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FACULTAD DE FARMACIA

DEPARTAMENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA

Providing scientific evidence for the use of dietary supplements: a case study in hepatoprotective plants

DOCTORAL THESIS

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Salamanca, 11 de enero de 2016

Celestino Santos-Buelga

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Lillian Bouçada de Barros

The studies developed within this PhD thesis led to the following articles, copies of which are included as annexes in the present memory:

[1] Pereira, C., Barros, L., Ferreira, I.C.F.R. 2015. Extraction, identification, fractionation and isolation of phenolic compounds in plants with hepatoprotective effects. DOI 10.1002/jsfa.7446

[2] Pereira, C., Barros, L., Ferreira, I.C.F.R. 2014b. Analytical tools used to distinguish chemical profiles of plants widely consumed as infusions and dietary supplements: artichoke, milk thistle and borututu. Food Anal. Methods 7, 1604-1611.

[3] Pereira, C., Calhelha R.C., Antonio A.L., Queiroz M.J.R.P., Barros L., Ferreira, I.C.F.R. 2014. Effects of gamma radiation on chemical and antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity of borututu. Innov. Food Sci. Emerg. Tech. 26, 271-277.

[4] Pereira, C., Calhelha, R.C., Barros, L., Ferreira, I.C.F.R. 2013b. Antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity of artichoke, milk thistle and borututu. Ind. Crop. Prod. 49, 61-65.

[5] Pereira, C., Calhelha, R.C., Barros, L., Queiroz, M.J.R.P., Ferreira, I.C.F.R. 2014a. Synergisms in antioxidant and anti-hepatocellular carcinoma activities of artichoke, milk thistle and borututu syrups. Ind. Crop. Prod. 52, 709-713.

[6] Pereira, C., Barreira, J.C.M., Calhelha, R.C., Queiroz, M.J.R.P., Barros, L., Ferreira, I.C.F.R. 2014. New insights into the effects of formulation type and compositional mixtures on the antioxidant and cytotoxic activities of dietary supplements based-on hepatoprotective plants. Food Funct. 5, 2052-2060.

[7] Pereira, C., Barreira, J.C.M., Calhelha, R.C., Lopes, M., Queiroz, M.J.R.P., Vilas-Boas, M., Barros, L., Ferreira, I.C.F.R. 2015. Is honey able to potentiate the antioxidant and cytotoxic properties of medicinal plants consumed as infusions for hepatoprotective effects? Food Funct. 6, 1435-1442.

[8] Pereira, C., Barros, L., Carvalho, A.M., Santos-Buelga, C., Ferreira, I.C.F.R. 2015. Infusions of artichoke and milk thistle represent a good source of phenolic acids and flavonoids. Food Funct. 6, 56-62.

[9] Pereira, C., Barros, L., Alves, M.J., Pereira, L., Santos-Buelga, C., Ferreira, I.C.F.R. 2015. Phenolic profile and antimicrobial activity of different dietary supplements based on *Cochlospermum angolensis* Welw. Ind. Crop. Prod. 74, 412-416.

Two more articles are in process of peer-revision:

[1] Pereira, C., Barros, L., Ferreira, I.C.F.R. Dietary supplements: foods, medicines, or both? A controversial designation with unspecific legislation.

[2] Pereira, C., Barros, L., Ferreira, I.C.F.R. Phenolic profile and antimicrobial activity of different dietary supplements based on *Cynara scolymus* L. and *Silybum marianum* (L.) Gaertn.

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ABBREVIATIONS

4-HNE	4-hydroxynonenal
AH	Aniline hydroxylase
ALAT	Alanine aminotransferase
Alb	Albumin
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ASAT	Aspartate aminotransferase
AST	Aspartate transaminase
BUN	Blood urea nitrogen
CA	Columbia agar
CAT	Catalase
COLOC	Correlation by means of long range coupling
COSY	Correlation spectroscopy
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DSHEA	Dietary Supplement Health and Education Act 1994
EC	European Commission
EDTA	Ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESBL	*Escherichia coli* spectrum extended producer of β-lactamases
ESI-MS	Electrospray ionization mass spectrometry
EU	European Union
FAB-MS	Fast atom bombardment mass spectrometry
FAME	Fatty acids methyl ester
FBS	Foetal bovine serum
FD&C Act	Food, Drug and Cosmetic Act
FDA	Food and Drug Administration
FSD	Food Supplements Directive
FT-IR	Fourier transform infrared spectroscopy
G-6-P	Glucose-6-phosphatase
GGT	γ-Glutamyltransferase

GSH	Glutathione
GSH-Px	Glutathione peroxidase
HBSS	Hank's balanced salt solution
HBV	Hepatitis B virus
HDL	High-density lipoprotein
HETCOR	Heteronuclear chemical shift correlation
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
HPLC-DAD	High-performance liquid chromatography with diode-array detection
HR-ESI-MS	High resolution electrospray ionisation mass spectrometry
INEPT	Insensitive nuclei enhanced by polarization transfer
INT	Iodonitrotetrazolium chloride
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LOOH	Lipid hydroperoxides
LPO	Lipid peroxidation
MDA	Malondialdehyde
MHB	Mueller Hinton broth
MPLC	Medium pressure liquid chromatography
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
N-de	Amidopyrine-*N*-demethylase
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
PBS	Phosphate buffered saline
PGT	Glutamic-pyruvic transaminase
PTLC	Preparative thin-layer chromatography
PUFA	Polyunsaturated fatty acids
SD	Standard deviation
SGOT	Serum glutamate oxaloacetate transaminase
SGPT	Serum glutamate pyruvate transaminase
SOD	Superoxide dismutase
SRB	Sulforhodamine B
TB	Total bilirubin
TBARS	Thiobarbituric acid reactive substances
TC	Total cholesterol

TCA	Trichloroacetic acid
TG	Triglyceride
THMP	Traditional Herbal Medicinal Products
TLC	Thin-layer chromatography
TP	Total protein
USA	United States of America
WCB	Wilkins–Chalgren broth
γ-GTP	γ-Glutamyl transpeptidase

ABSTRACT

Medicinal plants have been used worldwide for centuries for nutritional and medicinal purposes. Among them, artichoke, milk thistle, and borututu are used for hepatoprotective effects, in the prevention and treatment of liver diseases. These plants are available in several formulations to an easier consumption, but there were no scientific studies supporting their quality and efficiency. In this connection, the present study was designed to clarify the differences between these formulations regarding to antioxidant, anti-hepatocellular carcinoma, and antimicrobial activities, as well as to their chemical composition, namely in sugars, organic acids, tocopherols, fatty acids, and phenolic compounds. The bioactivity of several mixtures containing different proportions of the mentioned formulations, as well as the effect of honey addition, were also assessed. Moreover, a preservation technique was applied to borututu dry material, gamma irradiation, and the samples were analysed in order to verify the effects of radiation on the chemical composition and bioactivity of borututu.

In a general way, the studied plants revealed carbohydrates as the major components, with borututu revealing the highest energetic contribution with the highest content of carbohydrates and fat, sucrose and total sugars, shikimic and citric acids, α-, *β*-, δ- and total tocopherols. Artichoke had the highest ash and protein contents, oxalic acid, SFA (mainly palmitic acid acid), and γ-tocopherol, as also the best n6/n3 ratio. Milk thistle showed the highest levels of fructose and glucose, quinic acid and total organic acids, PUFA, mainly linoleic acid, and the best PUFA/SFA ratio. Gamma irradiation did not appreciably affect borututu chemical composition, but the highest energetic contribution, total sugars, organic acids, tocopherols, and PUFA contents were detected in the sample irradiated at maximum dose (10 kGy), which indicates a preservation effect of the radiation to these molecules. This sample also presented the highest levels of total phenolics and flavonoids and the highest antioxidant activity. Irradiated samples kept the anti-hepatocellular carcinoma activity, despite the decrease observed in the methanolic extract prepared from the above referred sample. All of the single plant infusions, pills, and syrups revealed antioxidant properties with EC_{50} values lower than the daily recommended dose, but infusions and syrups showed higher antioxidant activity than pills. Regarding to the prepared mixtures, all of them revealed synergistic effects for antioxidant activity assays, and also in several assays regarding

hepatocellular carcinoma toxicity, when compared to the activity of single plants. The results obtained in this work also proved the utility of honey addition to potentiate the antioxidant and cytoprotective properties of medicinal plant based infusions. Lastly, concerning the phenolic composition, these plants revealed to be a good source of these bioactive molecules, with prevalence of phenolic acids and flavonoids. Among the studied formulations, infusions presented the higher phenolic compounds amounts, with luteolin-7-*O*-glucuronide and luteolin-7-*O*-glucoside as the major flavonoids found in artichoke infusion, apigenin-7-*O*-glucuronide, luteolin-7-*O*-glucuronide, and apigenin-O-deoxyhexosyl-glucuronide as the main constituents of milk thistle infusion, and protocatechuic acid as the most abundant compound in borututu infusion. Accordingly, the antimicrobial activity of the three formulations followed the same tendency, with infusions presenting higher activity, which can be related to the recognized antimicrobial capacity of some phenolic compounds found in these formulations. The results obtained in this study might be a real asset in the choice of the best formulation of artichoke, borututu, and milk thistle, providing detailed information about the chemical composition of the plants and the bioactivity variation associated to the use of the different kinds of formulations, either as single and as combined preparations.

SUMARIO

Las plantas medicinales han sido ampliamente utilizadas por la humanidad con fines farmacológicos y nutricionales. Entre ellas, la alcachofa (*Cynara scolymus* L.), el cardo mariano (*Silybum marianum* (L.) Gaertn) y el borututu (*Cochlospermum angolensis* Welw.) se han empleado por sus putativos efectos hepatoprotectores para la prevención y tratamiento de enfermedades hepáticas. Diferentes preparaciones de estas plantas se encuentran comercializadas como suplementos dietéticos bajo diferentes formulaciones, como infusiones, cápsulas o jarabes. Sin embargo, existen pocas evidencias científicas que demuestren su calidad y eficacia. En el presente estudio se planteó como objetivo evaluar la bioactividad de formulaciones de estas plantas, solas y en diferentes mezclas, y, en particular, de sus propiedades antioxidantes, hepatoprotectoras y antimicrobianas, así como caracterizar su composición en azúcares, ácidos orgánicos, tocoferoles, ácidos grasos y compuestos fenólicos. Además, se evaluó el efecto de la adición de miel sobre la bioactividad, en el caso de las infusiones, y también se exploró la utilización de la irradiación con rayos gamma como técnica para la conservación de material desecado de borututu, valorando su efecto sobre su composición química y bioactividad.

En lo relativo a la composición química, las tres plantas presentaban los carbohidratos como componentes mayoritarios. Entre ellas, el borututu poseía los contenidos más elevados de carbohidratos, sacarosa y azúcares totales, grasas, ácidos cítrico y siquímico, y tocoferoles α-, *β*-, δ- y totales. La alcachofa mostraba los mayores contenidos de minerales, proteínas, ácido oxálico, γ-tocoferol y ácidos grasos saturados (SFA), principalmente ácido palmítico, así como la mejor relación entre ácidos grasos n6 y n3. Por su parte, el cardo mariano tenía los niveles más altos de fructosa y glucosa, ácido quínico, ácidos orgánicos totales y ácidos grasos poliinsaturados (PUFA), mayoritariamente ácido linoleico, junto con el mejor ratio PUFA/SFA.

El tratamiento con radiación gamma no afectó apreciablemente a la composición química del borututu, encontrándose incluso las mayores concentraciones totales de azúcares, ácidos orgánicos, tocoferoles y PUFA en la muestra irradiada a la concentración más elevada (10 kGy) entre las ensayadas, lo que sugería un efecto conservador de la irradiación sobre esas moléculas. Esa muestra presentaba también los

niveles más elevados de compuestos fenólicos y flavonoides totales y de actividad antioxidante. Además, las muestras irradiadas mantenían la actividad inhibitoria sobre el crecimiento en células HepG2 de carcinoma hepático.

Todas las formulaciones de cada una de las plantas mostraban una actividad antioxidante satisfactoria, mayor en las infusiones y jarabes que en las cápsulas. En las formulaciones elaboradas con mezclas de las tres plantas se observó un efecto sinérgico sobre la actividad antioxidante, y en algunos casos también sobre la actividad inhibitoria del crecimiento en células de carcinoma hepático, en relación con lo encontrado en las preparaciones de una única planta. Además, se encontró que la adición de miel a las infusiones potenciaba las propiedades antioxidantes y citoprotectoras.

Las tres plantas estudiadas demostraron ser buenas fuentes de compuestos fenólicos, tanto derivados de ácidos fenólicos como flavonoides. Entre las diferentes formulaciones, las infusiones fueron las que presentaron mayores concentraciones de compuestos fenólicos, siendo luteolina-7-*O*-glucurónido y luteolina-7-*O*-glucósido los flavonoides mayoritarios en la infusión de alcachofa, mientras que apigenina-7-*O*-glucurónido, luteolina-7-*O*-glucurónido y apigenina-*O*-desoxihexosil-glucurónido lo eran en la infusión de cardo mariano, y el ácido protocatéquico en la de borututu. En general, las formulaciones con mayores contenidos fenólicos eran las que presentaban la actividad antimicrobiana más elevada, lo que podría estar relacionado con los efectos antimicrobianos demostrados en otros estudios para diversos compuestos fenólicos.

En su conjunto, en este estudio se aporta información detallada cobre la composición nutricional y fitoquímica de tres plantas (alcachofa, cardo mariano y borututu), así como sobre su bioactividad asociada al uso de distintos tipos de formulaciones, ya sea basadas en una única planta como combinadas entre sí, apoyando de este modo su empleo con fines medicinales y como suplementos dietéticos.

CHAPTER 1

Background

1.1. Dietary supplements

1.1.1. Widespread use of dietary supplements

An adequate and balanced nutrition is considered the key to maintain the normal body functions, prevent diseases, and age healthily. Nevertheless, there are several cases of malnutrition due to insuficient, excessive or imbalanced intake of a range of nutrients present in food (Allen et al., 2013). This deficient nutrition can have different origins such as medical (anorexia, nausea and vomiting, gastrointestinal dysfunction, physical disability or inability to feed oneself, among others) and/or environmental causes (inadequate food quality or availability, irregular meal times, etc.), or altered requirements (modified substrate demands in critical illness, or increased energy expenditure) (Saunders et al., 2011). For that kind of reasons, or even just to improve or maintain the overall health, wellness and mental conditions, balance the diet, perk up the appearance, boost the performance or delay the onset of age-related diseases, the use of dietary supplements has tremendously increased in the last decades (Reay et al., 2005; Nichter and Thompson, 2006; Bailey et al., 2013). Nowadays, these supplements are widely used as a form of nutrition by both healthy people and individuals with a variety of health issues because they tend to be perceived as natural and safe; nonetheless, the most prevalent use remains in patient populations who are hospitalized or at risk of hospitalization, such as users of prescription medication, patients with chronic conditions, and the elderly (Gardiner et al., 2006). Among their various applications, the most common are weight loss (Chang and Chiou, 2014; Gambero and Ribeiro, 2015), diabetes (González-Ortiz et al., 2015), dementia (Allen et al., 2013), cognition (Clement et al., 2011), prostatic hyperplasia (Kim et al., 2012), epilepsy (Lee and Chung, 2010), gastrointestinal issues (Lino et al., 2014), sexual performance (Balayssac et al., 2012), and bones and articulations (Challoumas et al., 2015; LeBoff at al., 2015) improvement, among others.

The growing interest on dietary supplements has led to the production and commercialization of a wide range of formulations containing vitamins, minerals, herbal preparations, or similar ingredients and, consequently, has facilitated their distribution in several points with free availability like pharmacies, health food stores, department stores, groceries, on the Internet, and even on the black market (Radimer et al., 2004). Whereas

the easy access to these products offers obvious advantages by simplifying the buying process of effective professionally counselled treatments, the fact that anyone can easily access a variety of supplements without medical prescription or advice can have several associated risks. Indeed, negative interactions with prescribed drugs, side effects, and other adverse effects have been noted, and once complementary and alternative medicine is not currently integrated with conventional medicine, a specific legislation for dietary supplements with medicinal purposes is increasingly necessary (Eisenberg et al., 1993; Izzo and Ernst, 2009; Wallace and Paauw, 2015).

1.1.2. Dietary supplements on the borderline between food and medicine legislation

It is estimated that in the United States of America (USA) and the European Union (EU), nearly half of the population currently uses dietary supplements and that in Europe the prevalence of consumption is especially localized in the northern countries (Bailey at al., 2013; Skeie et al., 2009). Still, the scope and definition of these supplements remains unclear, with variations among the applied legislation among different countries.

Since ancient times, natural products such as plants and herbs have been used as healing agents, remaining the most common form of traditional medication worldwide, and with the organic chemistry advances, the industry has been able to prepare several synthetic medicines similar or identical to those found in natural sources, that is the case, for instance, of acetylsalicylic acid (Mahdi et al., 2006), immunosuppressive cyclosporines (Borel et al., 1990), anthracycline antibiotics (Nadas and Sun, 2006), or statins (Endo, 2004). Nonetheless, most of these natural products can also be incorporated in the human diet and, in this perspective, dietary supplements containing this sort of constituents do not exactly fit into neither medicine nor food scope, because pharmaceuticals are used to cure diseases or alleviate the symptoms of disease, whereas foods are primarily used to prevent diseases by providing the body with the optimal balance of macro and micronutrients needed for good health. Recently, the increased emergence of dietary supplements, and in particular those containing substances other that vitamins and minerals (that may exert both functions as nutrients and bioactives), on the market has blurred the distinction between pharmaceuticals and food, which place them in the borderline between food and drug legislation.

In order to provide an easier comparison between the USA and the EU legislation regarding dietary supplements, a thorough survey of the principal competent authorities' regulation documents (laws, regulations, directives, reports, etc.) has been conducted, with the aim of providing a definition of dietary supplements, the allowable substances on its manufacturing, and the marketing and labelling requirements depending on the area of marketing and on the classification of such products as food or medicines.

1.1.3. Definition of dietary supplements

In the USA, dietary supplements are mainly regulated by the Food and Drug Administration (FDA) (Figure 1) and have always been regulated as foods, with the enacting of the basic Food, Drug and Cosmetic Act (FD&C Act), in 1938. In this Act, Congress created a category of "foods for special dietary use" to include vitamin supplements, fortified foods or infant formula, which required a different label informing consumers about their special dietary properties. In 1941, the FDA proposed a new definition that was incorporated in Section 411 of the FD&C Act in 1976, under which special dietary uses included many specific functions like "supplying a vitamin, mineral, or other ingredient for use by man to supplement his diet by increasing the total dietary intake" (Food and Drug Administration, 1941; Public Law 94-278, 1976). Later, in order to formalise a number of regulatory statutes designed to ensure continued public access to a wide variety of dietary supplements and to provide consumers with more information about the intended use of dietary supplements, Dietary Supplement Health and Education Act 1994 (DSHEA) was enacted. According to DSHEA, a dietary supplement is "a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, an herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total dietary intake, or a concentrate, metabolite, constituent, extract, or combination of any of the aforementioned ingredients; it is deemed to be food, except for purposes of the drug definition". However, despite the modifications introduced by this Act, it did not change the longstanding classification of dietary supplements as a category of foods (Dietary Supplement Health and Education Act, 1994). Thus, hitherto the definition of this kind of supplements was considered by the Congress for three separate occasions: in 1938, in 1976, and in 1994, and on each occasion the conclusion was that the products were a subset of foods. Notwithstanding, the scope of these supplements have been

submitted to these reviews due to the fact that some of these products could be considered drugs if their labelling suggested that they could be used for "diagnosis, cure, mitigation, treatment, or prevention of disease", which is how drugs are defined in Section 201 (g) of the FD&C Act (www.fda.gov/RegulatoryInformation/Legislation/FederalFoodDrugandCosmeticActFDCAct/default.htm).

