetin μg.mL^{-1} (CAA 79.71%) exhibited the highest CAA values ($P < 0.05$). In this regard, quercetin, luteolin and rosmarinic acid have been reported as potent antioxidant molecules in different systems (Brewer, 2011; Rubió et al., 2013; Wolfe and Liu, 2007). Thus, it might be possible that based on the CAA of individual polyphenols, the antioxidant activity of our oregano samples could be due to a synergistic action of polyphenols (Seelinger et al., 2008).


A)
Cellular Antioxidant Activity (%)
0
20
40
60
80
100
c
b
a
b
c
b
a
b
c
a
a
b
Undigested
After Gastric Digestion
After Intestinal Digestion
25 ug/mL
50 ug/mL
100 ug/mL
200 ug/mL


B)
Cellular Antioxidant Activity (%)
0
20
40
60
80
100
c
a
b
d
d
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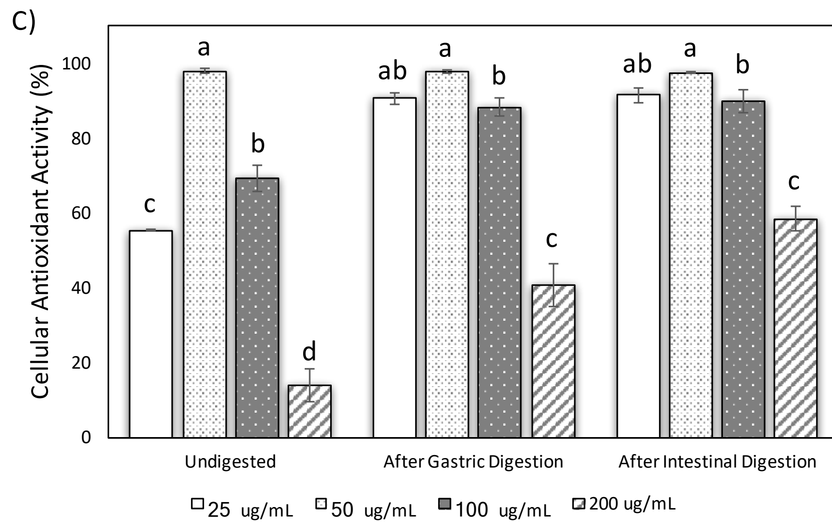
c
Undigested
After Gastric Digestion
After Intestinal Digestion
25 ug/mL
50 ug/mL
100 ug/mL
200 ug/mL


C)
Cellular Antioxidant Activity (%)
0
20
40
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Undigested
After Gastric Digestion
After Intestinal Digestion
25 ug/mL
50 ug/mL
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200 ug/mL

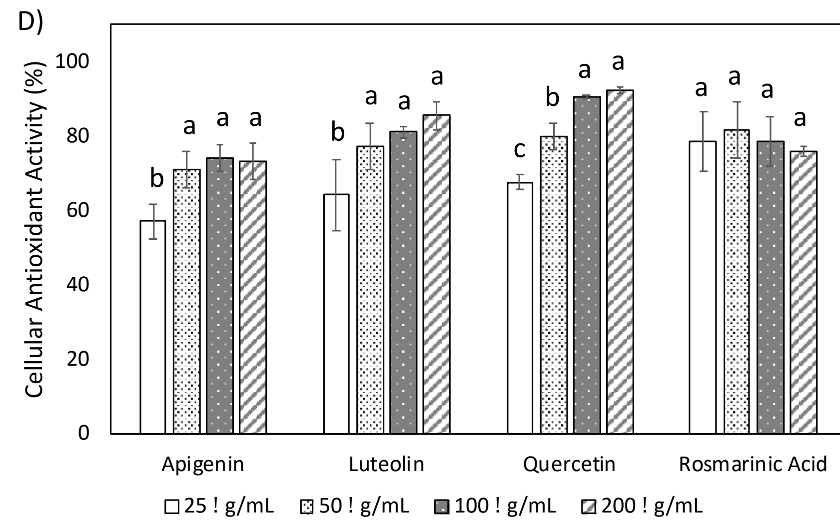
D)
Cellular Antioxidant Activity (%)
0
20
40
60
80
100
b
a
a
a
b
a
a
a
c
b
a
a
a
a
a
a
Apigenin
Luteolin
Quercetin
Rosmarinic Acid
25 ! g/mL
50 ! g/mL
100 ! g/mL
200 ! g/mL