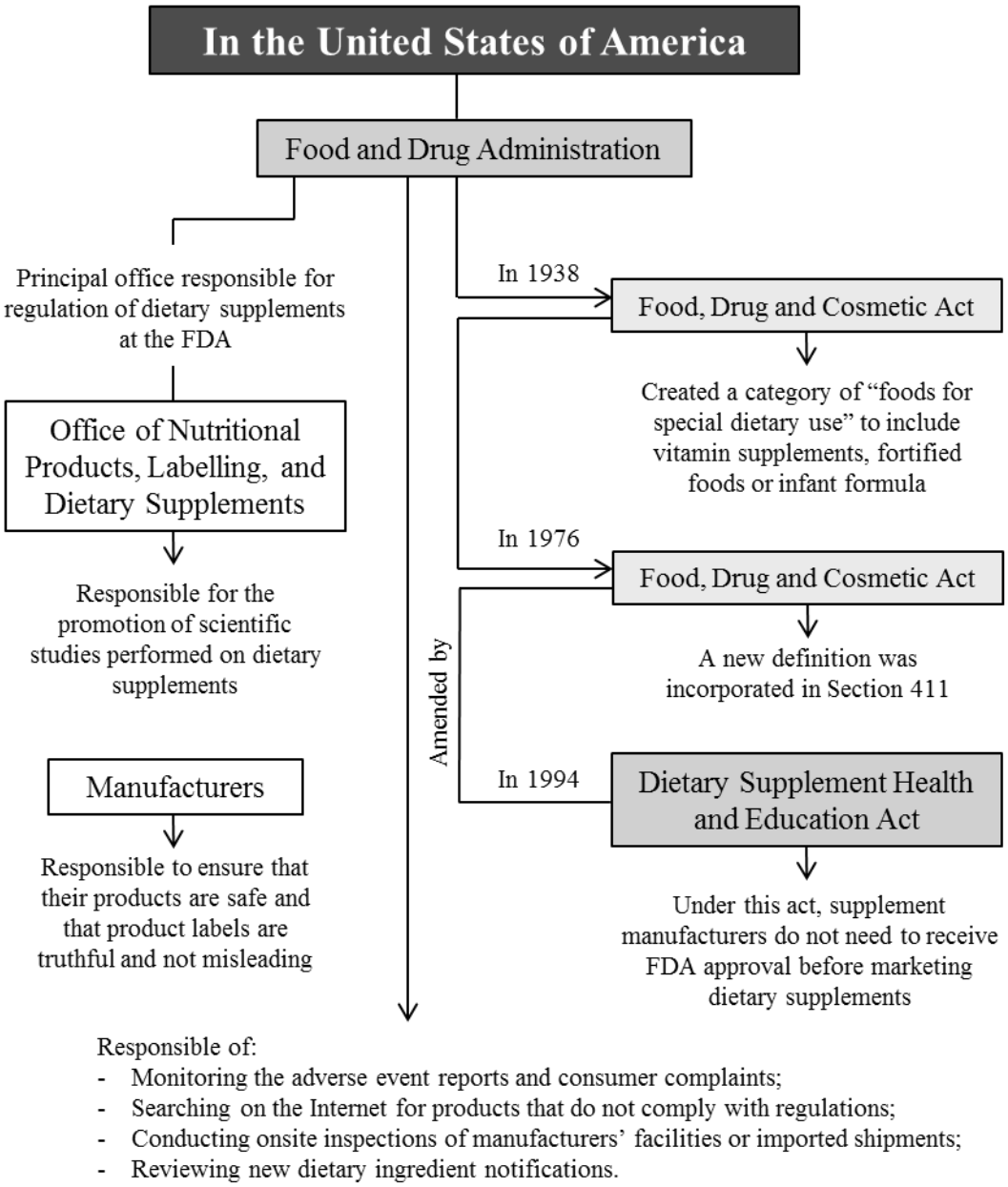


Figure 1. Overview of the legislation on dietay supplements in the USA.

In the EU (Figure 2), the European Food Safety Authority (EFSA) was set up in January 2002 as an independent source of scientific advice that produces opinions to be used by the European Commission (EC) to adopt legislation concerning dietary supplements, among others. The EFSA works in close collaboration with national authorities and in open consultation with its stakeholders (http://www.efsa.europa.eu/). With special relevance for companies in this sector, the EFSA contributed in the evaluation of proposals for the addition of vitamins and minerals to the Food Supplements Directive (FSD), Directive 2002/46/EC, which states that food supplements are "foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological function, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities" (Directive 2002/46/EC, 2002). On the other hand, dietary supplements containing herbal medicinal products are regulated by the Directive 2001/83/EC, which regulates the medicinal products for human use, and by the Traditional Herbal Medicinal Products (THMP) Directive 2004/24/EC that emerged to amend the Directive 2001/83/EC regarding the marketing of THMP on the EU. The Directive 2001/83/EC provides legal definitions for herbal medicinal products, herbal substances, and herbal preparations. Accordingly, a *herbal medicinal product* is “any medicinal product, exclusively containing as active ingredients one or more herbal substances or one or more herbal preparations, or one or more such herbal substances in combination with one or more such herbal preparations”. *Herbal substances* are “all mainly whole, fragmented or cut plants, plant parts, algae, fungi, lichen in an unprocessed, usually dried, form, but sometimes fresh, and also certain exudates that have not been subjected to a specific treatment”; while *herbal preparations* are “preparations obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation” that include “comminuted or powdered herbal substances, tinctures, extracts, essential oils, expressed juices and processed exudates” (Directive 2001/83/EC, 2001).

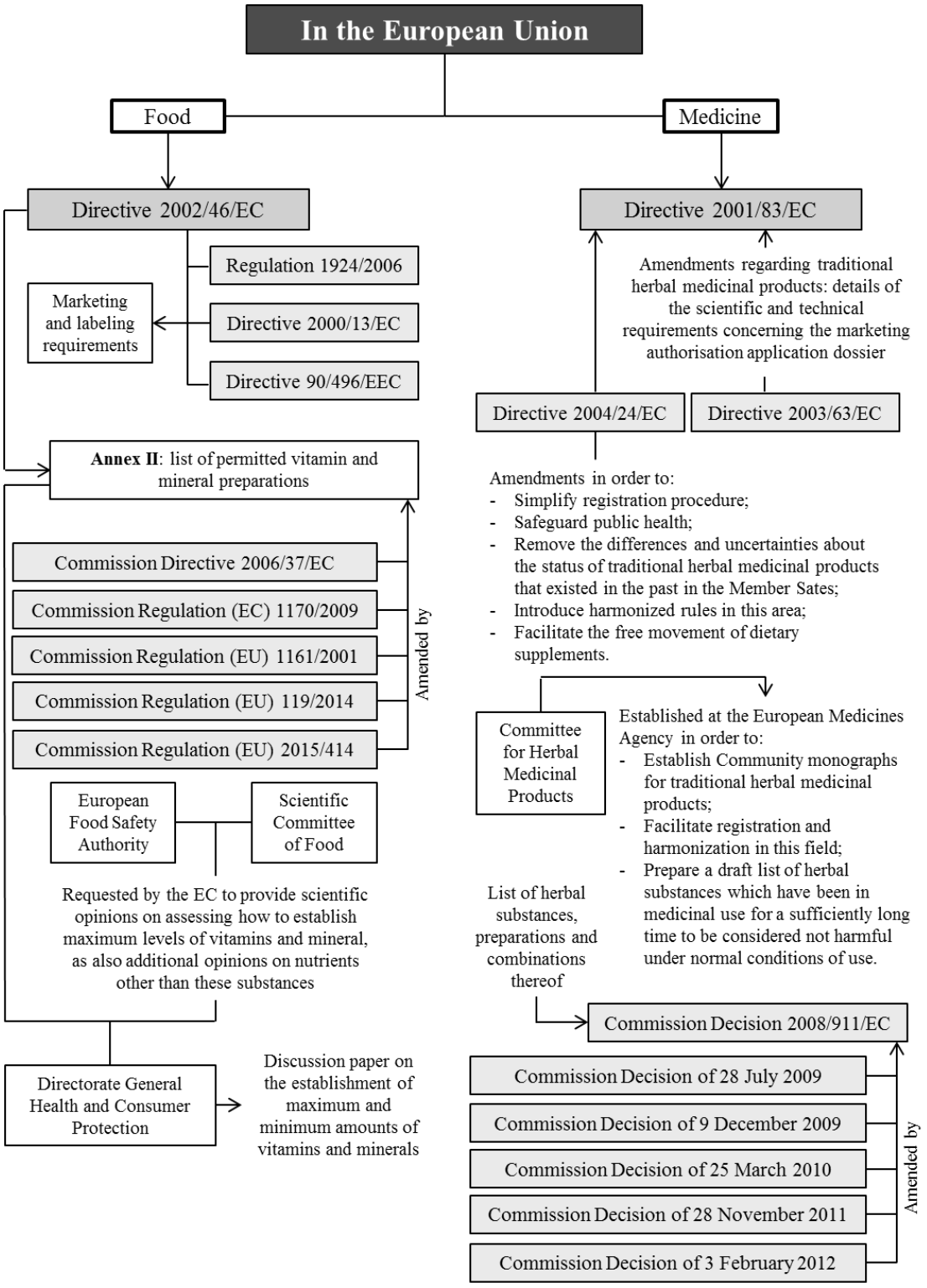


Figure 2. Overview of the legislation on dietary supplements in the EU.

1.1.4. Marketing requirements for dietary supplements

In the USA, according to the DSHEA, dietary supplements are considered as a category of food, which put them under different regulations than drugs. As such, they must be labelled as food and "be intended for ingestion, they must not be represented for use as conventional food or as a sole item of a meal or of the diet and cannot be approved or authorized for investigation as new drugs, antibiotics, or biologics, unless they were marketed as food or dietary supplements before such approval or authorisation" (Dietary Supplement Health and Education Act, 1994). The Office of Nutritional Products, Labelling, and Dietary Supplements is the principal office responsible for regulation of dietary supplements at the FDA. It regulates the marketing of dietary supplements and, therefore, can either refuse to allow new ingredients into or remove existing ingredients from the market for safety reasons. Nevertheless, FDA's authority to monitor structure/function claims of dietary supplements remains limited by law and has, currently, three main requirements to help ensure that such claims are truthful and not misleading: "substantiation to support the claims, which manufacturers must possess but need not submit to FDA; notification to FDA of claims within 30 days of first marketing the supplement with the claim; and a required disclaimer on supplement labels" (OEI-01-11-00210, 2002). Although manufacturers are not required to test new ingredients or supplements in clinical trials, the FDA can stop a company from marketing a dietary supplement if proven that the product is ineffective or unsafe. In order to ensure the quality of supplements, the conditions of preparation, packaging, and storage are issued by the FDA through Good Manufacturing Practices regulations, although they are primarily concerned with safety and sanitation rather than quality (Rapaka and Coates, 2006). The fact that the DSHEA does not require manufacturers to submit dietary supplements to the FDA for safety testing or approval prior to sale results in a lack of information, so that the FDA does not possess a comprehensive list of dietary supplements on the market. To ensure the safety of supplements marketed in the USA, the FDA monitors adverse event reports and consumer complaints, searches in the Internet for products that do not comply with regulations, conducts onsite inspections of manufacturers' facilities or imported shipments, and reviews new dietary ingredient notifications. This authority does not survey dietary supplements sold on retail establishments, but it does conduct limited surveillance of products sold on the Internet (Dietary Supplement Health and Education Act, 1994;

http://uscode.house.gov/view.xhtml?path=/prelim@title21/chapter9/subchapter4&edition=prelim) (Figure 3).

In the EU, according to the Food Supplements Directive, the fact that dietary supplements are subjected to different regulations in the various Member States "may impede their free movement, create unequal conditions of competition, and thus have a direct impact on the functioning of the internal market", which explains the need to "adopt Community rules on those products marketed as foodstuffs" (Directive 2002/46/EC, 2002). Indeed, some substances (in particular certain herbal extracts) are used both in food supplements and for manufacturing proprietary medicinal products, which justifies a dealt with on a case-by-case basis because the rules and procedures for the placing of medicinal products on the market and the marketing authorisation to be issued by the Member States' competent authority or, for certain types of medicinal product, at Community level, are laid down in the legislation on medicinal products (Commission of the European Communities, 2008). Anyway, a high level of protection for consumers and a facilitated choice must be ensured by putting on to the market safe products that bear adequate and appropriate labelling (Directive 2002/46/EC, 2002). Additional requirements to ensure the quality of medicinal products can be found in Directive 2003/63/EC, which amends Directive 2001/83/EC. This Directive contains details of the scientific and technical requirements regarding the marketing authorisation application dossier. Importantly, the unique aspects of herbal medicinal products quality are acknowledged in the preamble of this Directive that states that "herbal medicinal products differ substantially from conventional medicinal products in so far as they are intrinsically associated with the very particular notion of herbal substances and herbal preparations", and also refers that "it is therefore appropriate to determine specific requirements in respect of these products with regard to the standardised marketing authorisation requirements" (Directive 2003/63/EC, 2003). In 2004, the Directive 2004/24/EC attempted (i) to facilitate the registration of certain traditional herbal medicinal products, suggesting that "there should be the possibility of establishing a Community list of herbal substances that fulfill certain criteria, such as having been in medicinal use for a sufficiently long time, and hence are considered not to be harmful under normal conditions of use"; and (ii) to enhance harmonisation, proposing that "Member States should recognise registrations of traditional herbal medicinal products granted by another Member State based on Community herbal

monographs or consisting of substances, preparations or combinations thereof contained in a list to be established", and with respect to other products "Member States should take due account of such registrations" (Directive 2004/24/EC, 2004). Thus, in the amended Directive 2001/83/EC, a simplified registration procedure is established for these supplements that fulfill criteria such as (i) having indications exclusively appropriate to THMP, being intended and designed for use without the supervision of a medical practitioner for diagnostic purposes or for prescription or monitoring of treatment; (ii) being exclusively for administration in accordance with a specified strength and posology; (iii) being an oral, external and/or inhalation preparation; (iv) having been in medicinal use throughout a period of at least 30 years preceding the date of the application, including at least 15 years within the Community; and (v) proving to not be harmful in the specified conditions of use, with pharmacological effects or efficacy plausible on the basis of long-standing use and experience. Moreover, the amended Directive states that the applicant and registration holder shall be established in the Community and submit an application to the competent authority of the concerned Member State in order to obtain traditional-use registration (Directive 2004/24/EC, 2004). Nevertheless, the fact that the European Medicines Agency does not have a role in the registration of THMP means that applications for registration need to be submitted in each Member State where the product is to be marketed and are handled by the competent authority in each Member State. In this context, the Court of Justice has concluded on several occasions that the differences between the classification of products as foodstuffs or medicinal products in the different Member States will persist as long as there is no more complete harmonization of the measures necessary to ensure the protection of health. Thus, a product classified as foodstuff in another Member State can, at the same time, be marketed as medicinal product in the Member State of importation, if it displays the characteristics of such a product. In cases where a product fulfills the definition of food supplement (as laid down in FSD) but, simultaneously falls within one of the definitions of medicinal product (contained in the Directive 2001/83/EC), the provisions of the legislation applicable to medicinal products shall apply (Commission of the European Communities, 2008). When it regards to medicinal products, the competent authorities of the Member States are responsible for granting marketing authorisations for medicinal products placed on their markets, except for medicinal products that are authorized under Regulation (EC) 726/2004. In cases where national authorisations in more than one Member State are

required for the same medicinal product and the marketing authorisation holder has received a marketing authorisation in a Member State, this latest must submit an application in the concerned Member State using the procedure of mutual recognition. Then, the concerned Member States should recognize the marketing authorisation already granted by the reference Member State and, therefore, authorize the marketing of the product on their national territory. On the contrary, if there is no marketing authorisation in the Union for a given medicinal product, the applicant can submit an application in all the Member States where it intends to obtain a marketing authorisation at the same time, making use of a decentralized procedure, and choose one of them as reference Member State. The marketing authorisation should then be granted in accordance with the decision taken by the reference Member State and the concerned Member State taking into account the assessment report prepared by the reference Member State and any comments made by the concerned Member State. In order to obtain a Union authorisation, an application must be submitted to the European Medicines Agency (EMA) and its scientific evaluation is carried out within the Committee for Medicinal Products for Human Use of the EMA; a scientific opinion is then prepared and sent to the European Commission which drafts a Decision. This Decision is adopted by the Commission after consulting the Member States through the relevant Standing Committee, and the authorisation is granted. In any cases, the marketing authorisation must contain the summary of product characteristics in accordance to Article 11 of Directive 2001/83/EC (Directive 2001/83/EC, 2001). With the exception of the products having different therapeutic indications in national (decentralized/mutual recognition) and central marketing authorisations, the co-existence of both provided by the Communication is not allowed. Indeed, if the product falls under the optional scope of the centralised procedure (Article 3(2) of Regulation (EC) 726/2004), there is a possibility of choice of using either centralised or national procedure for the same product, but it does not allow the simultaneous co-existence of both marketing authorizations. Thus, if a central marketing authorization has been issued, there is no place for an additional scientific evaluation and regulation decision for the same product. According to Directive 2001/83/EC, “any additional strengths, pharmaceutical forms, administration routes, presentations, as well as any variations and extensions” shall also be granted an authorization or be included in the initial marketing authorisation, and all of these authorisations shall be considered as belonging to the same global marketing authorisation.

Meanwhile, dietary supplements considered as foodstuffs should comply with FSD and Regulation (EC) 1924/2006 on nutrition and health claims made on foods because they do not fulfill the definition of medicinal products. From that perspective, Member States shall ensure that food supplements only may be marketed within the Community if they comply with the rules laid down in the FSD. In agreement with that Directive, only vitamins and minerals listed in Annex I, and under the forms listed in Annex II, may be used to manufacture food supplements, and the purity criteria for these substances shall be adopted by the Commission. Apart from the vitamins and minerals listed in Annex I and forms listed in Annex II, Member States may allow in their territory the use of other substances, provided that they are used "in one or more food supplements marketed in the Community on the date of entry into force of this Directive" and the EFSA "has not given an unfavorable opinion in respect of the use of that substance, or its use in that form, in the manufacture of food supplements, on the basis of a dossier supporting use of the substance in question to be submitted to the Commission by the Member State" (Directive 2002/46/EC, 2002). Since May 2011, all unlicensed herbal medicinal products must either be marketed as medicines or be withdrawn from the market. They might be launched correctly labelled as food supplements, although carrying no claims unless those claims have been approved according to the Nutrition and Health Claims Regulation. However, the classification of some dietary supplements as food products has raised some concerns due to the fact that the food law is not regulated as strictly as the drug law, which contains additional regulatory provisions for protection of the consumer's health; and also because it is necessary to disclose the factual therapeutic properties of medicinal plants in form of disease-related indications and not in form of masked health-related claims (Quintus and Schweim, 2012) (Figure 3).

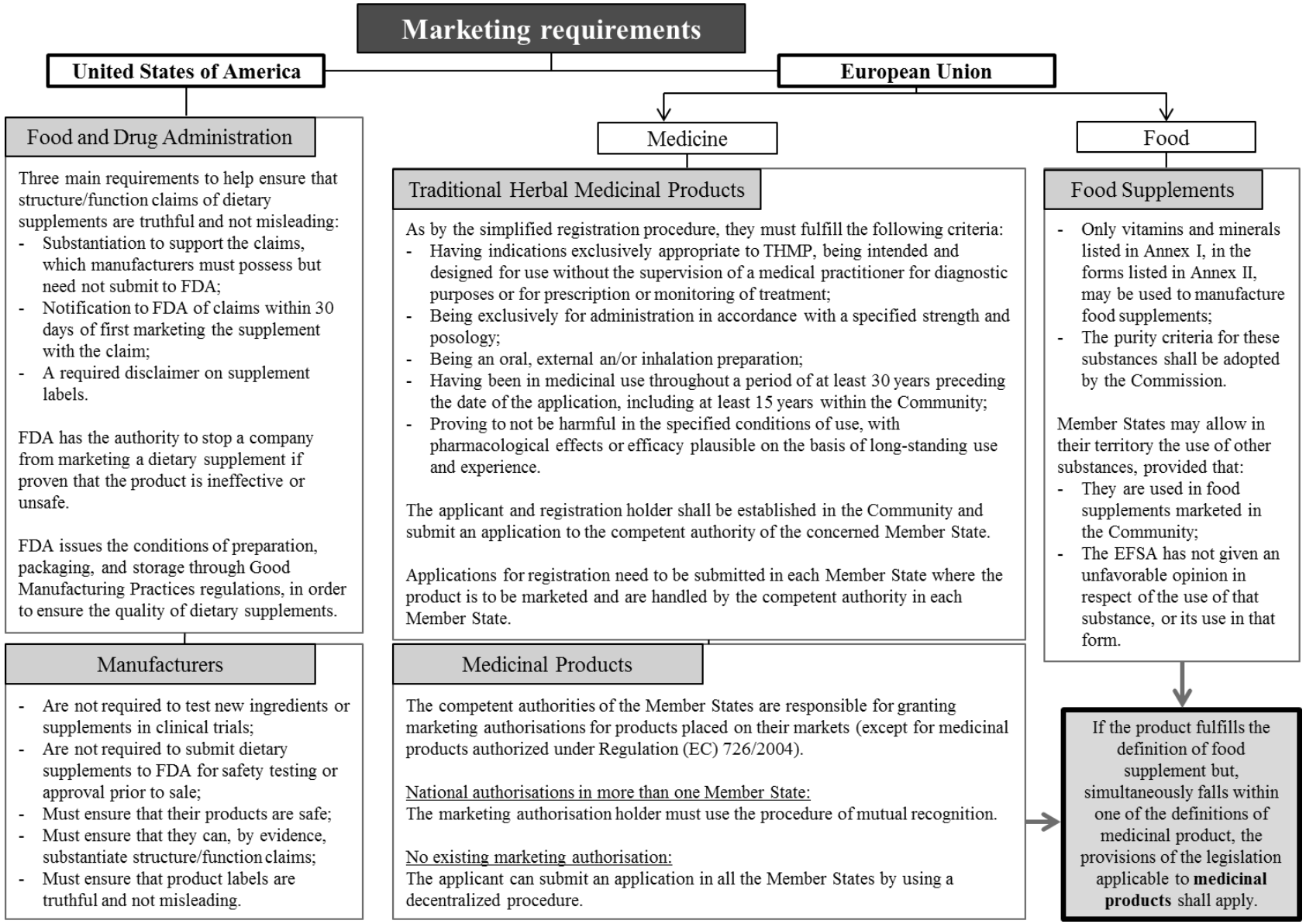


Figure 3. Marketing requirements for dietary supplements in the USA and the EU.

1.1.5. Allowable substances on dietary supplements

In the USA, with the adoption of the DSHEA, two different categories of dietary supplements ingredients were considered for pre-market safety notification: ingredients in the market before October 15, 1994, which are considered old dietary ingredients and presumed to be safe; and ingredients marketed after October 15, 1994 that are considered new dietary ingredients and, thus, require FDA pre-market review of safety (Dietary Supplement Health and Education Act, 1994). In a general way, a dietary supplement is considered safe until proven unsafe, and as can be read in FD&C Act, amended by DSHEA, a dietary supplement or ingredient is considered unsafe if (i) it "presents a significant or unreasonable risk of illness or injury under conditions of use recommended or suggested in labelling, or if no conditions of use are suggested or recommended in the labelling, under ordinary conditions of use"; (ii) it "is a new dietary ingredient for which there is inadequate information to provide reasonable assurance that such ingredient does not present a significant or unreasonable risk of illness or injury"; (iii) "the Secretary declares to pose an imminent hazard to public health or safety, except that the authority to make such declaration shall not be delegated and the Secretary shall promptly after such a declaration initiate a proceeding in accordance with sections 554 and 556 of title 5 to affirm or withdraw the declaration"; or (iv) it "is or contains a dietary ingredient that renders it adulterated under the conditions of use recommended or suggested in the labelling of such dietary supplement". If a dietary ingredient is considered safe by respecting the referred requirements, it can thus be introduced in dietary supplements to be marketed (Dietary Supplement Health and Education Act, 1994; http://uscode.house.gov/view.xhtml?path=/prelim@title21/chapter9/subchapter4&edition=prelim).

In the EU, specific rules on vitamins and minerals in food supplements were introduced by the FSD (Directive 2002/46/EC), which includes in its Annex II a list of permitted vitamin or mineral preparations that may be added for specific nutritional purposes in food supplements. The trade of products containing vitamins and minerals not listed in Annex II has been prohibited from the 1st of August 2005 (http://ec.europa.eu/food/food/labellingnutrition/supplements/index_en.htm).
Nevertheless, in order to include additional substances, the FSD has later been amended

by the Commission Directive 2006/37/EC, the Commission Regulation (EC) 1170/2009, the Commission Regulation (EU) 1161/2011, the Commission Regulation (EU) 119/2014 and the Commission Regulation (EU) 2015/414. Although the FSD calls for establishment of harmonized minimum and maximum dosage amounts through the Standing Committee procedures and lays down the criteria for their setting, this has yet to be done and remains a competence of EU Member States that are also responsible for the rules regulating substances other than vitamins and minerals, once they are not directly covered by the FSD. With this concern, the EFSA and the Scientific Committee of Food were requested by the EC to provide scientific opinions on assessing how to establish maximum levels of vitamins and mineral permitted in food supplements, as also additional opinions on nutrients other than these substances. Among these other substances are amino acids, enzymes, pre- and probiotics, essential fatty acids, botanicals and botanical extracts, and miscellaneous bioactive substances (Directive 2002/46/EC, 2002; http://www.efsa.europa.eu/). In June 2006, the Directorate General for Health and Consumer Protection published a discussion paper on the establishment of maximum and minimum amounts of vitamins and minerals in foods where the issues to be considered in this exercise were identified; furthermore, all interested parties were invited to provide their views, which are currently in analysis (Directorate E, 2006).

Regarding dietary supplements considered as medicinal products, including herbal medicinal products, a Committee for Herbal Medicinal Products, established at the EMA, was created to provide Community monographs for THMP and further facilitate the registration and harmonization in this field. A draft list was then prepared taking into account the substances used in traditional medicine for a sufficiently long time to be considered safe under normal conditions of use. The referred list is included in the Commission Decision 2008/91/EC that was, later, amended in order to include new substances, by several Commission Decisions (Commission Directive 2008/91/EC, 2008).

1.1.6. Labelling of dietary supplements

According to the FD&C Act and the respective amendments by the DSHEA, in the USA, dietary supplements must carry on the label the name and the quantity of each ingredient or with respect to a proprietary blend of such ingredients, the total quantity

of all ingredients in the blend. The label must also identify the product by using the term “dietary supplement”, which may be modified with the name of such an ingredient and, in cases where the supplement contains plants material, the labelling must identify the parts of the plant from which it was derived. Regarding supplement listing on the nutrition labelling, a dietary supplement shall comply with some labelling requirements, such as first listing the dietary ingredients that are present in the product in a significant amount and for which a recommendation for daily consumption has been established by the Secretary, and also listing any other dietary ingredient present and identified as having no such recommendation. The listing of dietary ingredients shall include the quantity of each ingredient per serving and may include the source of the ingredient; the nutrition information shall immediately precede the ingredient information, except that no ingredient identified shall be required to be identified a second time. Moreover, the label of a dietary supplement or food product may contain one of three types of claims: health claim, nutrient content claim, or structure/function claim. Nonetheless, the claims must not explicitly or implicitly claim to prevent, treat, mitigate, cure, or diagnose a disease; it can claim a benefit related to a classical nutrient deficiency disease with disclosed prevalence in the US, characterize the documented mechanism by which a nutrient or dietary ingredient acts in the maintenance of such structure or function, or describe well-being from consumption of a nutrient or dietary ingredient. In cases where this is not respected and a dietary supplement label contain a disease claim, the FDA treats the product as an unapproved drug and may take enforcement actions against the manufacturer or distributor. Among these actions, the FDA can issue a warning letter, seize the product, seek criminal prosecution, or prohibit the sale of the product through an injunction (Dietary Supplement Health and Education Act, 1994; http://uscode.house.gov/view.xhtml?path=/prelim@title21/chapter9/subchapter4&edition=prelim) (Figure 4).

In the EU, the labelling and the package leaflet of dietary supplements considered as medicinal products must respect Title V of the Directive 2001/83/EC. Accordingly, some particulars shall appear on the outer packaging or, where it does not apply, on the immediate packaging, such as “the name of the medicinal product followed by its strength and pharmaceutical form, and, if appropriate, whether it is intended for babies, children or adults”; “a statement of the active substances expressed qualitatively and

quantitatively per dosage unit or according to the form of administration for a given volume or weight, using their common names"; "the pharmaceutical form and the contents by weight, by volume or by number of doses of the product"; "a list of those excipients known to have a recognized action or effect"; "the method of administration and, if necessary, the route of administration"; "a special warning that the medicinal product must be stored out of the reach and sight of children"; "a special warning, if this is necessary for the medicinal product"; "the expiry date in clear terms (month/year)"; "special storage precautions, if any"; among many others. The Directive also contains recommendations on the labelling of products subject and not subject to prescription, stating that the National competent authorities must notify the Commission of non-prescription medicinal products which they judge to be at risk of falsification and may inform the Commission of medicinal products which they deem not to be at risk according to criteria also described in the Directive, and specifies how the Member States may proceed regarding to reimbursement purposes (Article 54a) (Directive 2001/83/EC, 2001).

The labelling requirements applied to dietary supplements considered as foodstuffs are described in the FSD, in accordance with the Article 5(1) of Directive 2000/13/EC, relating to the labelling, presentation and advertising of foodstuffs, whereby the name under which products covered by this Directive are sold shall be "food supplement". Moreover, these supplements must not carry on the label, presentation and advertising the property of preventing, treating or curing a human disease, but should carry some particulars such as (i) "the names of the categories of nutrients or substances that characterize the product or an indication of the nature of those nutrients or substances"; (ii) "the portion of the product recommended for daily consumption"; (iii) "a warning not to exceed the stated recommended daily dose"; (iv) "a statement to the effect that food supplements should not be used as a substitute for a varied diet"; and (v) "a statement to the effect that the products should be stored out of the reach of young children". Also, the label should not present any mention stating or implying that the appropriate quantities of nutrients cannot be provided by a balanced and varied diet, and the amount of those nutrients or substances with nutritional or physiological effect shall be declared in numerical form (expressed in the units specified in Annex II of the FSD), per portion of the product as recommended for daily consumption. For vitamins and minerals, the amounts shall also be indicated in percentage of the reference values

mentioned in the Annex to Directive 90/496/EEC, which may also be given in graphical form. In order to facilitate the efficient monitoring of food supplements, the person placing the product on the market may be required (by the Member State in which the product is placed on the market) to forward a model of the label used for the product to the competent authority (Directive 90/496/EEC, 1990; Directive 2000/13/EC, 2000; Directive 2002/46/EC, 2002). Later, the general principles of this directive were complemented by the Regulation (EC) 1924/2006 that lay down specific provisions concerning the use of nutrition and health claims regarding foods to be delivered as such to the consumer (Regulation (EC) No 1924/2006, 2006) (Figure 4).

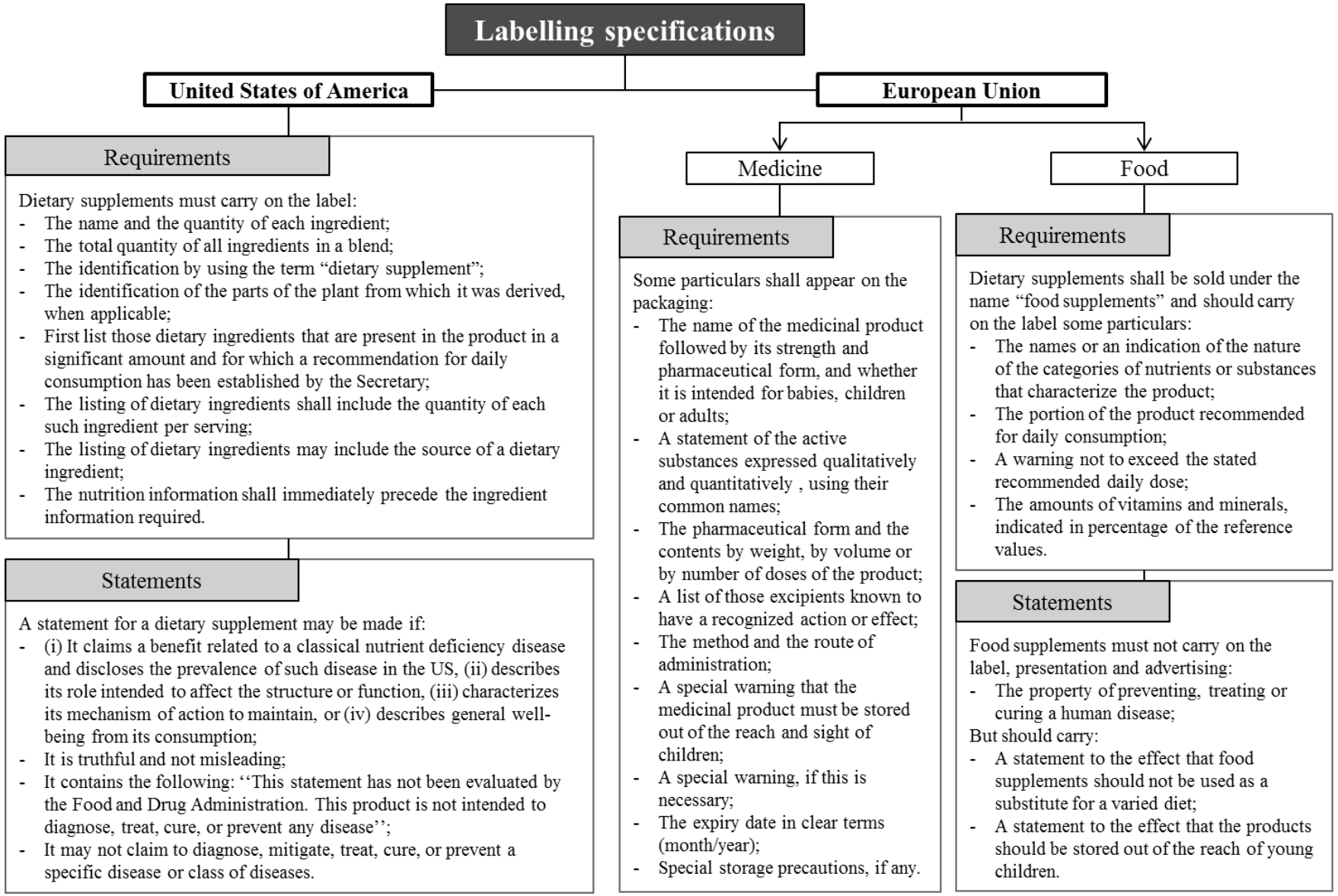


Figure 4. Labelling specifications for dietary supplements in the USA and the EU.

1.2. Hepatoprotective plants used in dietary supplements

1.2.1. Oxidative stress and liver injuries

The liver is the largest organ in human body and has a pivotal role in regulation of physiological processes. It is involved in several vital functions related to digestion, metabolism, immunity, and storage of nutrients, also helping in the maintenance, performance and regulation of homeostasis of the body (Ahsan et al., 2009). Furthermore, the liver is responsible for the excretion of waste metabolites and detoxification of a variety of drugs, xenobiotics and toxins, which makes it a vulnerable target of injury caused by toxic chemicals and free radicals capable of binding to cellular macromolecules such as deoxyribonucleic acid (DNA), lipids, proteins, or carbohydrates and produce major interrelated derangement of cell metabolism (Bodakhe and Ram, 2007; Hsu et al., 2010). Ideally, these radicals are neutralized by cellular antioxidant defenses and the maintenance of this equilibrium is crucial to normal organism functioning. Nonetheless, this balance can be compromised by the excessive increase of oxidative metabolites resulting from high chemical drugs intake, among others, and organ-related localized oxidative stress can initiate a systemic inflammatory response syndrome leading directly to severe cell damage (Ferreira et al., 2009; Lichtenstern et al., 2011).

In the liver, the most frequent consequence of oxidative stress is the initiation of lipid peroxidation that occurs by the attack on a fatty acid or fatty acyl side chain of any chemical species having sufficient reactivity to abstract a hydrogen atom from a methylene carbon in the aliphatic chain. This mechanism is facilitated by the presence of double bonds in the chain, which explains the particular susceptibility of polyunsaturated fatty acids. The free radical resulting from the molecule to which the hydrogen atom was removed can then undergo molecular rearrangement and react with molecular oxygen originating a peroxyl radical. This radical can have different fates like combine with each other, attack membrane proteins (causing structural changes in membrane and signaling of membrane bound proteins), or abstract hydrogen atoms from adjacent fatty acid side chains, propagating the chain reaction of lipid peroxidation. This propagation is influenced by many factors, being for instance boosted by a high ratio protein/lipid content of a membrane, and hampered by the presence of chain-breaking antioxidants within the membrane that interrupt the reaction

chain (donating a hydrogen to peroxyl radicals), avoiding the propagation of the lipid peroxidation reactions (Halliwell and Chirino, 1993; Ferreira et al., 2009).

The main primary end products of lipid peroxidation (LPO) processes are lipid hydroperoxides (LOOH), notwithstanding, secondary products formed during LPO, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), generated by decomposition of arachidonic acid and larger polyunsaturated fatty acids (PUFA), are described as the most mutagenic and toxic products of lipid peroxidation, respectively. MDA is reported by acting as a signaling messenger, regulating gene expression, and particularly in hepatic stellate cells, inducing collagen-gene expression. Moreover, it has a high capacity to react with proteins or DNA, among many other biomolecules, leading to adducts formation (Ayala et al., 2014).

Depending on the position and degree of hydroxylation, the polarity, the solubility and the reducing potential of phenolic compounds (Elliott et al., 1992; Ferrali et al., 1997; Cos et al., 1998; Hirano et al., 2001), these molecules can have different antioxidant capacities, being classified as chain breaking antioxidants, known for quenching free radicals by donating a hydrogen atom and/or an electron to free radicals by means of concerted or stepwise mechanisms. Beyond the described effects, flavonoids have also gained pharmacological interest due to their capacity to chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases. These compounds can, therefore, interfere not only with the propagation reactions of the free radical, but also with the formation of the radicals, either by chelating the transition metal, or by inhibiting the enzymes involved in the initiation reaction (Figure 5) (Ferrali et al., 1997; Rice-Evans et al., 1996).

Liver injuries are mainly caused by toxic substances, infections and autoimmune disorder and are among the most serious ailments, remaining one of the major threats to public health (Asha and Pushpangadan, 1998). However, the treatment options for usual liver diseases such as cirrhosis, fatty liver and chronic hepatitis are still problematic due to side effects caused by chemicals commonly used in their therapy (Wendel et al., 1987). Thereby, there is an emerging need for effective therapeutic agents with low incidents of side effect, which has triggered off extensive research in the field of hepatoprotective medicinal plants.

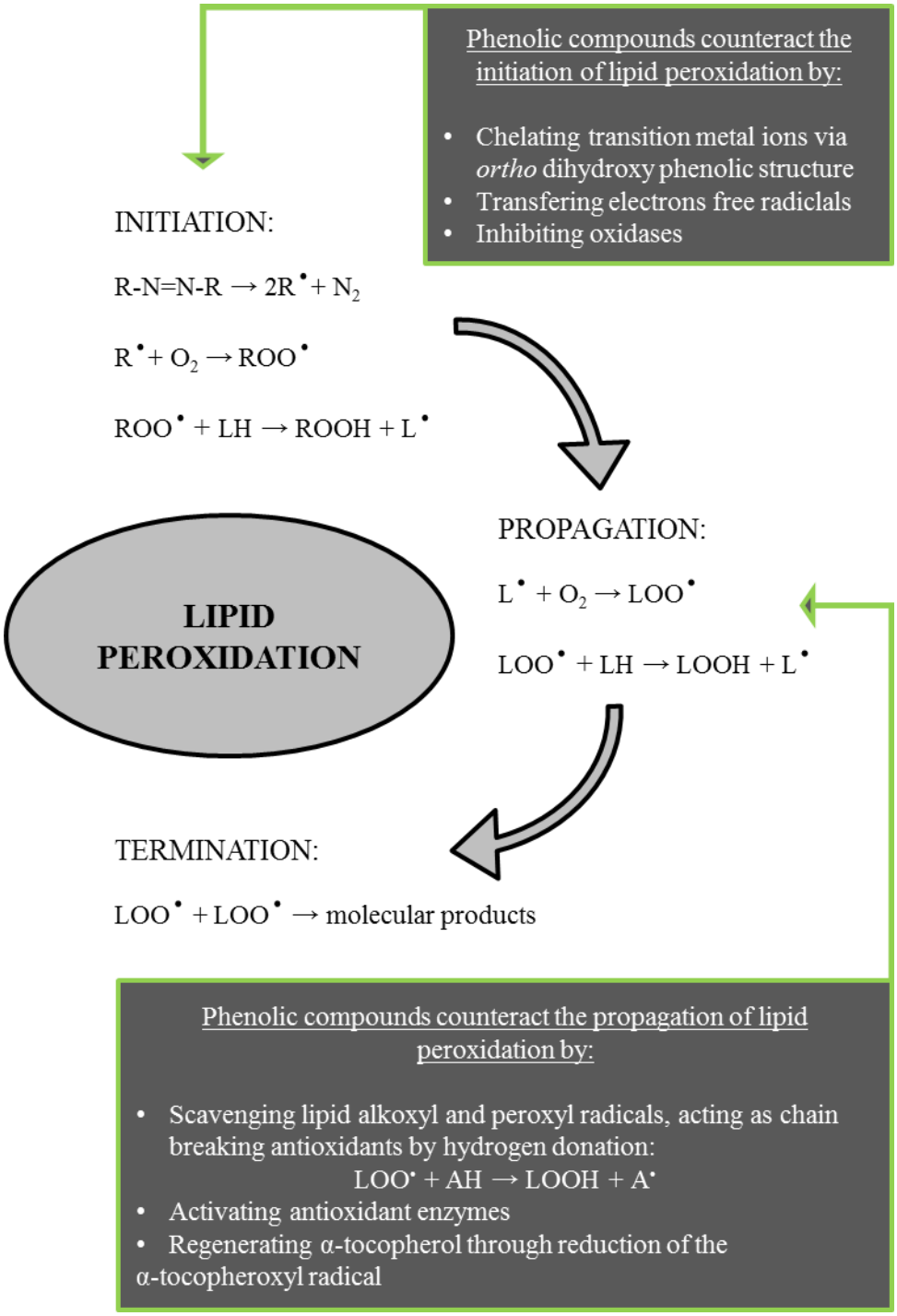


Figure 5. The role of phenolic compounds in lipid peroxidation inhibition, the major mechanism of action related with their hepatoprotective effects.