Figure 10. Cellular antioxidant activity of polyphenol-rich extracts of A) *Hedeoma patens*, B) *Lippia graveolens*, C) *Lippia palmeri* before and after *in vitro* digestion at different concentrations, and D) Common standard polyphenols. Caco-2 cells were pre-incubated for 20 min with the polyphenol extracts of the oregano species *Hedeoma patens*, *Lippia graveolens*, and *Lippia palmeri* at 25, 50, 100 and 200 μg dried extract.mL^{-1}. We assessed the effect of the concentration of polyphenolic extract at each digestive phase in cell-based antioxidant activity reported as antioxidant percentage (%). Data obtained from three biological repeats (n =3) are shown as mean ± SD values. Different letters correspond to significant differences ($P < 0.05$) by one-way ANOVA followed by Tukey´s test.

3.6. Inhibition of Enzymes Involved in Carbohydrate and Lipid Metabolism.

The inhibition of pancreatic lipase, α-glucosidase and α-amylase is one the pharmacological effects of commonly used drugs against metabolic syndrome factors, such as high cholesterol, hyperglycemia or diabetes (Alberti et al., 2006).

3.6.1. Inhibition of α-Glucosidase

α-Glucosidase hydrolyzes the terminal, non-reducing 1,4-linked α-D-glucose residues of disaccharides, which releases α-glucose and hence enables gastrointestinal absorption (Hubatsch et al., 2007). The inhibitory capacity of polyphenolic extracts from three oregano species (HP, LG, and LP) at a concentration of 400 μg of dried extract on α-glucosidase is presented in Table 12 and is expressed as a percentage of inhibition (%).

Both the oregano species and the gastrointestinal digestion process had a significant effect ($P < 0.05$) on the inhibitory properties against α-glucosidase. The highest inhibition was revealed for the undigested samples from HP (63.332%) and LG (43.408%) followed by the gastric fraction from LP (19.7767%) ($P < 0.05$).

Overall, the α-glucosidase inhibitory activity of HP, LG and LP polyphenols significantly ($P < 0.05$) decreased during simulated gastrointestinal digestion with the exception of the gastric fraction from LP extracts. Regarding HP polyphenols, the inhibitory activity decreased significantly ($P < 0.05$) to 13.17% and 9.46% in the gastric and intestinal fractions, respectively. Regarding the digested samples of LG polyphenols, the inhibitory α-glucosidase activity decreased significantly ($P < 0.05$) to 16% and 7.4% in the gastric and intestinal fractions, respectively. Furthermore, the LP digested samples increased from 12.185% to 19.7767% in the gastric fraction follow by a decrease to 5.7663% in the intestinal fraction.

3.6.2. Inhibition of α-Amylase.

The inhibitory capacity of polyphenolic extracts from three oregano species (HP, LG, and LP) on pancreatic α-amylase is presented in Table 12 and is expressed as a percentage of inhibition (%). The type of oregano species and the simulated digestion process had a significant effect ($P < 0.05$) on α-amylase inhibition. In this regard, the oregano species and digestive fraction with the highest inhibition of α-amylase ($P < 0.05$) was Hedeoma patens and the intestinal fraction, respectively. Moreover, in contrast with the results of the inhibitory activity of α-glucosidase, α-amylase inhibition increased ($P < 0.05$) at the end of the intestinal fraction. Our results demonstrate the highest inhibitory rate of α-amylase for the intestinal fraction from HP extracts (52.53%) followed by the non-digested extract from LG (39.57%) and its corresponding intestinal fraction (39.1%) ($P < 0.05$).

3.6.3. Inhibition of Pancreatic Lipase.

Inhibiting the uptake of triglycerides is the main therapy for managing obesity. Thus, the inhibition of pancreatic lipase, a key enzyme in lipid metabolism, is crucial (Bello et al., 2017; Costamagna et al., 2016). The inhibitory capacity of polyphenols against pancreatic lipase is presented in Table 12. In accordance with the α-glucosidase and α-amylase inhibitory assays, the type of oregano species and GID had significant effects ($P < 0.05$) on the inhibition of pancreatic lipase. The highest inhibitory lipase activity was demonstrated for the intestinal fraction of Hedeoma patens (54.54%) followed by the intestinal fractions of LP and LG (49.73% and 47.37%, respectively). Interestingly, contrary to the α-glucosidase inhibitory activity, pancreatic lipase inhibition increased for HP, LG and LP polyphenolic extracts at the end of the intestinal fraction.