1.2.2. Hepatoprotective plants

Despite the worldwide popularity of herbal medicines, with about 80% of the world population relying on their various effects on living systems (sedatives, analgesics, antipyretics, cardioprotectives, antibacterial, antipyretic, and antiprotozoal, among others), there is still a lack of efficient treatment modalities for liver diseases (Olaleye et al., 2006). Some possible limiting factors are the unscientifically exploitation and/or improper utilization of these herbal drugs, which justify the need of detailed studies in the light of modern science. Nevertheless, owing to the explicit inadequacy of reliable chemical hepatic drugs, the search for natural herbal drugs has intensified in the recent decades and plant derived products are ungdergoing a comeback with up to 65% of liver patients, in Europe and the United States, taking herbal preparations. Medicinal plant remedies are increasingly perceived as safe alternatives to synthetic drugs, fitting into the image of a gentle and, therefore, harmless kind of treatment by entailing less toxicity, better therapeutic effect, good patient compliance and cost effectiveness (Al-Asmari et al., 2014).

Several plants have been reported as hepatoprotective, such as *Abrus mollis* Hance, *Aloe barbadensis* Mill, *Artemisia capillaris* Thunb., *Bauhinia variegata* L., *Butea monosperma (lam), Cardiospermum halicacabum* Linn., *Cineraria abyssinica* Sch. Bip. exA. Rich., *Cochlospermum vitifolium* (Willd.), *Cochlospermum angolensis* Welw., *Equisetum arvense* L., *Erycibe hainanesis* Merr., *Ficus ingens* (Miq.) Miq., *Gentiana olivieri* Griseb., *Hippophae rhamnoides* L., *Lactuca indica* L., *Laggera pterodonta* (DC). Benth, *Nelumbo nucifera* Gaertn., *Ocimum gratissimum* Linn., *Peltiphyllum peltatum* Engl., *Phyllanthus amarus* Schum. & Thonn., and *Schisandra chinensis* (Turcz.) Baill. These plants are used, among others, for the treatment of different hepatic complaints such as liver enlargement (*A. barbadensis*) (The Wealth of India, 2000), inhibition of hepatitis virus (*A. capillaris* (Wu et al., 2001); *C. vitifolium* (Sánchez-Salgado et al., 2007); *P. amarus* (Sane et al., 1997); *E. arvense* (Editorial Committee of Chinese Medicinal Herbs); *A. mollis* (Zhou and Li, 2005)), inhibition of tumour growth in hepatocarcinoma cell (*A. capillaris* (Zhao et al., 2014); *L. indica* (Kim et al., 2007); *E. arvense* (Oh et al., 2004); *P. amarus* (Krithika et al., 2009); *E. hainanesis* (Feng et al., 2014)) and in animal models (*C. angolensis* (Bousserouel et al., 2012); *L. pterodonta* (Wu et al., 2007), and protection against chronic liver fibrosis and cirrhosis (*B. monosperma, B. variegata, O. gratissimum*) (Gupta et al., 2013).

Medicinal plants containing phenolic compounds have been intensively explored in what concerns their hepatoprotective capacity against chemically induced damage, either *in vivo* or *in vitro*, because herbs natural constituents seem to overcome liver injuries often caused by the described above oxidative reactions that promote lipid peroxidation in hepatic tissues. Among these constituents, flavonoids and phenolic acids have received a special attention for their high antioxidant activity (Rice-Evans et al., 1996).

Herein, an attempt was made to find studies where hepatoprotective plants, revealing the presence of phenolic compounds, were tested for their hepatoprotective activity using well-established experimental models, such as those collected in Figure 6. In those studies, well-reported hepatotoxic chemicals have been used, including carbon tetrachloride (Jeyadevi et al., 2013), paracetamol, sodium fluoride (Nabavi et al., 2013), thioacetamide (Wu et al., 2007), tacrine (Oh et al., 2004), *tert*-butyl hydroperoxides (Wu et al., 2007), or D- and DL-galactosamine (Feng et al., 2014). These chemicals are known as inducers of liver injury by promoting inflammation processes and hepatic parenchyma damage that lead to deleterious effects on liver physiochemical functions through the generation of reactive oxygen species (Jeyadevi et al., 2013). For instance, carbon tetrachloride, which is the most commonly used toxic substance in those experiments, is metabolized by P4502E1 (CYP2E1) to the trichloromethyl ($CCl_3^{\bullet}$) and proxytrichloromethyl ($OOCCl_3^{\bullet}$) radicals, being responsible for the initiation of free radical-mediated lipid peroxidation in the liver (Poli et al., 1987). On the other hand, *tert*-butyl hydroperoxide can be metabolized to free radical intermediates by cytochrome P450, and subsequently initiate lipid peroxidation, affecting cell membrane integrity (Duh et al., 2010).

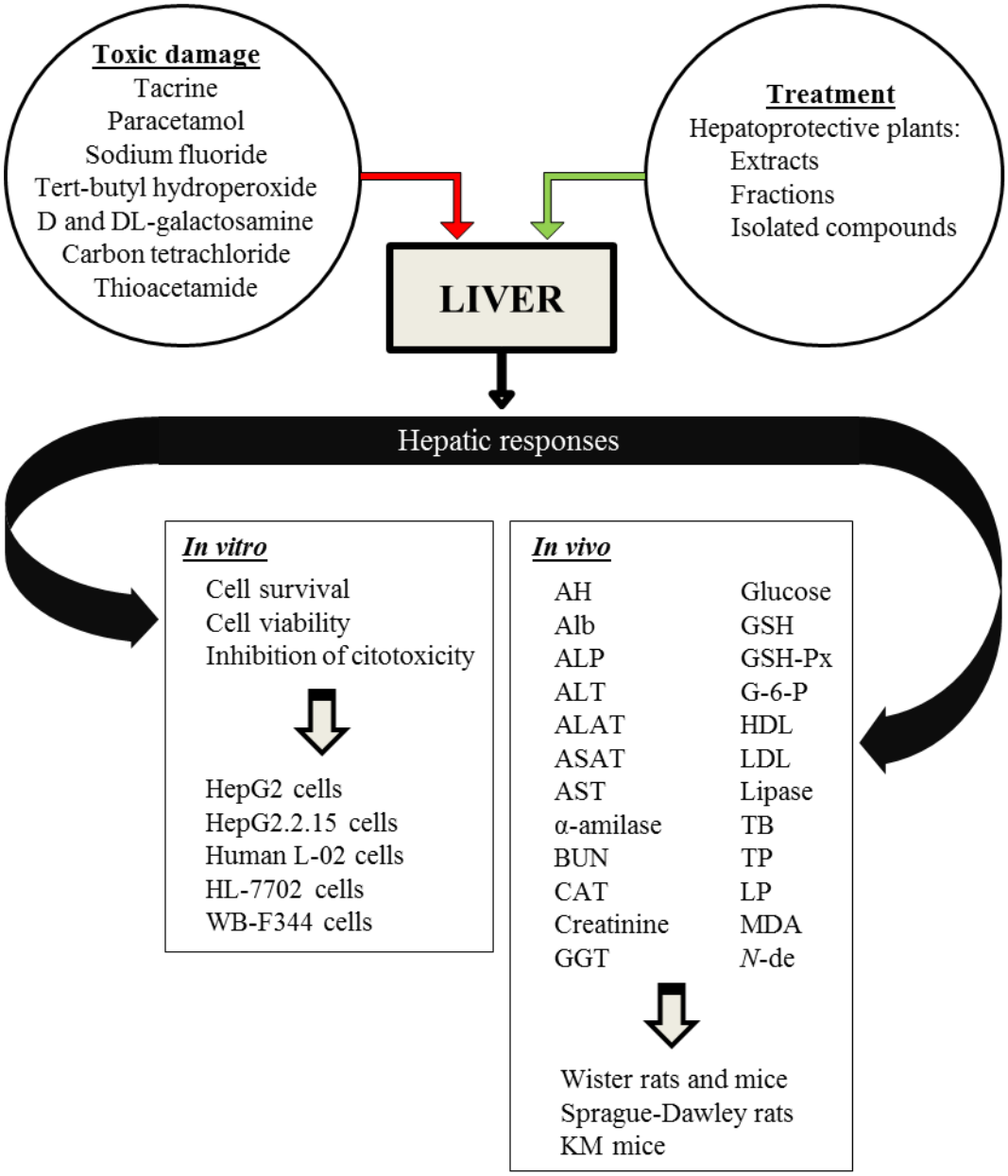


Figure 6. Overview of the in vitro and in vivo assays used to evaluate the hepatoprotective effects of plant phenolic compounds (extracts, fractions and isolated compounds).

In reaction to those injuries, the body initiates an effective mechanism to neutralize the chemical induced damage, and a set of endogenous antioxidant enzymes are activated (Wang et al., 2012). For example, superoxide dismutase (SOD) is known to convert superoxide anion into H_2O_2 and O_2, whereas catalase (CAT) reduces H_2O_2 to H_2O, resulting in a decrease of the pool of free radicals (Paoletti and Mocali, 1990); there is, thus, a relationship between these enzymes in regulating intracellular and extracellular levels of superoxide (Duh et al., 2010). Also, elevated γ-glutamyltransferase (GGT) results from fatty liver disease, both alcoholic and nonalcoholic, cholestatic liver

disease, and induction by drugs; while alanine transaminase (ALT) is considered a specific marker for liver injury, often associated to mortality (Ruhl and Everhart, 2009). On the contrary, reduced glutathione (GSH) is presumed to be an important endogenous defense against peroxidative destruction of cellular membranes (Elliott et al., 1992). Increased levels of ALT, alanine aminotransferase (ALAT), aspartate transaminase (AST), aspartate aminotransferase (ASAT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in the blood stream indicate severe hepatic cell necrosis, and the accumulation of triglycerides (TG) leads to fatty liver (Chandan et al., 2007; Wu et al., 2007). Moreover, the production of secondary oxidation products (including MDA), measured by the formation of thiobarbituric acid reactive substances (TBARS), is enhanced upon lipid peroxidation in hepatic tissue and causes cellular damage and disruption of cell membrane when endogenous antioxidants are depleted (Karthikesan et al., 2010). In this connection, the levels of aniline hydroxylase (AH), albumin (Alb), ALP, ALT, ALAT, ASAT, AST, α-amylase, blood urea nitrogen (BUN), CAT, creatinine, GGT, glucose, GSH, glutathione peroxidase (GSH-Px), glucose-6-phosphatase (G-6-P), high-density lipoproteins (HDL), LDH, low-density lipoproteins (LDL), lipase, LPO, MDA, amidopyrine-*N*-demethylase (*N*-de), glutamic-pyruvic transaminase (PGT), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), SOD, total bilirubin (TB), TBARS, total cholesterol (TC), TG, total protein (TP), triacylglycerol and γ-glutamyl transpeptidase (γ-GTP) have been used to measure liver injury in *in vivo* systems, namely Wister rats and mice, Sprague-Dawley rats and KM mice, whereas cell survival and cell viability have been tested *in vitro* in some cell cultures such as HepG2, HepG2.2.15, human L-02, WB-F344 and HL-7702 cells (Jain et al., 2013).

Innumerous other medicinal plants were studied regarding the same parameters, but herein we have restricted the available information to works where the screening of phenolic compounds was also performed. In order to collect such results, a thorough survey of the literature on the studies performed on hepatoprotective plants was undertaken by searching the published data for the period between 2000 and 2014 with the aid of "Science Direct", "Pubmed", "Web of Science" and "Google Scholar" search engines.

1.2.3. Phenolic extracts and fractions as bioactives

With the described search we were able to gather several studies that have been carried out on herbal extracts with hepatoprotective purposes. Table 1 summarises the studies performed with aqueous, ethanol or methanol extracts from aerial parts, leaves, barks, flowering herbs and pollen of different plants, as well as some syrups containing plant extracts. The aqueous extracts were obtained by extracting with distilled water and lyophilizing in freeze drier (Chandan et al., 2007) or by boiling the plant material for 30 minutes followed by cooling, filtering and lyophilizing (Sintayehu et al., 2012). The ethanol extractions were performed with different concentrations of alcohol (from 50 to 100%), under reflux for two times (2 or 3 hours each time) (Cheng et al., 2013; Zhao et al., 2014); using a soxhlet apparatus and evaporating the solvent under reduced pressure at 45 °C (Jeyadevi et al., 2013); by percolating in ethanol for 72 hours and concentrating under reduced pressure at a temperature not exceeding 45 °C (Donia et al., 2013); by macerating with the solvent for 3 hours, in continuous stirring at room temperature, and evaporating to dryness under reduced pressure (Orhan et al., 2003); by suspending in ethanol at 70 °C for three times, filtering and lyophilizing; by decocting for 2 h at 80 °C, filtering and evaporating at 35 °C (Huang et al., 2010); or by extracting at room temperature and evaporating under reduced pressure (Krithika et al., 2009). The methanol extracts were prepared by macerating the plant with methanol (at 80% and 100%) for 3 times (72 hours each), filtrating and evaporating the solvent at 40 °C (Sánchez-Salgado et al., 2007; Sintayehu et al., 2012). Regarding the assessed syrups, their concentration of plants extracts was about 2.3% to 35%.

The hepatotoxicity assays performed gave, in a general way, similar results among all the extracts, with reduction of serum liver enzymes (*in vivo*) and increase of cell viability and survival (*in vitro*). In the studies performed *in vitro* by using cell cultures the concentrations of extracts that allowed the inhibition of cells proliferation could be compared. *A. capillaris* ethanol extract revealed good hepatoprotective activity with IC_{50} values of 76.10 µg/mL (on HepG2.2.15 cells), while *L. pterodonta* and *P. amarus* reduced liver enzyme levels and increased cell viability in concentrations of 1-100 µg/mL (on primary hepatocytes) and 200-600 µg/mL (on HepG2 cells), respectively (Wu et al., 2007; Krithika et al., 2009). Among the extracts studied *in vivo*, those that presented beneficial effects on liver parameters in lower concentrations were *S. chinensis* (10-40 mg/kg) and *G. olivieri* (62.5-250 mg/kg) ethanol extracts (Orhan et

al., 2003; Cheng et al., 2013), followed by *C. vitifolium* methanol extract (100 mg/kg) (Sánchez-Salgado et al., 2007), *F. ingens* (100-400 mg/kg) and *N. nucifera* (100-500 mg/kg) ethanol axtracts (Huang et al., 2010; Donia et al., 2013), *A. barbadensis* aqueous extract (125-500 mg/kg) (Chandan et al., 2007), *C. abyssinica* aqueous and methanol extracts (200 mg/kg) and *C. halicacabum* ethanol extract (250-500 mg/kg) (Sintayehu et al., 2012; Jeyadevi et al., 2013).

A similar search was conducted on studies that assessed different kinds of fractions extracted from hepatoprotective herbs and, once more, the number of reports was not negligible. Nonetheless, within those investigations, only a few focused on preliminary research regarding phenolic compounds-containing fractions, which explains the limited results here reported (Table 2). Despite the paucity of studies found in this scope, information concerning the hepatoprotective effects of ethanol, ethyl acetate, n-butanol, acetone, dichloromethane and methanol fractions obtained from aerial parts, barks, leaves and flowering herbs extracts was collected. For the obtainment of the fractions different procedures were adopted: i) the plants were sequentially extracted with solvents of increasing polarity using a soxhlet apparatus (Sintayehu et al., 2012; Gupta et al., 2013); ii) the extracts described in the previous section (Orhan et al., 2003) or obtained by soaking in the solvent (at room temperature for 3 times, each for 24 hours) (Maheshwari et al., 2011) were dissolved in distilled water and fractionated through successive extractions with increasing polarity solvents; iii) previously prepared extracts were fractionated by column chromatography using a macroporous resin D101 (Zhao et al., 2014).

A. capillaris revealed hepatoprotective activity in HepG2.2.15 cells with IC_{50} values of 23.20 µg/mL for the 50% ethanol fraction, and 108.20 µg/mL for the 90% ethanol fraction, which confered them anti-Hepatitis B antigen secretion activity (Zhao et al., 2014). The other fractions were tested *in vivo* and, among them, an ethyl acetate fraction of *H. rhamnoides* can be highlighted, with a hepatoprotective activity at concentrations of 25-75 mg/kg in rat models (Maheshwari et al., 2011), closely followed by *B variegata* (mixture of ethyl acetate and n-butanol fractions), *B. monosperma* (acetone fraction), and *O. gratissimum* (dichloromethane and ethyl acetate fractions), at concentrations of 50-100 mg/kg in assays performed in rats (Gupta et al., 2013). *G. olivieri* (ethyl acetate fraction) and *C. abyssinica* (methanol fraction) were also able to

protect liver tissues of rats from chemically induced damage at concentrations of 125-250 and 200 mg/kg, respectively (Orhan et al., 2003; Sintayehu et al., 2012).

Table 1. Plant extracts with phenolic compounds showing hepatoprotective effects.

Plant	Origin	Part used	Extract	Extraction Method	Tested concentration	Hepatoprotective effects		Identified compounds	Reference
Aloe barbadensis Mill.	India	Aerial parts	Aqueous	Distilled water extraction of the plant marc and lyophilisation in freeze drier	125-500 mg/kg	*In vivo*	↓TG, ↓ALP, ↓ALT, ↓LDH, ↓TB, ↓AST, ↑AH, ↑G-6-P, ↑protein, ↓LPO, ↑*N*-de, ↓TG	Barbaloin	Chandan et al., 2007
Artemisia capillaris Thunb.	China	Aerial parts	90% Ethanol	Ethanol extraction of the dried plant and *in vacuo* concentration	-	*In vitro*	IC_{50} of 76.10 µg/mL on HepG2.2.15 cells	Chlorogenic acid; cryptochlorogenic acid; neochlorogenic acid; 3,5-dicaffeoylquinic acid; 4,5-dicaffeoylquinic acid; 3,4-dicaffeoylquinic acid; chlorogenic acid methyl ester; cryptochlorogenic acid methyl ester; neochlorogenic acid methyl ester	Zhao et al., 2014
Cardiospermum halicacabum Linn.	India	Leaves	99% Ethanol	Ethanol extraction of the lyophilized powder in a soxhlet apparatus and *in vacuo* concentration	250-500 mg/kg	*In vivo*	↓SGOT, ↓SGPT, ↓ALP, ↓TG, ↓TC, ↓LDL, ↓BUN, ↓creatinine, ↓TB, ↑HDL	Luteolin 7-*O*-glucuronide; apigenin 7-*O*-glucuronide and chrysoeriol 7-*O*-glucuronide	Jeyadevi et al., 2013

Plant	Origin	Part used	Extract	Extraction Method	Tested concentration	Hepatoprotective effects		Identified compounds	Reference
Cineraria abyssinica Sch. Bip. exA. Rich.	Ethiopia	Leaves	Aqueous	Boiling water extraction of the powdered shade-dried plant and lyophilisation	200 mg/kg	*In vivo*	↓ALP, ↓ALT, ↓AST	Rutin	Sintayehu et al., 2012
Cineraria abyssinica Sch. Bip. exA. Rich.	Ethiopia	Leaves	80% Methanol	Methanol maceration of the powdered shade-dried plant and *in vacuo* concentration	200 mg/kg	*In vivo*	↓ALP, ↓ALT, ↓AST	Rutin	Sintayehu et al., 2012
Cochlospermum vitifolium (Willd.) Sprengel	Mexico	Bark	Methanol	Methanol maceration of the dried plant and *in vacuo* concentration	100 mg/kg	*In vivo*	↓PGT, ↓ALP, ↑γ-GTP	Naringenin	Sánchez-Salgado et al., 2007
Ficus ingens (Miq.) Miq.	Saudi Arabia	Aerial parts	70% Ethanol	Ethanol percolation of the powdered dried plant, *in vacuo* concentration	100-400 mg/kg	*In vivo*	↓ALT, ↓AST, ↓ALP, ↓LDH, ↓TB, ↑TP, ↑albumin, ↑SOD, ↑GSH-Px, ↑CAT, ↑GSH, ↓MDA	*β*-Sitosterol glucoside; 7-hydroxy-2,5-dimethyl-chromen-4-one; chrysophanol; quercetin; aloe emodin glucoside; rutin and patuletin-3-*O*-methyl-3-*O*-rutinoside	Donia et al., 2013
Gentiana olivieri Griseb.	Turkey	Flowering herbs	80% Ethanol	Ethanol maceration of the powdered dried plant and *in vacuo* concentration	62.5-250 mg/kg	*In vivo*	↓Tissue MDA, ↓plasma MDA, ↑Tissue GSH, ↓ALT, ↓AST	Isoorienti	Orhan et al., 2003

Plant	Origin	Part used	Extract	Extraction Method	Tested concentration	Hepatoprotective effects		Identified compounds	Reference
Laggera pterodonta (DC). Benth	China	Aerial parts	75% Ethanol	Ethanol suspension of the powdered dried plant, filtration and lyophilisation	1-100 µg/mL	*In vitro*	↓ASAT, ↓ALAT, ↑Cell survival on primary hepatocytes	3,4-*O*-dicaffeoylquinic acid; 3,5-*O*-dicaffeoylquinic acid; 4,5-*O*-dicaffeoylquinic acid	Wu et al., 2007
Nelumbo nucifera Gaertn.	China	Leaves	60% Ethanol	Ethanol decoction of the powdered dried plant, filtration and *in vacuo* concentration	100-500 mg/kg	*In vivo*	↓ALT, ↓AST, ↓ALP, ↓GGT, ↓TB	Catechin rhamnoside; miricitrin-3-*O*-glucoside; hyperin; isoquercitrin; quercetin-3-*O*-rhamnoside; astragalin	Huang et al., 2010
Phyllanthus amarus Schum. & Thonn.	India	Aerial parts	50% Ethanol	Ethanol extraction of the powdered shade-dried plant and *in vacuo* concentration	200-600 µg/mL	*In vitro*	↓ALT, ↓LDH, ↓MDA, ↑GSH, ↑cell viability on HepG2 cells	Phyllanthin	Krithika et al., 2009
Schisandra chinensis (Turcz.) Baill.	China	Pollen	70% Ethanol	Ethanol extraction under reflux, centrifugation, filtration and *in vacuo* concentration	10-40 mg/kg	*In vivo*	↓AST, ↓ALT, ↓MDA, ↑SOD, ↑GSH-Px	Gallic acid; protocatechuic acid; vanillic acid; *p*-coumaric acid; resveratrol; quercetin; hesperetin; kaempferol; galangin	Cheng et al., 2013

Table 2. Plant fractions with phenolic compounds showing hepatoprotective effects.

Plant	Origin	Part used	Fraction provenance	Fraction	Fractionation method	Tested concentration	Hepatoprotective effects		Identified compounds	Reference
Artemisia capillaris Thunb.	China	Aerial parts	90% Ethanol extract	50% Ethanol	The 90% ethanol extract was subjected to macroporous resin D101 column chromatography with 50% ethanol	-	*In vitro*	IC_{50} of 23.20 µg/mL on HepG2.2.15 cells	Chlorogenic acid; cryptochlorogenic acid; neochlorogenic acid; 3,5-*O*-dicaffeoylquinic acid; 4,5-*O*-dicaffeoylquinic acid; 3,4-*O*-dicaffeoylquinic acid; chlorogenic acid methyl ester; cryptochlorogenic acid methyl ester; neochlorogenic acid methyl ester	Zhao et al., 2014
Artemisia capillaris Thunb.	China	Aerial parts	90% Ethanol extract	90% Ethanol	The 90% ethanol extract was subjected to macroporous resin D101 column chromatography with 90% ethanol	-	*In vitro*	IC_{50} of 108.20 µg/mL on HepG2.2.15 cells	Chlorogenic acid; cryptochlorogenic acid; neochlorogenic acid; 3,5-*O*-dicaffeoylquinic acid; 4,5-*O*-dicaffeoylquinic acid; 3,4-*O*-dicaffeoylquinic acid; chlorogenic acid methyl ester; cryptochlorogenic acid methyl ester; neochlorogenic acid methyl ester	Zhao et al., 2014

Plant	Origin	Part used	Fraction provenance	Fraction	Fractionation method	Tested concentration	Hepatoprotective effects		Identified compounds	Reference
Bauhinia variegata L., *Butea monosperma (lam)*, and *Ocimum gratissimum* Linn.	India	Barks (*B. variegata* and *B. monosperma*) and leaves (*O. gratissimum*)	Ethanol extract (*B. monosperma*), methanol extract (*B. Variegata* and *O. gratissimum*)	Ethyl acetate (*B. variegata*) + n-butanol (*B. variegata*) + acetone (*B. monosperma*) + dichloromethane (*O. gratissimum*) + ethyl acetate (*O. gratissimum*)	Sequentially extracted with petroleum ether, benzene, chloroform and acetone (*B. monosperma*); ethyl acetate and n-butanol (*B. Variegate*); hexane, dichloromethane, ethyl acetate and methanol (*O. gratissimum*)	50-100 mg/kg	*In vivo*	↓SGPT, ↓SGOT, ↓ALP, ↓TB	Flavonoids; tannins;other phenolic compounds	Gupta et al., 2013
Cineraria abyssinica Sch. Bip. exA. Rich.	Ethiopia	Leaves	-	Methanol	Successively extracted in a Soxhlet apparatus with chloroform, acetone and methanol	200 mg/kg	*In vivo*	↓ALP, ↓ALT, ↓AST	Rutin	Sintayehu et al., 2012
Gentiana olivieri Griseb.	Turkey	Flowering herbs	80% Ethanol extract	Ethyl acetate	Sequentially extracted with chloroform, ethylacetate and n-butanol/saturated with water	125-250 mg/kg	*In vivo*	↓Tissue MDA, ↓plasma MDA, ↑tissue GSH, ↓ALT, ↓AST	Isoorienti	Orhan et al., 2003
Hippophae rhamnoides L.	India	Leaves	70% Ethanol extract	Ethyl acetate	Sequentially extracted with hexane and ethyl acetate	25-75 mg/kg	*In vivo*	↑protein, ↓AST, ↓ALT, ↓TB, ↑GGT	Gallic acid; myricetin; quercetin; kaempferol; isorhamnetin	Maheshwari et al., 2011

1.2.4. Phenolic compounds as bioactives

Phenolic compounds constitute one of the most numerous and ubiquitously distributed group of plant secondary metabolites being present in plant leaves, seeds, bark, roots and flowers, with several thousand phenolic structures currently known. These compounds can range from simple molecules with low molecular weight (phenolic acids, phenylpropanoids, flavonoids) to highly polymerized compounds (lignins, lignans, melanins, tannins). The flavonoids represent the most common and widely distributed phenolic sub-group, comprising more than 8000 identified molecules (Andersen and Markham, 2006) sharing a common C6-C3-C6 phenylchromane skeleton (Figure 7). Based on the oxidation level of the ring C, different flavonoids classes are distinguished such as flavones, flavonols, flavanones, flavan-3-ols, anthocyanins, dihydroflavonols and isoflavones (Table 7). Other minor flavonoid groups can also be found in plants and foods, such as the opened chalcone and dihydrochalcone forms (Figure 7), flavan-3,4-diols or aurones (Santos-Buelga and González-Paramás, 2014).

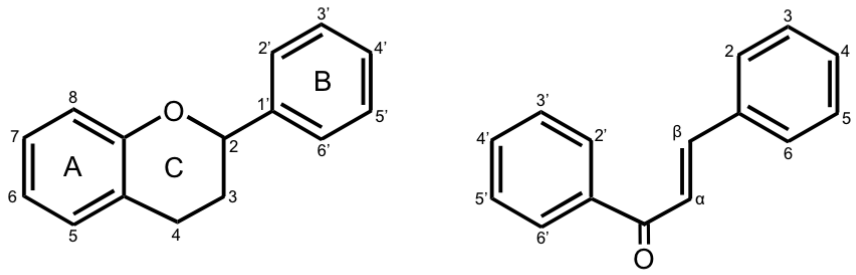
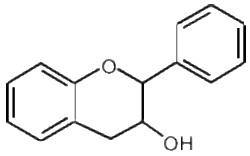
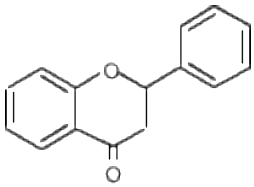
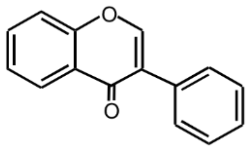
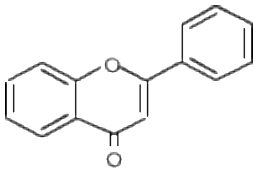
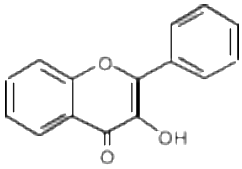
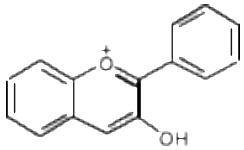
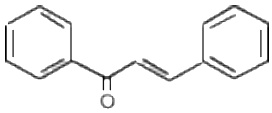


Figure 7. Basic structure and numbering of flavonoids: phenylchromane (left) and chalcone (right) forms.

On the other hand, lignans are one of the most characteristic groups of phenylpropanoids, representing their most abundant biogenetically related and structurally defined group (Harmatha and Dinan, 2003); these compounds are known to possess various biological activities such as antibacterial, antifungal, antiviral, anticancer, anti-inflammatory and antioxidant effects, by acting in the prevention of diseases linked to reactive oxygen species (MacRae and Towers, 1984). Beyond these

compounds, phenolic acids are well-known for their action on the autoxidation of linoleic acid micelles through the direct inhibition of *trans*, *trans*-conjugated diene hydroperoxide formation associated to the hydrogen donation ability of the phenol (Torel et al., 1986; Chimi et al., 1991).

Table 3. Basic structures of the main flavonoid classes (Adapted from Santos-Buelga and González-Paramás, 2014).

Flavonoid class	Core structure	Examples
Flavan-3-ols		(Epi)catechin, (epi)gallocatechin proanthocyanindins
Flavanones		Naringenin, hesperidin, taxifolin, eriodictyol
Isoflavones		Genistein, daidzein, biochanin A, puerarin
Flavones		Apigenin, luteolin, chrysin, chrysoeriol
Flavonols		Quercetin, kaempferol, myricetin, isorhamnetin
Anthocyanins		Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin
Chalcones		Naringenin chalcone, phloretin, arbutin

Tables 4-7 present a collection of results obtained in studies that focused on the isolation and characterization of phenolic compounds extracted from several hepatoprotective plants. In order to facilitate the interpretation of the studies that have been carried out in this field, the isolated molecules were organized by family, including flavonoids (Table 4), lignan compounds (Table 5), phenolic acids (Table 6), and other phenolic compounds (Table 7).

This sort of research has been greatly assisted by modern physico-chemical techniques of isolation and structural elucidation namely nuclear magnetic resonance (NMR), such as ^{1}H NMR, ^{13}C NMR, homonuclear two-dimensional NMR correlation spectroscopy (COSY), homonuclear decoupling, attached-proton-test (APT), heteronuclear chemical shift correlation (HETCOR), nuclear verhauser effect (NOE) difference, selective insensitive nuclei enhanced by polarization transfer (INEPT), and correlation by means of long range coupling (COLOC) experiments; some of which were applied in the presented studies.

The phenolic compounds were isolated from hepatoprotective plants aerial parts, flowering herbs, leaves, roots, and rhizomes extracts and fractions prepared as described in the previous sections (or using similar procedures). The isolation of the compounds was achieved by silica gel, C18, LH-20 and macroporous column chromatography, reversed-phase high-performance liquid chromatography (HPLC) and medium pressure liquid chromatography (MPLC), high-performance liquid chromatography with diode-array detection (HPLC-DAD), thin-layer chromatography (TLC), preparative thin-layer chromatography (PTLC and HPTLC); and further identification of the compounds was performed by different analytical approaches, such as NMR, fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS), high resolution electrospray ionisation mass spectrometry (HR-ESI-MS), Fourier transform infrared spectroscopy (FT-IR), or HPLC.

In the *in vitro* assays, the tested concentration of flavonoids ranged from 1 to 390 µM, with luteolin present in *E. arvense* revealing strong hepatoprotective effects at very low concentrations, with EC_{50} values of 20.20 µM in HepG2 cells (Oh et al., 2004). 5,2'-Dihydroxy-7-*O*-β-D-glucuronylflavone, luteolin 7-*O*-β-D-glucuronide, quercetin 3-*O*-

β-D-glucopyranoside; quercetin 5-*O*-β-D-glucopyranoside, and quercetin 3-*O*-a-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside isolated from *L. indica* revealed the capacity to inhibit hepatitis B virus HBV secretion from HepG2.2.15 cells, and viral DNA replication or transcription (Kim et al., 2007). On the other hand, isoorientin from *G. olivieri* and rutin from *C. abussinica* showed *in vivo* hepatoprotection by decreasing the levels of MDA, ALT, ALP, AST, and LDH (Table 4) (Sintayehu et al., 2012).

In what concerns the lignan compounds, 4-{erythro-2-[3-(4-hydroxyl-3,5-dimethoxyphenyl)-3-*O*-β-D-glucopyranosyl-propan-1-ol]}-*O*-syringaresinol isolated from *E. hainanesis* was tested *in vitro* and revealed the capacity to increase cell survival and inhibit cytotoxicity on WB-F344 cells (Feng et al., 2014), whereas phyllanthin, isolated from *P. amarus*, was able to decrease ALT, LDH, and MDA levels (on HepG2 cells), and increase cell viability (Table 5) (Krithika et al., 2009).

Different phenolic acids and derivatives were isolated from hepatoprotective plants. 3-*O*-Caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoyl-muco-quinic acid, 4,5-di-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, and 5-*O*-(*E*)-*p*-coumaroylquinic acid were isolated from *L. indica* and revealed the ability to inhibit HBV secretion from HepG2.2.15 cells and HBV replication and transcription (Kim et al., 2007). Gallic acid, obtained from *P. peltatum*, was able to decrease TBARS, ALP, AST, ALT, triacylglycerol, TB, glucose, and cholesterol levels and increase SOD, CAT, GSH, protein, albumin, lipase, and α-amylase concentration, in *in vivo* systems (Table 6) (Nabavi et al., 2013).

In addition to these molecules, other phenolic compounds were found in medicinal plants, like hydrangesides A, C, and D, onitin, 1-*O*-[2-*O*-(5-*O*-syringoyl-β-D-apiofuranosyl)-β-D-gluco-pyranosyl]-isoamyl alcohol, 7-*O*-[2-*O*-(5-*O*-vanilloyl-β-D-apiofuranosyl)-β-D-glucopyranosyl]-phenylmethanol, and 1-*O*-[6-*O*-(5-*O*-vanilloyl-β-D-apiofuranosyl)-β-D-glucopyranosyl]-3,4,5-trimethoxybenzene. The hepatoprotective activity of those compounds was tested *in vitro* and while onitin presented EC_{50} values of 85.8 M in HepG2 cells (Oh et al., 2004), the remaining compounds increased cell survival and inhibited chemically-induced cytotoxicity in WB-F344 (Feng et al., 2014) and HL-7702 (Shi et al., 2014) cells (Table 7).

Table 4. Plant flavonoids showing hepatoprotective effects.

Flavonoid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
5,2'-Dihydroxy-7-*O*-β-D-glucuronylflavone	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	390 µM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007
Isoorientin	*Gentiana olivieri* Griseb.	Turkey	Flowering herbs	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with chloroform, ethylacetate and n-butanol/saturated with water	The ethylacetate fraction was subjected to column chromatography, TLC	NMR and FAB-MS	15 mg/kg	*In vivo*	↓Tissue MDA, ↓plasma MDA, ↑Tissue GSH, ↓ALT, ↓AST	Orhan et al., 2003
Luteolin	*Equisetum arvense* L.	Korea	Aerial parts	Methanol, 7 days, and sequentially partitioned with n-hexane, ethylacetate, and butanol	Bioactive ethylacetate fraction purification by Chromatography on silica gel column and reversed-phase HPLC on column	NMR and MS	1, 10, 50 and 100 µM	*In vitro*	EC_{50} of 20.20 µM in Hep G2 cells	Oh et al., 2004

Flavonoid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
Luteolin 7-*O*-β-D-glucuronide	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	390 μM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑Iinhibition of HBV DNA replication or transcription	Kim et al., 2007
Quercetin 5-*O*-β-D-glucopyranoside	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	390 μM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007

Flavonoid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
Quercetin 3-*O*-a-L-rhamnopyranosyl(1→6)-β-ɒ-glucopyranoside	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	390 µM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007
Rutin	*Cineraria abyssinica* Sch. Bip. exA. Rich.	Ethiopia	Leaves	Successively extracted in a a Soxhlet apparatus with chloroform, acetone and methanol	Bioactive methanolic fraction purification by PTLC on silica column, LH-20 column chromatography and TLC	NMR and ESI-MS	100 mg/kg	*In vivo*	↓ALP, ↓ALT, ↓AST	Sintayehu et al., 2012

Table 5. Plant lignan compounds showing hepatoprotective effects.

Lignan compound	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
4-{Erythro-2-[3-(4-hydroxyl-3,5-dimethoxyphenyl)-3-*O*-β-D-glucopyranosyl-propan-1-ol]}-*O*-syringaresinol	*Erycibe hainanesis* Merr.	China	Roots	95% Ethanol extract, and sequentially partitioned with petroleum ether, ethylacetate and n-butanol	The n-butanol extract was subjected to column chromatography over macroporous resin, and purified using a normal-phase silica gel column, HPLC-DAD	NMR and ESI-MS	1×10^{-5} M	*In vitro*	↑Cell survival (WB-F344 cells), ↑inhibition of citotoxicity	Feng et al., 2014
Phyllanthin	*Phyllanthus amarus* Schum. & Thonn.	India	Aerial parts	50% Ethanol extract	The ethanol extract was purified using a silica gel column, TLC, HPTLC, reversed-phase HPLC	NMR and FT-IR	10-30 µM	*In vitro*	↓ALT, ↓LDH, ↓MDA, ↑GSH, ↑cell viability on HepG2 cells	Krithika et al., 2009

Table 6. Plant phenolic acids showing hepatoprotective effects.

Phenolic acid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
3-*O*-Caffeoylquinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-Butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007
3,4-di-*O*-Caffeoylquinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007

Phenolic acid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
3,5-di-*O*-Caffeoylquinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007
3,5-di-*O*-Caffeoyl-*muco*-quinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007

Phenolic acid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
4,5-di-*O*-Caffeoylquinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007
5-*O*-Caffeoylquinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n- butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007

Phenolic acid	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration		Hepatoprotective effects	Reference
5-*O*-(*E*)-*p*-Coumaroylquinic acid	*Lactuca indica* L.	Korea	Aerial parts	The ethanol extract was dissolved in distilled water and fractionated through successive extractions with n-hexane, chloroform and n-butanol	The n-butanol fraction was subjected to silica gel column chromatography, purified by reversed-phase HPLC, reversed-phase MPLC	NMR and MS	0.39 mM	*In vitro*	↑Inhibition of HBV secretion from HepG2.2.15 cells ↑ inhibition of HBV DNA replication or transcription	Kim et al., 2007
Gallic acid	*Peltiphyllum peltatum* Engl.	England	Leaves and rhizomes	The ethanol extract was fractionated through successive extractions with petroleum ether, chloroform, ethyl acetate, n-butanol and water	The ethyl acetate fraction was subjected to C18 column and flash chromatography, and repurified by LH-20 column chromatography	HPLC	10-20 mg/kg	*In vivo*	↓TBARS, ↑SOD, ↑CAT, ↑GSH, ↓ALP, ↓AST, ↓ALT, ↓triacylglycerol, ↓TB, ↑protein, ↑albumin, ↓glucose, ↑lipase, ↑α-amylase, ↓cholesterol	Nabavi et al., 2013

Table 7. Other plant phenolic compounds showing hepatoprotective effects.