Table 12. Inhibitory effect of polyphenols from *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri* on enzymes related to carbohydrate and fat metabolism.

Oregano	Phase	α-Glucosidase Inhibition (%)	α-Amylase inhibition (%)	Lipase inhibition (%)
Hedeoma patens	Undigested	63.33 ± 2.04 a f	23.93 ± 1.18 a	21.95 ± 0.99 a f
	Gastric	13.17 ± 0.02 b	25.43 ± 1.12 a	33.70 ± 3.72 b
	Intestinal	9.47 ± 0.62 c	52.53 ± 1.52 b f	54.54 ± 0.47 c
Lippia graveolens	Undigested	43.41 ± 4.44 a f	39.57 ± 1.42 a	17.93 ± 3.84 a f
	Gastric	16.01 ± 0.53 b	20.30 ± 1.07 b f	31.88 ± 2.05 b
	Intestinal	7.40 ± 0.34 c	39.10 ± 3.79 a	47.37 ± 2.40 c
Lippia palmeri	Undigested	12.19 ± 0.46 a f	31.52 ± 0.47 a f	14.36 ± 1.97 a f
	Gastric	19.78 ± 1.21 b	25.95 ± 0,36 b	11.86 ± 0.15 a f
	Intestinal	5.77 ± 0.23 b	36.26 ± 3.08 c	49.73 ± 3.13 b
1 mM Acarbose		43.25 ± 2.46	76.31 ± 0.76	
400 µg Acarbose		26.62 ± 2.95		
Orlistat				95.55 ± 1.53

[a,b,c] Means that do not share a letter are significantly different by Tukey´s test ($P < 0.05$)

[f,g,h] Means that do not share a letter are significantly different by Dunnett´s test (Control: undigested sample; $P < 0.05$)

4. Discussion

Oregano polyphenols have been the object of numerous studies due to their attributed bioactive properties. In this sense, the use of HPLC-DAD and LC-TOF techniques is a valuable tool in the identification of the main constituents of plant extracts (Zhou et al., 2009). The main polyphenolic compounds identified in *Hedeoma patens*, *Lippia graveolens* and *Lippia palmeri* are derivatives of flavones, flavonols and phenolic acids. This finding is consistent with our previous reports (Gutiérrez-Grijalva et al., 2017; Leyva-López et al., 2016) and that reported for different oregano species (Lin et al., 2007; Martins et al., 2014). However, for polyphenols to exert any biological activity, they need to be bioaccessible in the gastrointestinal tract and then be absorbed in the small intestine to reach the systemic circulation, target tissues and organs within the body (Crozier et al., 2010; Gutiérrez-Grijalva et al., 2016; Manach et al., 2004). Therefore, we evaluated the HPLC-DAD and LC-TOF-MS profiles of oregano polyphenols after an *in vitro* gastrointestinal digestion process as a method to assess the bioaccessibility of oregano polyphenols.

Our analysis revealed that oregano polyphenols exhibited low *in vitro* bioaccessibility given that only 3 – 18% of luteolin-7-glucoside-3'-glucuronide was present in the intestinal fraction in HP, LG and LP samples. It has been reported that the gastrointestinal tract is a site with high oxidative stress due to its increased exposure to dietary pro-oxidants, such as iron, copper, lipid peroxides, and nitrosamines, that can lead to the formation of reactive species that cause oxidative damage (Halliwell et al., 2000). Hence, the presence of polyphenols from oregano in the gastric fraction could exert antioxidant activity and prevent the formation of damaging reactive species. Moreover, our data suggest that the decreased content of polyphenols after the *in vitro* GID process could be caused by pH changes during the gastrointestinal process, which has been reported to cause oxidation and racemization of polyphenolic compounds (Bermúdez-Soto et al., 2007; Chen et al., 2015; Gutiérrez-Grijalva et al., 2017; Wootton-

Beard et al., 2011). However, new strategies are currently being developed to avoid degradation of polyphenols during digestion, such as micro- and nanoencapsulation with different polymers (Lewandowska et al., 2013). Further research on this matter using oregano polyphenols is needed.