Phenolic compound	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
Hydrangeside A	*Hydrangea paniculata* Sieb.	China	Stems	Aqueous extract, passed through macroporous resin column and eluted with water, 30% ethanol, 70% ethanol and 95% ethanol	The 70% ethanol fraction was subjected to silica gel column chromatography, separated by reversed-phase silica MPLC and purified by preparative HPLC	NMR and HR-ESI-MS	10 μM	*In vitro*	↑Cell survival (HL-7702 cells), ↑inhibition of citotoxicity	Shi et al., 2014
Hydrangeside C	*Hydrangea paniculata* Sieb.	China	Stems	Aqueous extract, passed through macroporous resin column and eluted with water, 30% ethanol, 70% ethanol and 95% ethanol	The 70% ethanol fraction was subjected to silica gel column chromatography, separated by reversed-phase silica MPLC and purified by preparative HPLC	NMR and HR-ESI-MS	10 μM	*In vitro*	↑Cell survival (HL-7702 cells), ↑inhibition of citotoxicity	Shi et al., 2014

Phenolic compound	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
Hydrangeside D	*Hydrangea paniculata* Sieb.	China	Stems	Aqueous extract, passed through macroporous resin column and eluted with water, 30% ethanol, 70% ethanol and 95% ethanol	The 70% ethanol fraction was subjected to silica gel column chromatography, separated by reversed-phase silica MPLC and purified by preparative HPLC	NMR and HR-ESI-MS	10 µM	*In vitro*	↑Cell survival (HL-7702 cells), ↑inhibition of citotoxicity	Shi et al., 2014
Onitin	*Equisetum arvense* L.	Korea	Aerial parts	Methanol, 7 days, and sequentially partitioned with n-hexane, ethylacetate, and butanol	Bioactive ethylacetate fraction purification by chromatography on silica gel column and reversed-phase HPLC on C18 column	NMR and MS	1, 10, 50 and 100 µM	*In vitro*	EC_{50} of 85.8 M in HepG2 cells	Oh et al., 2004

Phenolic compound	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
1-*O*-[2-*O*-(5-*O*-Syringoyl-β-D-apiofuranosyl)-β-D-gluco-pyranosyl]-isoamyl alcohol	*Erycibe hainanesis* Merr.	China	Roots	95% Ethanol extract, and sequentially and successively partitioned with petroleum ether, ethylacetate and n-butanol	The n-butanol extract was subjected to column chromatography over macroporous resin, and purified using a normal-phase silica gel column, HPLC-DAD	NMR and ESI-MS	1×10^{-5} M	*In vitro*	↑Cell survival (WB-F344 cells), ↑inhibition of citotoxicity	Feng et bal., 2014
7-*O*-[2-*O*-(5-*O*-Vanilloyl-β-D-apiofuranosyl)-β-D-glucopyranosyl]-phenylmethanol	*Erycibe hainanesis* Merr.	China	Roots	95% Ethanol extract, and sequentially and successively partitioned with petroleum ether, ethylacetate and n-butanol	The n-butanol extract was subjected to column chromatography over macroporous resin, and purified using a normal-phase silica gel column, HPLC-DAD	NMR and ESI-MS	1×10^{-5} M	*In vitro*	↑Cell survival (WB-F344 cells), ↑inhibition of citotoxicity	Feng et bal., 2014

Phenolic compound	Source	Origin	Part used	Extraction method	Isolation method	Identification method	Tested concentration	Hepatoprotective effects		Reference
1-*O*-[6-*O*-(5-*O*-Vanilloyl-β-D-apiofuranosyl)-β-D-glucopyranosyl]-3,4,5-trimethoxybenzene	*Erycibe hainanesis* Merr.	China	Roots	95% Ethanol extract, and sequentially and successively partitioned with petroleum ether, ethylacetate and n-butanol	The n-butanol extract was subjected to column chromatography over macroporous resin, and purified using a normal-phase silica gel column, HPLC-DAD	NMR and ESI-MS	1×10^{-5} M	*In vitro*	↑Cell survival (WB-F344 cells), ↑inhibition of citotoxicity	Feng et bal., 2014

1.3. The particular case of artichoke, borututu and milk thistle

1.3.1. Botanical characterization and hepatoprotective uses

Artichoke (*Cynara scolymus* L.; Figure 8) is an annual to biennal plant belonging to the Asteraceae family, native to the Mediterranean region. It grows until 1.4 to 2 m high, with arching, deeply lobed, silvery, glaucous-green leaves of 50 to 82 cm long. The stems are about 105 cm tall, erect, simple, and branched at the top, with circular section, longitudinally ribbed. The bracts are fleshy and usually blunt or notched at the apex (though cultivars with acute ans spiny bracts do exist) and enclose the flower heads and large immature inflorescences, known as capitula or head, with an edible bud about 8 to 15 cm of diameter with numerous triangular scales. The flowers are thistle-like with globular heads, from which mauve-purple tufts into a flat-topped inflorescence. The edible portions of the buds consist primarily of the fleshy lower portions of the involucral bracts and the base, known as the heart; the mass of immature florets in the center of the bud is called the choke or beard. These are inedible in older, larger flowers.

Milk thistle (*Silybum marianum* (L.) Gaertn; Figure 8) is an annual or biennal Mediterranean herb that also belongs to the Asteraceae family. It is a biennial herb, with 0.3 to 2 m tall, that possesses ribbed stems, sparely villous, emerging from a rosette of leaves. The leaves are pale green, mottled along the veins, and the rosette leaves are obovate in outline, deeply triangular-lobed, ranging from 10 to 50 cm long and 5 to 25 cm wide, with a petiolate base, withered when flowering. The cauline leaves are slightly lobed, ovate-lanceolat in outline, with 2 to 20 cm of length and 0.7 to 8 cm wide, the base auriculate and amplexicaul, and the margins of all leaves are strongly spiny-dentate and sparsely pilose. It has a terminal and solitary capitula, an involucre of 2.5 to 4 cm long, phyllaries with an oppressed ovate base that widens to an ovate appendage that recurves in the outer phyllaries, 1 to 4.5 cm of length, with acuminate pungent apex. The corolla is blue-violet, with a filiform tube of 22 to 25 mm long and lobes of 7 to 9 mm lond, erect. The achenes are brown, with black streaks, obovoid, 6 to 7 mm long, slightly compressed. Pappi are 15 to 22 mm long and connate into a basal ring, falling as a unit.

Borututu (*Cochlospermum angolensis* Welw.; Figure 8) is a widespread tree in Angola belonging to the Cochlospermaceae family. It can attain a height of 3 to 6 m with a

trunk of about 22.9 cm of diameter at the base and divaricate branches. The leaves are rather coriaceous and deeply divided into five lanceolate or oblong-lanceolate, acuminate segments, nearly entire or serrulate above, glabrous in both sides or with pubescent nerves beneath, the segments usually overlap each other at the base. The larger leaves are 15.2 to 17.8 cm broad, 8.9 to 10.2 cm long, with central lobe about 3.2 to 4.5 cm broad, and a petiole of 5.1 to 10.2 cm long. Flowers are deep yellow and appear about three together, with 7.6 to 1.5 cm of diameter. The pedicels are puberulous, with around 2.5 cm of length, and the sepals are nearly or quite glabrous. The anthers are dehiscing by a single minute terminal slit, and the ovary is densely and softly villous. It has fruits about 7.6 cm long and 5.1 cm of diameter, generally ellipsoidal or obovoid, depressed at the top, which can be separated into four valves when ripe. The seeds are reniform, black, and shining; they are enveloped in deciduous cotton.

Figure 8. Artichoke (left), borututu (centre), and milk thistle (right).

Plant names are according to Flora Iberica (http://www.floraiberica.es) and to The Plant List (http://www.theplantlist.org).

These three medicinal plants are widely used to prevent oxidative stress and different liver diseases (Adzet et al., 1987; Campos et al., 1989; Fehér et al., 1990; Gebhardt, 1997; Ferreres et al., 2013), as previously detailed in *section 1.2*. In this connection, and given the wide range and availability of supplements based on these plant species, (such as infusions, capsules, pills, and syrups, among others) they were chosen to undertake this study.

1.3.2. Chemical composition

Consumed raw, boiled, steamed or fried, artichoke is today widely cultivated all over the world for its large fleshy immature inflorescences. It is known since ancient times as a tasty plant that can be used in soups, stews and salads, being perceived as a nutritious and healthy vegetable (Lattanzio et al., 2009) due to its antioxidant and hepatoprotective effects (Gebhardt and Fausel, 1997; Zapolska-Downar et al., 2002; Jiménez-Escrig et al., 2003; Wang et al., 2003; Falleh et al., 2008; Kubić et al., 2008; Gouveia and Castilho, 2012). This plant contains very little fat and high levels of minerals, vitamin C, fibre, inulin, polyphenols hydoxycinnamates and flavones, but most of its activity could be related to the polyphenolic fraction (Schütz et al., 2004; Falleh et al., 2008; Lutz et al., 2011; Pandino et al., 2011a and b; Gouveia and Castilho, 2012). In previous works, artichoke hydroalcoholic extracts proved to be a good source of flavonoids such as luteolin and apigenin glycosides, and mono-/di-caffeoylquinic acids and derivatives (Abu-Reidah et al., 2013; Jun et al., 2007).

As far as we know nothing was reported on milk thistle and borututu nutritional value and primary metabolites, but there are several studies regarding their phytochemical composition in non-nutrients such as phenolic compounds. For instance, milk thistle is known to contain a standard mixture of flavonoligans, called silymarin, wich is indicated as the main responsible for its terapeutical effects (Giese, 2001; Zuber et al., 2002; Doehmer et al., 2011) and is composed of diastereomeric and/or constitutional isomers of silybin A, silybin B, isosylibin A, isosylibin B, silychristin, isosilychristin, and silydianin (Wang et al., 2010; Calani et al., 2012; Althagafy et al., 2013). On the other hand, the phenolic composition of borututu hydromethanolic and aqueous extracts were recently characterized and revealed high levels of ellagic acid and methyl ellagic acid and their derivatives, with methyl an ellagic acid pentoside isomer as the major compound (Ferreres et al., 2013).

1.3.3. Bioactive properties

Artichoke, borututu, and milk thistle have numerous pharmacological effects, such as antioxidant and hepatoprotective activities, as described in different studies. Milk thistle silymarin (present in the seeds) seems to be the main pharmacological active ingredient present in this plant, with reported effects against hepatotoxicity, acute and

chronic liver diseases (Giese, 2001; Zuber et al., 2002; Doehmer et al., 2011), and in the prevention of spleen and gallbladder disorders (Rainone, 2005). This flavonoid complex also has been reported as inhibitor of tumour growth in hepatocarcinoma in cell (Brandon-Warner et al., 2010) and animal models (Bousserouel et al., 2012).

Borututu bark infusion is used in the traditional medicine of Angola for the treatment of hepatic diseases and for the prophylaxis of malaria (Poppendieck, 1981; Silva et al., 2011), wereas the infusion of its dry roots showed high 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity (Costa et al., 2012), inhibited *Plasmodium falciparum*, and depressed the DNA synthesis of mice erythrocytes infected by *Plasmodium berghei* (Presber et al., 1991)

On its turn, artichoke leaves are used for their cholagogue, choleretic and choliokinetic actions, and also for treatment of dyspepsia and as anti-diabetics (Koubaa et al., 1999).

Despite the described hepatoprotective effects of the three mentioned plants and a few reports on their anti-hepatocellular carcinoma activity (Miccadei et al., 2008; Brandon-Warner et al., 2010), studies with the most consumed forms (infusions and dietary supplements) are scarce. Hepatocellular carcinoma is a major health problem with more than 660,000 new cases per year worldwide, being a rapid fatal disease with a life expectancy of about 6 months from the time of diagnostics; it has the third highest mortality rate among all cancers (Jemal et al., 2011).

1.4. Objectives and Working Plan

There has been an intensive scientific effort to validate the effectiveness of herbal formulations, since the preparation of dietary supplements/nutraceuticals and some pharmaceutical products are based on the extraction of bioactive compounds from natural products (Dai and Mumper, 2010). In the present study, three plant species, namely artichoke, borututu, and milk thistle, which are commonly present in nutraceutical formulations/dietary supplements, were selected due to their availability in different formulations.

Given the fact that these plants are consumed with medical/functional purposes and incorporated in different dietary supplements (borututu and milk thistle are directly used in the form of pills), and that artichoke and milk thistle can also be eaten (Lattanzio et al. 2009; Vaknin et al. 2008), it becomes important to know their nutritional/energetic contribution. Thus, in the present Thesis, these three plant species have been characterized in terms of nutritional properties and energetic contribution, and their chemical composition has also been studied regarding their hydrophilic (sugars and organic acids) and lipophilic (tocopherols and fatty acids) components. Besides, the phenolic profile of different formulations and extracts from the parts most commonly used of these plants was also analyzed, once phenolics are pointed out as their most active compounds (Razali et al., 2012).

The bioactivity screening of plant derived products is often supported by evaluating their antioxidant activity, as a preliminary approach. In the present study, four different assays were used: DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition. Also, HepG2 human cell line was used to assess anti-hepatocellular carcinoma activity, while a primary culture of porcine liver cells was established to evaluate hepatotoxicity. In fact, since some potential effects of compounds naturally present in plants are difficult to anticipate, the assessment of the safety of a plant extract used as a food or a medicine by the population is completely mandatory (Jacociunas et al., 2013). Moreover, the anti-microbial activity against clinical isolates of multiresistant bacteria (*Escherichia coli*, *Escherichia coli* spectrum extended producer of β-lactamases (ESBL), *Proteus mirabilis*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*) was also assessed.

Besides aiming studying the influence of the formulation type in the chemical profile and bioactivity, this study was designed also to evaluate supposed differences resulting from using different percentages of these plant species in the formulations, as well as the effect of adding honey to their infusions (with different proportions of each plant). In addition, an attempt was made to correlate the bioactivity of the samples with their phenolic composition, bearing in mind that such compounds are considered as the main responsible for the beneficial properties of these plant products (Razali et al., 2012).

Furthermore, the effect of gamma irradiation, one of the most promising decontamination methods for many foodstuffs, at different doses (1 and 10 kGy) on borututu regarding its chemical characterization, antioxidant and anti-hepatocellular carcinoma activities, was tested in order to conclude about the effectiveness of this preservation technique when applied to plant dry material.

Figures 9 and 10 present a schematic representation of the working plan followed to accomplish the proposed objectives regarding to chemical composition and bioactive properties of the different samples, respectively.

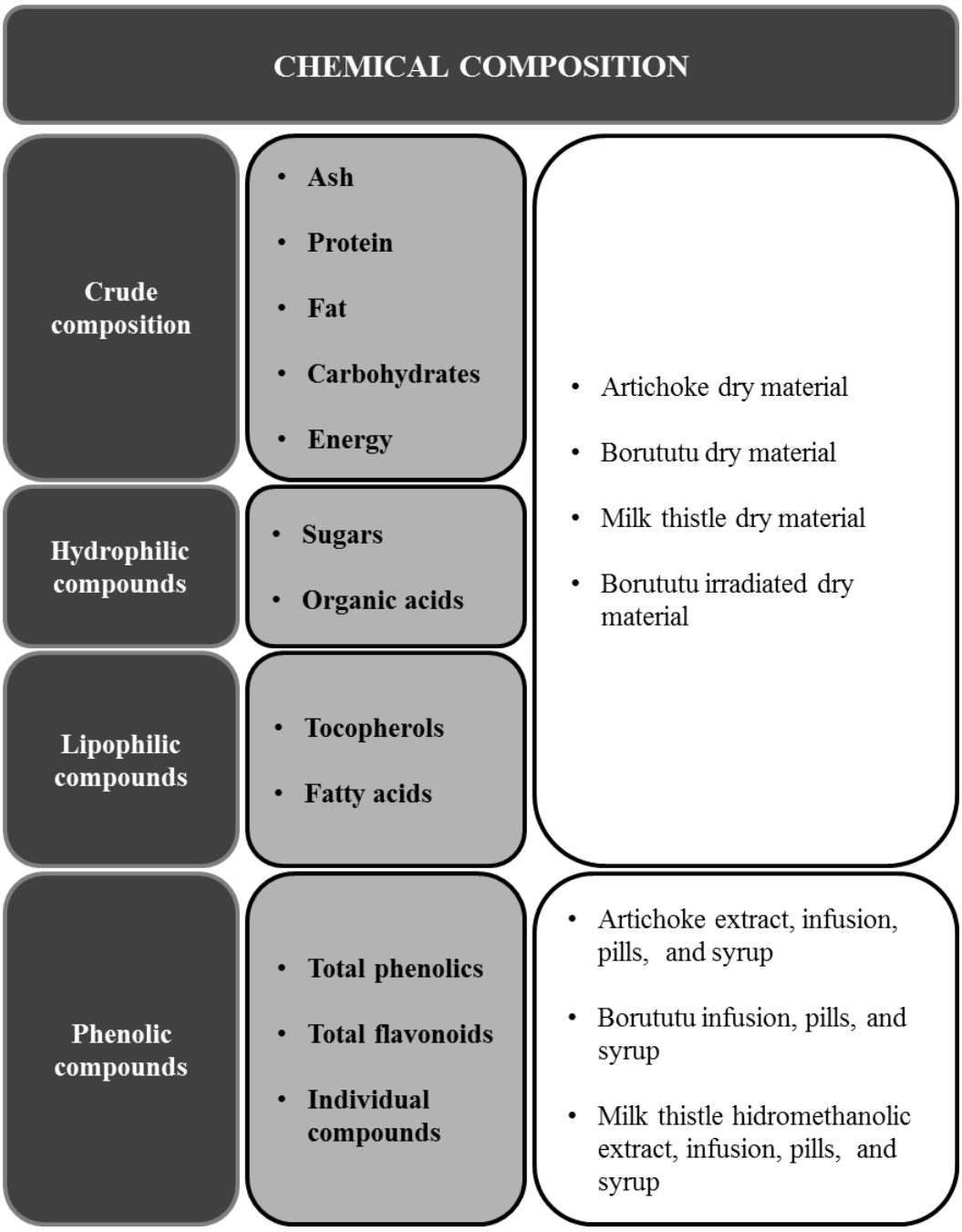


Figure 9. Schematic representation of the working plan regarding to chemical composition of the studied samples.

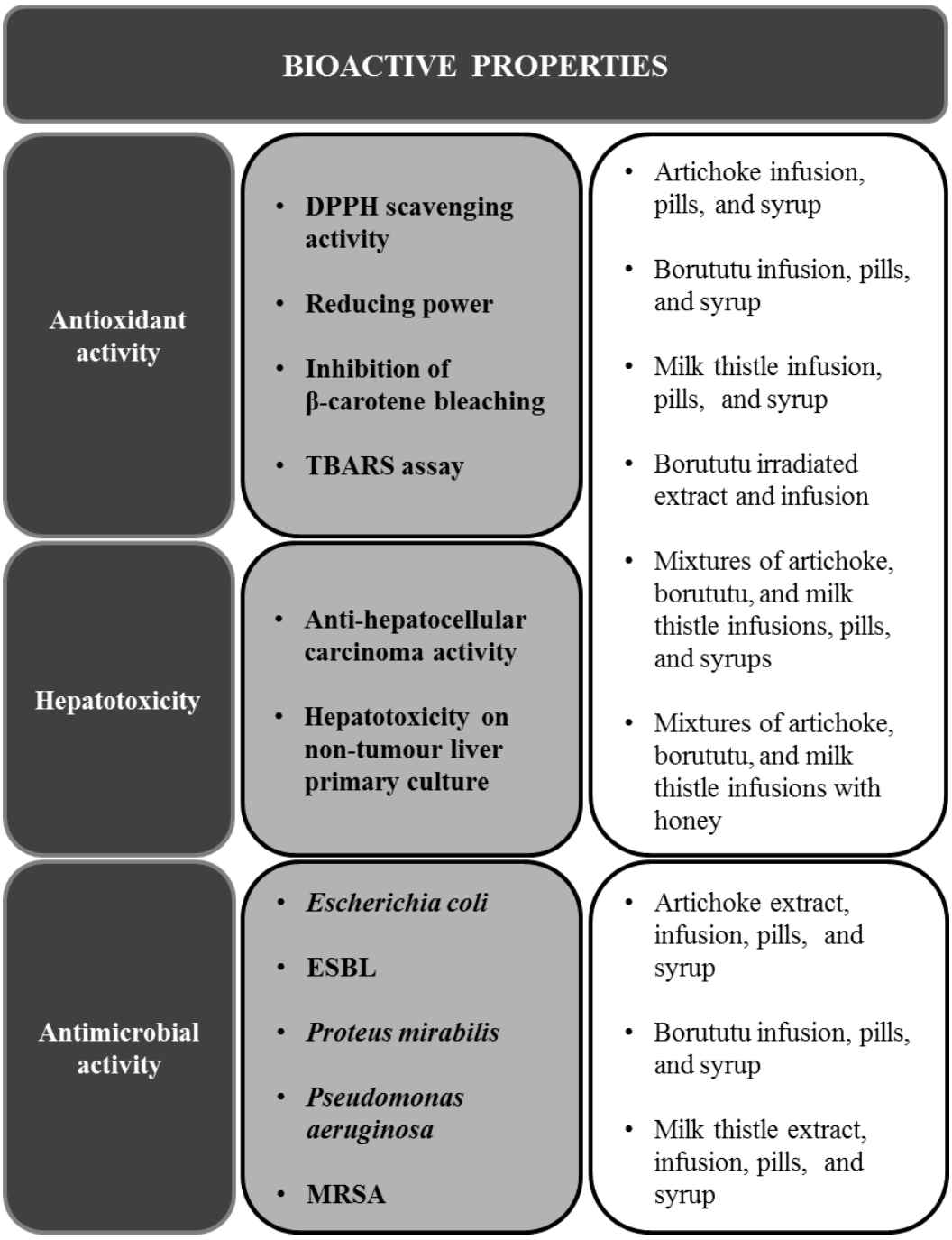


Figure 10. Schematic representation of the working plan regarding to bioactive properties of the studied samples.

CHAPTER 2

Material and Methods

2.1. Standards and reagents

For chemical analyses. Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). Phenolic standards were from Extrasynthèse (Genay, France). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

For antioxidant activity evaluation. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Sigma (St. Louis, MO, USA).

For anti-hepatocellular carcinoma activity and hepatotoxicity evaluation. Ellipticine, phosphate buffered saline (PBS), acetic acid, sulforhodamine B (SRB), trichloroacetic acid (TCA) and Tris were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediamine tetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) and DMEM (Dulbecco's Modified Eagle Medium) were from Hyclone (Logan, Utah, USA).

For antimicrobial activity evaluation. The culture media Mueller Hinton broth (MHB), Wilkins–Chalgren broth (WCB) and Columbia agar (CA) with 5% horse blood were obtained from Biomerieux (Marcy l'Etoile, France). The dye p-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St Louis, MO, USA) to be used as microbial growth indicator.

For irradiation assays. To estimate the dose and dose rate of irradiation, a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Company, UK) were used. To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with

purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

For honey quality analysis. For hydroxymethylfurfural (HMF) determination Carrez´s I and II reagents were used and obtained from Panreac (Barcelona, Spain). Phadebas was acquired from Magle AB (Lund, Sweden).

2.2. Plant materials and irradiation procedure

2.2.1. Dry material of artichoke, borututu and milk thistle

Artichoke, milk thistle and borututu were obtained from a herbalist shop in Bragança (Portugal), as dry materials for infusions preparation. The materials were composed of artichoke leaves, milk thistle plant, and borututu barks.

For subsequent analysis or preparation of extracts, the different samples were reduced to a fine dried powder (20 mesh) using a grinding mill, and mixed to obtain homogenized samples.

2.2.2. Irradiation of borututu dry material

For the irradiation procedure, borututu was obtained from a herbalist shop in Alcanede (Portugal), imported from Angola, as dry barks. The samples were divided into three groups: control (non-irradiated, 0 kGy), sample irradiated at 1 kGy, and sample irradiated at 10 kGy, where 1 kGy and 10 kGy were the predicted doses.

The irradiation of the samples was performed in a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd.) with four sources, total activity 177 TBq (4.78 kCi), in September 2013, and the dose rate for the irradiation position was obtained with Fricke dosimeter. During the irradiation process, the dose was estimated using Amber Perspex routine dosimeters (batch V, from Harwell Company), following the procedure previously described by Fernandes et al. (2013a). The estimated doses, dose rates and dose uniformity ratios (D_{max}/D_{min}) were, respectively: 1.20±0.07 kGy, 2.57±0.15 kGy h^{-1}, 1.20 for sample irradiated at 1 kGy and 8.93±0.14 kGy, 1.91±0.03 kGy h^{-1}, 1.02 for sample irradiated at 10 kGy. For simplicity, in the text and tables we considered the values 0, 1 and 10 kGy, for the doses of non-irradiated and irradiated samples 1 and 2, respectively.

After irradiation, the samples were reduced to a fine dried powder and mixed to obtain homogenized samples for analysis or preparation of infusions/extracts.

2.3. Nutritional characterization of the artichoke, borututu and milk thistle dry material

2.3.1. Crude composition

The samples were analyzed for chemical composition (protein, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 2005). Crude protein content (N×6.25) was calculated by the macro-Kjeldahl method; the crude fat was determined using a Soxhlet apparatus by extracting a known weight of sample with petroleum ether; the ash content was determined by incineration at 600±15 °C. Total carbohydrates were calculated by difference, and total energy was calculated according to the following equation:

$$\text{Energy (kcal)} = (\text{protein mass (g)} + \text{carbohydrates mass (g)}) \times 4 + \text{fat mass (g)} \times 9$$

2.3.2. Sugars composition

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by Reis et al. (2014) using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH2 column (5 µm, 4.6×250 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with commercial standards of each sugar. Quantification was based on the refraction signal response of each standard, using the IS method and by using calibration curves obtained from commercial standards of each compound: fructose (y=0.864x; R^2=0.999); glucose (y=0.909x; R^2=0.999); sucrose (y=0.892x; R^2=0.999); trehalose (y=0.953x; R^2=0.999); raffinose (y=0.847x; R^2=0.999). Sugar contents were further expressed in g per 100 g of dry weight.

2.3.3. Organic acids composition

Organic acids were determined following a procedure previously described (Pereira et al. 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation). Separation was achieved on a SphereClone (Phenomenex) reverse phase C18 column (5 μm, 4.6×250 mm i.d.) thermostatted at 35 ºC. The elution was performed with sulfuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the areas of their peaks recorded at 215 nm or 245 nm with calibration curves obtained from commercial standards of each compound. For quantitative analysis, calibration curves were prepared for: oxalic acid ($y=1x10^7x+96178$; $R^2=0.999$), quinic acid ($y=601768x+8853.2$; $R^2=1$), malic acid ($y=952269x+17803$; $R^2=1$), citric acid ($y=1x10^6+4170.6$; $R^2=1$), fumaric acid ($y=172760x+52193$; $R^2=0.999$), and shikimic acid ($y=8x10^7+55079$; $R^2=0.999$). The results were expressed in g per 100 g of dry weight.

2.3.4. Fatty acids composition

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column after derivatization to their fatty acid methyl esters (FAME) as described by Reis et al. (2014). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey–Nagel column (30 m × 0.32 mm i.d. × 0.25 μm df). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

2.3.5. Tocopherols composition

Tocopherols were determined following the procedure described by Barros et al. (2013). Analysis was performed by HPLC (Knauer equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (5µm, 250× 4.6 mm i.d.) normal-phase column from YMC Waters operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with commercial standards of each compound. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound: α-tocopherol (y=1.295x; R^2=0.991); β-tocopherol (y=0.396x; R^2=0.992); γ-tocopherol (y=0.567x; R^2=0.991); δ-tocopherol (y=0.678x; R^2=0.992). The results were expressed in mg per 100 g of dry weight.

2.4. Preparation of dietary supplements based on artichoke, borututu and milk thistle

2.4.1. Extracts

For phenolic individual compounds profiles: artichoke and milk thistle dry material (1 g) was extracted by stirring with 25 mL of methanol:water (80:20 *v:v*, 25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional 25 mL of methanol:water (80:20 *v:v*) for another hour. The combined extracts were evaporated at 40 ºC in a rotary evaporator (Büchi R-210, Flawil, Switzerland), frozen, lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), and re-dissolved in water:methanol (80:20, *v:v*) to a final concentration of 20 mg/mL.

For antioxidant properties, anti-hepatocellular carcinoma activity, and hepatotoxicity evaluation: methanolic extracts of borututu (non-irradiated and irradiated samples 1 and 2) were prepared. A dry weight (1 g) of each sample was stirred with methanol (30 mL) at 25 °C at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic

extracts were evaporated under reduced pressure and re-dissolved in methanol at a final concentration of 10 mg/mL.

2.4.2. Infusions

For phenolic individual compounds profiling and antimicrobial activity evaluation: infusions of artichoke, borututu, and milk thistle were prepared. Each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure; afterwards the obtained infusions were frozen and lyophilized. For phenolic compounds analysis, the lyophilized aqueous extracts were re-dissolved in water to a final concentration of 20 mg/mL for artichoke and milk thistle, and 30 mg/mL for borututu. For antimicrobial activity, solutions of 1000 mg/mL were prepared for the three plants, by re-dissolving in water. The remaining liophilized material was used to prepare the mixtures described in *section 2.4.5*.

For antioxidant properties, anti-hepatocellular carcinoma activity, and hepatotoxicity evaluation: a dry weight of each material (20 g of artichoke and 8.5 g of milk thistle corresponding to the recommended 4 spoons; and 10 g of borututu) was added to 1 L or 0.5 L (in the case of borututu) of boiling distilled water, left to stand at room temperature for 10 min, filtered under reduced pressure, frozen, lyophilized and re-dissolved in distilled water at a final concentration of 10 mg/mL.

In the case of borututu (non-irradiated and irradiated samples 1 and 2), the different samples (1 g) were added to 200 mL of boiling distilled water, left to stand at room temperature for 5 min, and filtered through Whatman No. 4 paper (final concentration 5 mg/mL).

2.4.3. Pills

For phenolic individual compounds profiling: the pills (1.5 g) were reduced to powder and submitted to hydromethanolic extraction by stirring with 25 mL of methanol:water (80:20 *v:v*, 25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional 25 mL of methanol:water (80:20 *v:v*) for another hour. The combined extracts were dried and purified using a C_{18} SepPak® Vac 3 cc cartridge (Phenomenex), previously activated with methanol

followed by water; sugars and more polar substances were removed by passing through 10 mL of water and the purified samples were further eluted with 5 mL of methanol. The extract was concentrated under vacuum and totally re-dissolved in methanol:water (20:80 *v:v*, 2 mL); the final concentrations of artichoke, borututu, and milk thistle pills extracts were 3, 14, and 59 mg/mL, respectively.

For antioxidant properties, anti-hepatocellular carcinoma activity, and hepatotoxicity evaluation: three pills of each sample (1500 mg) were dissolved in 100 mL of distilled water (final concentration 15 mg/mL).

For antimicrobial activity evaluation: the pills were reduced to powder and dissolved in distilled water (final concentration 150 mg/mL).

2.4.4. Syrups

For phenolic individual compounds profiling: the syrups of artichoke and borututu (5 mL) were submitted to a purification following the procedure described for the pills. The extracts were, then, concentrated under vacuum and totally re-dissolved in methanol:water (20:80 *v:v*, 2 mL) to a final concentration of 7, 23, and 16 mg/mL, for artichoke, borututu, and milk thistle, respectively.

For antioxidant properties, anti-hepatocellular carcinoma activity, and hepatotoxicity evaluation: the individual syrups (1000, 100, and 26.4 mg/mL, for artichoke, borututu, and milk thistle, according with the label information) and the syrup with the three plants (artichoke and borututu at 150 mg/mL and milk thistle at 350 mg/mL, according with the label information) were directly used. Also, a mixture with the syrups of each plant was prepared at the same concentrations indicated for the commercial syrup containing the three plants (artichoke and borututu at 150 mg/mL and milk thistle at 350 mg/mL).

For antimicrobial activity evaluation: the syrups were directly used (1000, 100, and 26.4 mg/mL, for artichoke, borututu, and milk thistle).

2.4.5. Mixtures of the dietary supplements

Mixtures containing different proportions (1:1:1, 2:1:1, 1:2:1, and 1:1:2, *m:m:m*) of artichoke, borututu and milk thistle were prepared from each formulation (infusion, pills and syrup), and further dissolved in distilled water to a final concentration of 6

mg/mL. For the preparation of the mixtures of infusions, the lyophilized extracts, described in *section 2.4.2*, were used. Twelve stock solutions were thus obtained: four mixtures of infusions, four mixtures of pills and four mixtures of syrups.

2.4.6. Mixtures of the dietary supplements with honey

Infusions were prepared by adding 1 g of plant material (1 g of each plant for individual infusions, 0.5 g of each plant for mixtures of two plants, and 0.33 g of each plant for mixtures containing the three plants) to 100 mL of boiling distilled water and filtering after 5 min of standing. For the infusions containing honey, the same procedure was followed, but 5 g (the equivalent to a teaspoon) of honey were added after the filtration process.

Thus, the following samples were studied: *i)* eight control samples (plants or honey separately): three individual infusions (artichoke, borututu or milk thistle), three infusions containing two plants (artichoke+borututu, artichoke+milk thistle and borututu+milk thistle), one infusion containing the three plants (artichoke+borututu+milk thistle), and honey dissolved in boiled water (5 g in 100 mL); *ii)* seven mixtures of plants and honey: three individual infusions with honey (artichoke+honey, borututu+honey or milk thistle+honey), three infusions containing two plants with honey (artichoke+borututu+honey, artichoke+milk thistle+honey and borututu+milk thistle+honey), and one infusion containing the three plants with honey (artichoke+borututu+milk thistle+honey).

The concentrations for the control infusions and honey were: 10 mg/mL of dried plant material (5 and 3.33 mg/mL for each plant in the infusions containing two and three plants, respectively) and 47.6 mg/mL of honey. For the mixtures containing the plant infusions and honey, the concentrations were 9.5 mg/mL of dried plant material (4.8 and 3.2 mg/mL for each plant in the mixtures containing infusions of two and three plants, respectively) and 47.6 mg/mL of honey (Table 8).

Table 8. Concentrations of the components included in each sample/mixture.

Sample/Mixture	Concentration (mg/mL of solution)*			
	H	A	B	M
Honey (H)	47.6	-	-	-
Artichoke (A)	-	10	-	-
Borututu (B)	-	-	10	-
Milk thistle (M)	-	-	-	10
AH	47.6	9.5	-	-
BH	47.6	-	9.5	-
MH	47.6	-	-	9.5
AB	-	5	5	-
AM	-	5	-	5
BM	-	-	5	5
ABH	47.6	4.8	4.8	-
AMH	47.6	4.8	-	4.8
BMH	47.6	-	4.8	4.8
ABM	-	3.3	3.3	3.3
ABMH	47.6	3.2	3.2	3.2

*Mixtures containing honey were considered as having a total volume of 105 mL.

2.5. Analysis of phenolic phytochemicals in dietary supplements

2.5.1. Total phenolics

Total phenolics were estimated by Folin-Ciocalteu colorimetric assay according to procedures previously described (Batista et al., 2011) and the results were expressed as mg of gallic acid equivalents (GAE) per g of sample (lyophilized infusion or pill).

2.5.2. Total flavonoids

Total flavonoids were determined by a colorimetric assay using aluminum trichloride, following procedures previously reported (Batista et al., 2011); the results were expressed as mg of (+)-catechin equivalents (CE) per g of sample (lyophilized infusion or pill).

2.5.3. Individual compounds profiles

The previously described hydromethanolic extracts were analysed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, US) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 μm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was: isocratic 15% B for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% B for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1700.

The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, calibration curves for different phenolic standards were obtained based on the areas of the peakes recorded at 280 nm or 370 nm depending on the compound class at their maximum wavelength:

apigenin-7-*O*-glucoside (y=159.62x+7.5025; R^2=0.999); caffeic acid (y=611.9x-4.5733; R^2=0.999); chlorogenic acid (y=313.03x-58.2; R^2=0.999); *p*-coumaric (y=884.6x+184.49; R^2=0.999); ferulic acid (y=505.97x-64.578); isorhamnetin-3-*O*-rutinoside (y=284.12x+67.055; R^2=1); isorhamnetin-3-*O*-glucoside (y=218.26x-0.98; R^2=1); kaempferol-3-*O*-glucoside (y=288.55x-4.0503; R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587; R^2=1); luteolin-7-*O*-glucoside (y=80.829x-21.291; R^2=0.999); protocatechuic acid (y=291.1x-6.4558; R^2=0.999); quercetin-3-*O*-glucoside (y=363.45x+117.86; R^2=0.999); quercetin-3-*O*-rutinoside (y=281.98x-0.3459; R^2=1), taxifolin (y=224.31x+148.41; R^2=0.999) and vanillic acid (y=394.49x+423.86; R^2=0.998). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compound from the same phenolic group. The results were expressed in mg per g of lyophilized extract.

2.6. Bioactivity evaluation

2.6.1. General

The solutions prepared from the different dietary supplements were diluted to different concentrations to be submitted to distinct bioactivity evaluation in *in vitro* assays. The results were expressed in: *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumor activity, or *iii)* MIC values (lowest sample concentration that exhibited complete inhibition of bacterial growth) for antimicrobial activity.

2.6.2. Antioxidant properties

The antioxidant properties were evaluated by four different tests as there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively: DPPH radical-scavenging activity, reducing power, inhibition of *β*-carotene bleaching and inhibition of lipid peroxidation using TBARS (thiobarbituric acid reactive substances) in brain homogenates (Rafael et al., 2011). Trolox was used as positive control.

DPPH radical-scavenging activity. This assay was performed using an ELX800 microplate Reader (Bio-Tek Instruments, Inc; Winooski, USA). The reaction mixture on 96 well plate consisted of sample solutions (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the following equation, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution:

$$\%\ \mathrm{RSA} = \frac{A_{DPPH} - A_S}{A_{DPPH}} \times 100$$

Reducing power. The sample solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL) were then incorporated, and the absorbance was measured at 690 nm in the Microplate Reader mentioned above.

Inhibition of β*-carotene bleaching*. A solution of *β*-carotene was prepared by dissolving *β*-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing sample solutions (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. *β*-Carotene bleaching inhibition was measured by the formula:

$$\%\ \beta\text{-Carotene bleaching inhibition} = \frac{A_{\beta\text{-Carotene after 2 h}}}{A_{\beta\text{-Carotene}}} \times 100$$

TBARS assay. Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate that was centrifuged at 3000*g* for 10 min. An aliquot (100 μL) of the supernatant was incubated with the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1

mM; 100 µl) at 37 °C for 1h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 µL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 µL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000*g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the following formula, where $A_{Control}$ and A_{Sample} were the absorbance of the control and the sample solution, respectively:

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

2.6.3. Anti-hepatocellular carcinoma activity

The anti-hepatocellular carcinoma activity was evaluated using HepG2, which is the most widely used tumour cell line and generally regarded as a good hepatocellular carcinoma model. HepG2 cells were routinely maintained as adherent cell cultures in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, at 37 ºC, in a humidified air incubator containing 5% CO_2. The cell line was plated at 1.0×10^4 cells/well in 96-well plates. Cells were then treated for 48h with the sample solutions. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 min at 4 ºC. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 µL) was added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 µL, pH 7.4) and the absorbance was measured at 540 nm (Guimarães et al., 2013) in the microplate reader mentioned above. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.6.4. Hepatotoxicity

The hepatotoxicity was assessed using a non-tumour liver primary culture established in our laboratory (PLP2). The cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse. Once in the lab, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U/mL penicillin, 100

μg/mL streptomycin and divided into 1×1 mm^3 explants. Five explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% FBS, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Guimarães et al., 2013). Cells were treated for 48h with the sample solutions and the same procedure described in the previous section for SRB assay was followed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.6.5. Antimicrobial activity

The microorganisms used to screen the antimicrobial activity were clinical isolates from patients hospitalized in various departments of the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Four Gram-negative bacteria (*Escherichia coli* isolated from urine, *Escherichia coli* spectrum extended producer of *β*-lactamases (ESBL) isolated from blood culture, *Proteus mirabilis* isolated from wound exudates and *Pseudomonas aeruginosa* isolated from urine) and one Gram-positive bacterium (methicillin-resistant *Staphylococcus aureus* (MRSA) isolated from wound exudates) were used. All strains were identified using the MicroScan® panels automated methodology (Siemens).

Minimum inhibitory concentration (MIC) determinations were performed by the microdilution method and the rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay following the methodology previously described by Alves et al. (2012).

Initially, 50 μL of each extract (1000, 150 and 100 mg/mL for the infusion, the pills and the syrup, respectively) was diluted in 450 μL of MHB (final concentration of 100, 15 and 10 mg/mL, respectively) and then, 200 μL of this extract solution was added in each well (96-well microplate). Dilutions were carried out over the wells with 100 μL of MHB and, afterwards, 10 μL of inoculum (1×10^8 cfu/mL) were added to all the wells. Two negative (one with MHB and the other with the extract) and one positive

(with MHB and the inoculum) controls were prepared. The plates were incubated at 37 °C, for 24 h, in an oven (Jouan).

The MIC's of the samples were determined after adding INT (0.2 mg/mL, 40 μL) and incubating at 37 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink color. MIC was defined as the lowest extract concentration that prevented this change and exhibited complete inhibition of bacterial growth.