The aim of the total reducing capacity and total flavonoid content assays was to determine the total reducing capacity and total flavonoid content of the oregano extracts used for the enzyme inhibitory assays at 400 μg of dried extract. The decreased reducing capacity and flavonoid content evaluated by the colorimetric methods can be associated with the loss of polyphenols during the gastrointestinal digestion process (Gutiérrez-Grijalva et al., 2017; Wootton-Beard et al., 2011).

Contrary to chemical antioxidant assays (DPPH, ABTS, ORAC, etc.), the measurement of antioxidant capacity using mammal cells, such as HepG2 and Caco-2, takes into consideration physiological conditions, such as pH, temperature, bioavailability, cellular metabolism and metabolism of the tested antioxidant compounds (Wolfe and Liu, 2007). Additionally, to our knowledge, this is the first report on cellular antioxidant activity on oregano polyphenols during simulated gastrointestinal digestion. Our results revealed that the effects of the *in vitro* GID process on the CAA of the three oregano species were similar to those on the chemical-based antioxidant assays previously reported by our group (Gutiérrez-Grijalva et al., 2017). As the CAA of oregano polyphenols was enhanced by the gastrointestinal process, these phenomena have been commonly attributed to the possible formation of new compounds with improved antioxidant activity as a result of the pH shift in each digestive phase (Friedman and Jürgens, 2000; Wootton-Beard et al., 2011). This outcome has also been reported in Sea buckthorn berries by Guo et al. (2017), where the increased CAA during the GID was attributed to the formation of flavonoid aglycones from glycosylated compounds. Similar results were reported by Huang et al. (2014) in Chinese bayberry. Specifically, increased CAA values were noted after the digestion process and were correlated with the presence of more bioactive phenolics. In addition, our CAA evaluation of the polyphenol standards quercetin, luteolin, rosmarinic acid and apigenin could suggest that these compounds

might be somewhat related to the CAA properties of oregano polyphenolic extracts, whereas a possible synergistic action has been proposed in several studies regarding the antioxidant capacity of polyphenol-enriched extracts. Further studies are needed to assess this possibility (Brewer, 2011; Seelinger et al., 2008). Remarkably, as previously mentioned in Section 3.5, some of our oregano samples exhibited greater CAA values than quercetin, which has been recommended as a standard in this assay given its high antioxidant activity due to the strong conjugative stabilization of its heterocyclic ring (Wolfe and Liu, 2007; Yuwang et al., 2018). This finding could suggest the potential use of oregano polyphenols as a rich source of antioxidants.

As mentioned in Section 3.6, simulated gastrointestinal digestion significantly affected ($P < 0.05$) CAA and inhibition of enzymes involved in carbohydrate and fat metabolism, which are typically targeted in the management of metabolic syndrome, such as hyperglycemia, diabetes and high cholesterol. Our results demonstrated that the inhibitory potential of HP, LG and LP polyphenols against α-amylase and pancreatic lipase is enhanced at the end of the intestinal fraction. In this regard, some authors, such as Costamagna et al. (2016), have suggested that hydrolyzation of phenolic compounds during digestion leads to the accumulation of shorter phenolic groups, which could hinder or enhance their biological properties. This finding could explain the high cellular antioxidant activity and inhibition of α-amylase and pancreatic lipase in the intestinal fractions. However, at this stage, it is not possible to determine the specific compounds responsible for this activity.

5. Conclusion

Together, our data provide *in vitro* evidence of the low bioaccessibility of oregano polyphenols given that most polyphenols from HP, LG, and LP could not be identified at the end of the intestinal fraction with the exception of luteolin-7-glucoside-3’-glucuronide. However, our results suggest that the oregano species *Hedeoma patens*,

Lippia graveolens and *Lippia palmeri* have prominent cellular antioxidant activity and are able to *in vitro* inhibit enzymes involved in carbohydrate and lipid metabolism. These findings could suggest the potential use of oregano polyphenols as hypoglycemic and hypolipidemic agents. Additionally, these properties are enhanced in an *in vitro* gastrointestinal digestion model. However, further *in vitro* and *in vivo* research is needed to escalate the use of oregano polyphenols in human health in the prevention/treatment of metabolic syndrome conditions, such as hyperglycemia, hyperlipidemia, and diabetes, and its comorbidities.

Conflict of Interest

The authors declare that there are no conflicts of interest

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