2.6.6. Synergistic effects

Classification of additive, synergistic or antagonistic effects. The classification in additive (AD), synergistic (SN) or antagonistic (negative synergistic; AN) effects was performed as follow: AD: theoretical and experimental values reveal differences lower than 5%; SN: experimental values are more than 5% lower than theoretical values; AN: experimental values are more than 5% higher than theoretical values. For each case, the effect was calculated by applying the formula:

$$E = \frac{\text{Theoretical value} - \text{Practical value}}{\text{Theoretical value}}$$

The effect was further classified as synergistic (SN): $E \geq 0.05$; additive (AD): $-0.05 < E < 0.05$, or antagonistic: $E \leq -0.05$ (Queirós et al., 2009).

Theoretical values calculation. For the syrup containing the three plants and the syrup prepared, at the same concentration, from the individual syrups (described in *section 2.4.4*), theoretical values for antioxidant activity of the mixtures were calculated as weighted mean experimental EC_{50} values of the individual syrups and considering additive contributions of individual species in each percentage (AS-artichoke syrup, BS-borututu syrup, MS-milk thistle syrup):

$$EC_{50} = EC_{50\,(AS)} \times 0.23 + EC_{50\,(BS)} \times 0.23 + EC_{50\,(MS)} \times 0.54$$

The theoretical value for hepatocellular carcinoma activity is the EC_{50} value of MS since AS and BS did not show activity.

For the mixtures of dietary supplements referred to in *section 2.4.5*, the theoretical values for antioxidant and anti-hepatocellular carcinoma activities of the mixtures were calculated as weighted mean experimental EC_{50} or GI_{50} values of the individual samples (Petrović et al., 2014) and considering additive contributions of individual species in each percentage; for instance, mixture 2:1:1:

$$EC_{50} = EC_{50\,(A)} \times 0.5 + EC_{50\,(B)} \times 0.25 + EC_{50\,(M)} \times 0.25$$

For the mixtures of dietary supplements with honey described in *section 2.4.6*, the theoretical values were calculated from the EC_{50} values (Table 24) obtained for preparations without honey and for the samples containing only honey (H), considering the exact concentration of each component (Queirós et al., 2009). For instance, the theoretical values for ABH were calculated as:

$$\frac{EC_{50\,(AB)} \times \frac{10}{9.52} + EC_{50\,(H)}}{2}$$

Where, 10 is the concentration of the solution before adding the 5 g of honey, and 9.52 is the concentration afterwards; the concentration of honey was considered as being maintained unaltered due to the negligible contribution of the extract mass to the total mass of the solution.

2.7. Statistical analysis

In general: For all the experiments three samples (n=3) were analysed and all the assays were carried out in triplicate. The results are expressed as mean values±standard deviation (SD). The differences between the different samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. This treatment was carried out using SPSS v. 18.0 and v. 20.0 programs. The regression analysis between total phenolic or flavonoid contents, and antioxidant activity EC_{50} values and anti-hepatocellular carcinoma activity GI_{50} values used the same statistical package.

For the mixtures of the dietary supplements: An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors F and R. In this case, when a statistically significant interaction (F×R) is detected, the two factors should be evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction is verified, means might be compared using, for instance, Tukey's honestly significant difference (HSD) multiple comparison test.

Furthermore, a linear discriminant analysis (LDA) was used to compare the effect of F and R on antioxidant activity and extracted bioactive compounds. A stepwise

technique, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination processes, where the inclusion of a new variable is preceded by ensuring that all variables selected previously remain significant (Maroco, 2003; López et al., 2008; Zielinski et al., 2014). With this approach, it is possible to identify the significant variables obtained for each sample. To verify the significance of canonical discriminant functions, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

For the mixtures of the dietary supplements with honey: all statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp.).

The differences between the infusions were analyzed using one-way analysis of variance (ANOVA). The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

As described above for the mixtures of dietary supplements, a linear discriminant analysis (LDA) was used to study the combined effect on the antioxidant activity and hepatotoxicity of the infusions prepared with the addition of honey; and for the variable selection and significance of canonical discriminant functions, the same methods were applied.

CHAPTER 3

Results and Discussion

3.1. Functional characterization of dry plant materials

3.1.1. Nutritional characterization of artichoke, borututu and milk thistle dry material

Nutritional characterization. The proximate composition and energetic values of artichoke, milk thistle and borututu are shown in Table 9. Among the three studied plants, artichoke had the highest ash (24.5 g/100 g) and protein (5.9 g/100 g) contents and the lowest fat and carbohydrate levels, which is in accordance with a previous study that reported the high content of proteins and low amount of fat in this plant (Lattanzio et al., 2009). On the other hand, borututu possessed the highest carbohydrate (87.93 g/100 g) and fat (2.48 g/100 g) concentrations with, consequently, the highest energetic contribution (384.2 g/100 g); it also revealed the lowest protein content. Overall, all the samples contained carbohydrates and fat as the major and the minor components, respectively.

Table 9. Proximate composition and energetic contribution of artichoke, borututu and milk thistle.

	Artichoke	Borututu	Milk thistle
Ash (g/100 g dw)	24.5 ± 0.6^{a}	7.05 ± 0.07^{b}	6.9 ± 0.3^{b}
Proteins (g/100 g dw)	5.9 ± 0.3^{a}	2.54 ± 0.01^{c}	4.44 ± 0.07^{b}
Fat (g/100 g dw)	1.02 ± 0.03^{c}	2.48 ± 0.04^{a}	1.46 ± 0.01^{b}
Carbohydrates (g/100 g dw)	68.6 ± 0.4^{c}	87.93 ± 0.02^{a}	87.2 ± 0.3^{b}
Energy (kcal/100 g dw)	307 ± 2^{c}	384.2 ± 0.3^{a}	379.6 ± 0.9^{b}

In each row different letters mean significant differences ($p<0.05$).

Hydrophilic compounds. The results obtained for hydrophilic compounds are presented in Table 10. Regarding sugars, milk thistle showed the highest contents of fructose (2.16 g/100 g) and glucose (0.97 g/100 g), whereas borututu revealed the highest sucrose (1.1 g/100 g) and total sugars levels (4.1 g/100 g); trehalose (0.98 g/100 g) was only detected in this plant (Figure 11), among the analyzed ones. Fructose was the only sugar found in artichoke (2.0 g/100 g).

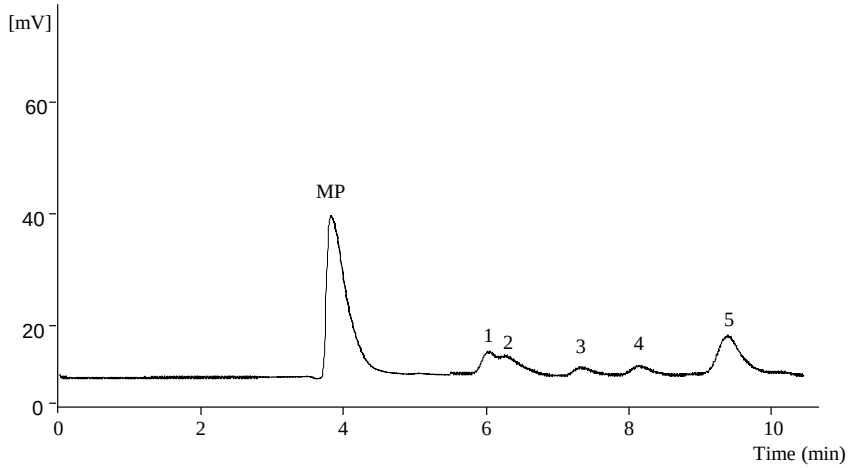


Figure 11. HPLC-RI chromatogram showing the profiles of individual sugars in a sample of borututu. 1- fructose; 2- glucose; 3- sucrose; 4-trehalose; 5- melezitose (IS).

Carbohydrates are the most abundant organic molecules found in nature and all organisms synthesize and metabolize these compounds. Glucose (present in borututu and milk thistle) is a common monosaccharide that is oxidized to provide energy for important cellular processes (protein synthesis, growth, development and transport). Furthermore, cell surface glycans are involved in many physiologically important functions, acting as signaling, recognition and adhesion molecules due to their structural variability and complexity (Sharon and Lis, 1993).

Regarding organic acids, artichoke revealed the highest levels of oxalic acid (1.95 g/100 g), milk thistle of quinic acid (2.8 g/100 g) and borututu of shikimic (0.010 g/100 g) and citric (0.57 g/100 g) acids. Malic and fumaric acids contents were similar in artichoke and milk thistle and the latest was also present in similar amount in borututu. The chromatographic profile of organic acids in milk thistle, which presented the highest total content of these compounds (5.4 g/100 g) among the analyzed plants, can be observed in Figure 12.

Organic acids are widely present in fruits and derived products and some of them have many applications in food industry: citric acid, for example, is largely used as a food additive in different kinds of beverages, soft drinks and wines. A moderate intake of organic acids can promote appetite, help digestion, and may be beneficial to human health (Cameron and Campbell, 1974). These compounds are very important at the cellular level as they are involved in different biochemical pathways, such as energy

production or formation of precursors for amino-acid biosynthesis, and at the whole plant level in modulating adaptation to the environment (López-Bucio et al., 2000).

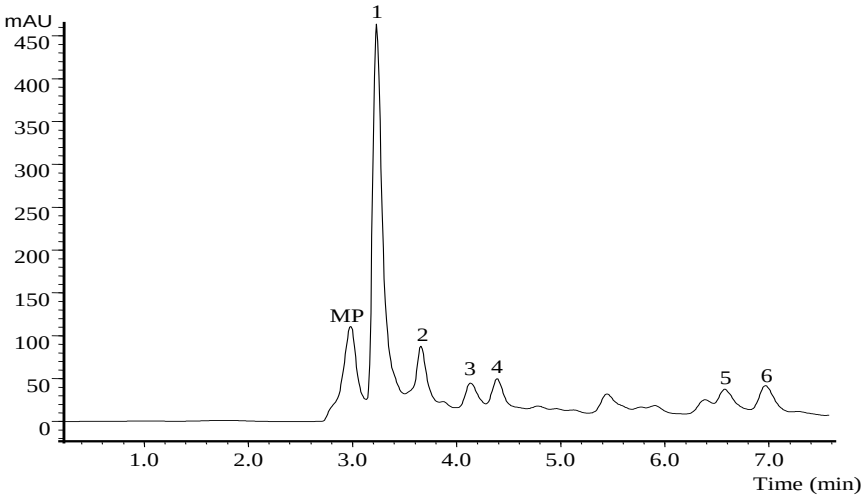


Figure 12. HPLC chromatogram with the profile of organic acids in Milk thistle.
1- oxalic acid; 2- quinic acid; 3- malic acid; 4- shikimic acid; 5- citric acid; 6- fumaric acid. MP- Mobile phase front peak.

Table 10. Hydrophilic compounds in artichoke, borututu and milk thistle.

	Artichoke	Borututu	Milk thistle
Fructose (g/100 g dw)	2.0±0.1[b]	1.30±0.07[c]	2.16±0.04[a]
Glucose (g/100 g dw)	nd	0.79±0.02[b]	0.97±0.07[a]
Sucrose (g/100 g dw)	nd	1.1±0.1[a]	0.47±0.08[b]
Trehalose (g/100 g dw)	nd	0.98±0.05	nd
Total sugars (g/100 g dw)	2.0±0.1[c]	4.1±0.2[a]	3.6±0.1[b]
Oxalic acid (g/100 g dw)	1.95±0.09[a]	0.70±0.04[c]	1.39±0.05[b]
Quinic acid (g/100 g dw)	1.32±0.04[b]	nd	2.8±0.2[a]
Malic acid (g/100 g dw)	1.03±0.07[a]	0.63±0.03[b]	0.96±0.05[a]
Shikimic acid (g/100 g dw)	nd	0.010±0.001[a]	0.006±0.001[b]
Citric acid (g/100 g dw)	0.33±0.02[b]	0.57±0.03[a]	0.24±0.02[c]
Fumaric acid (g/100 g dw)	0.004±0.001[a]	0.008±0.004[a]	0.006±0.001[a]
Total organic acids (g/100 g dw)	4.6±0.2[b]	1.9±0.1[c]	5.4±0.2[a]

nd- not detected. In each row different letters mean significant differences (p<0.05).

Lipophilic compounds. The results for fatty acids composition, total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), ratios of PUFA/SFA and n-6/n-3, and the tocopherols content of the studied herbals are shown in Table 11.

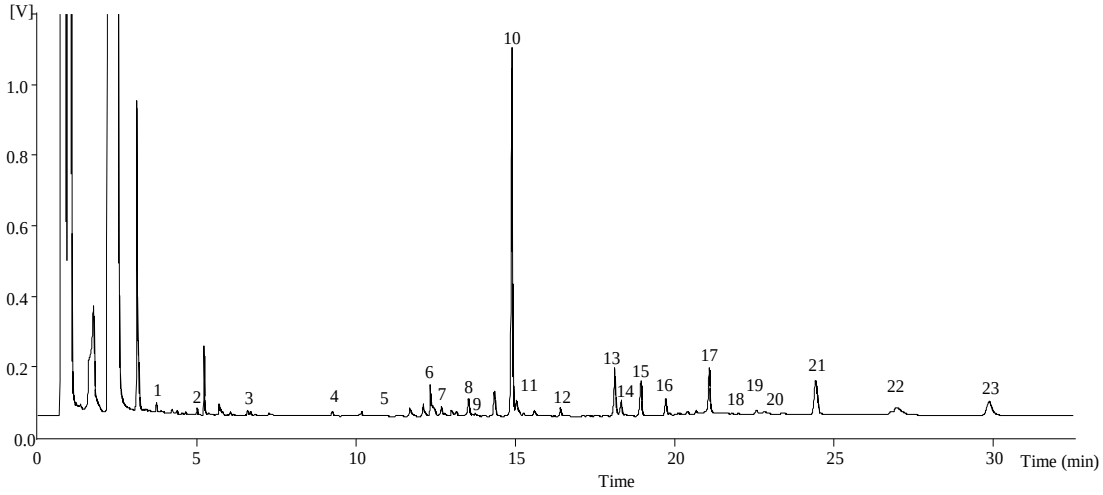


Figure 13. GC-FID profile of individual fatty acids in artichoke.
1- C6:0; 2- C8:0; 3- C10:0; 4- C12:0; 5- C13:0; 6- C14:0; 7- C14:1; 8- C15:0; 9- C15:1; 10- C16:0; 11- C16:1; 12- C17:0; 13- C18:0; 14- C18:1n9; 15- C18:2n6; 16- C18:3n3; 17- C20:0; 18-C20:1; 19- C20:2; 20- C20:3n3+C21:0; 21- C22:0; 22- C23:0; 23- C24:0.

The fatty acids profile of artichoke is shown in Figure 13. It revealed the highest levels of SFA (88%) among the analysed samples, with palmitic acid (C16:0; 47.2%) as the main fatty acid, followed by behenic acid (C22:0; 9%), stearic acid (C18:0; 8.6%), arachidic acid (C20:0; 7.5%) and lignoceric acid (C24:0; 7%).

Tocopherols have a confirmed superb antioxidant activity in different food systems (Ko et al., 2010) being considered the most powerful natural antioxidants. Their most important function in biological membranes is that they act as recyclable chain reaction scavengers of PUFA free radicals generated by lipid oxidation (Schneider, 2005; Fryer, 1992); they have also been suggested to play a major role in the maintenance and protection of the photosynthetic machinery in the plant (Collakova and DellaPena, 2003).

Table 11. Fatty acids and tocopherols composition in artichoke, borututu and milk thistle.

	Artichoke	Borututu	Milk thistle
C6:0	0.39±0.01	0.06±0.02	0.61±0.02
C8:0	0.25±0.01	0.29±0.03	0.16±0.03
C10:0	0.21±0.03	0.26±0.04	0.17±0.04
C11:0	nd	0.07±0.01	nd
C12:0	0.46±0.01	2.05±0.02	0.21±0.03
C13:0	0.176±0.002	0.158±0.002	0.142±0.003
C14:0	2.8±0.6	4.7±0.1	0.58±0.09
C14:1	0.72±0.08	0.12±0.01	0.16±0.03
C15:0	1.51±0.05	0.82±0.01	0.43±0.06
C15:1	0.13±0.01	0.13±0.01	0.044±0.002
C16:0	47.2±0.5	26.0±0.1	20±1
C16:1	nd	0.625±0.002	0.35±0.01
C17:0	1.88±0.01	1.34±0.04	0.46±0.04
C18:0	8.6±0.2	2.9±0.1	5.8±0.2
C18:1n9	2.1±0.2	19.96±0.03	18.52±0.03
C18:2n6	6±1	24.11±0.06	42±1
C18:3n6	0.17±0.02	nd	nd
C18:3n3	2.3±0.5	6.4±0.1	2.72±0.02
C20:0	7.5±0.3	6.7±0.3	2.9±0.1
C20:1	nd	nd	0.62±0.03
C20:2	0.12±0.02	0.22±0.02	0.058±0.003
C20:3n3+C21:0	0.69±0.05	0.23±0.01	0.21±0.01
C22:0	9±1	1.26±0.01	2.0±0.2
C23:0	0.8±0.2	0.38±0.04	0.22±0.01
C24:0	7±1	1.30±0.01	1.35±0.05
Total SFA	88±2^{a}	48.2±0.2^{b}	35±1^{c}
Total MUFA	3.0±0.3^{b}	20.84±0.03^{a}	19.70±0.01^{a}
Total PUFA	9±2^{c}	30.9±0.2^{b}	45±1^{a}
PUFA/SFA	0.10±0.02^{c}	0.64±0.01^{b}	1.30±0.09^{a}
n-6/n-3	2.01±0.06^{c}	3.65±0.05^{b}	14.5±0.6^{a}
α-tocopherol (mg/100 g dw)	0.073±0.005^{c}	3.7±0.2^{a}	0.42±0.01^{b}
β-tocopherol (mg/100 g dw)	0.87±0.03^{b}	597±14^{a}	nd
γ-tocopherol (mg/100 g dw)	12.3±0.3^{a}	2.0±0.1^{b}	0.88±0.01^{c}
δ-tocopherol (mg/100 g dw)	nd	43±2	nd
Total tocopherols (mg/100 g dw)	13.3 ± 0.3^{b}	646.1±15.9^{a}	1.30±0.01^{b}

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecanoic acid (C11:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); *cis*-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); γ-Linolenic acid (C18:3n6); α-Linolenic acid (C18:3n3); Arachidic acid (C20:0); Eicosenoic acid (C20:1); *cis*-11,14-Eicosadienoic acid (C20:2); *cis*-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3 + C21:0); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). nd - not detected. The results for fatty acids are expressed in percentage. In each row different letters mean significant differences (p<0.05).

Borututu also showed prevalence of SFA (48.2%) due to the main contribution of palmitic acid (C16:0; 26.0%) and arachidic acid (C20:0; 6.7%), but also relevant percentages of PUFA (30.9%) mostly due to linoleic acid (C18:2n6; 24.11%) and α-

linolenic acid (C18:3n3; 6.4%), and MUFA (20.84%), namely oleic acid (C18:1n9; 19.96%). Lastly, milk thistle presented the highest percentages of PUFA (45%) followed by significant proportions of SFA (35%) and MUFA (19.70%) with linoleic acid (C18:2n6; 42%), palmitic acid (C16:0; 20%) and oleic acid (C18:1n9; 18.52%) as main contributors. Essential fatty acids, i.e., linoleic acid (18:2n6) and α–linolenic acids (18:3n3, ALA), play important nutritional roles in growth, reproduction and health, for the prevention of cardiovascular diseases (Simopoulos and Ordovas, 2004; von Schacky and Harris, 2006) and maintenance of the homeostasis in the human body (Psota et al. 2006). These PUFA, which are present in the three studied plants, cannot be synthesized by humans (Din et al., 2004; Siddiqui et al., 2008), thus identifying new sources for them, and especially for PUFAs of the n3 series, within medicinal plants is of great importance, so that they could be included in the diet.

For "good nutritional quality", including health beneficial effects, the PUFA/SFA ratio should be higher than 0.45, while n-6/n-3 fatty acids ratio should be lower than 4.0 (Guil et al., 1996). As observed in Table 11, borututu was the only species that presented both ratios within the cited values (0.64 and 3.65, respectively). Artichoke showed the lowest n6/n-3 ratio (2.01), wile milk thistle revealed the highest PUFA/SFA ratio (1.30).

The four vitamers of tocopherols were only detected in borututu, which presented much higher levels of total tocopherols (646.1 mg/100 g) than the other two analysed plants, especially due to the noticeable contents of β-tocopherol (597 mg/100 g); γ-tocopherol was present in higher levels is artichoke (12.3 mg/100 g), whereas δ-tocopherol was neither detected in artichoke nor in milk thistle that did not reveal β-tocopherol, either. The individual profile of tocopherols in borututu can be observed in Figure 14.

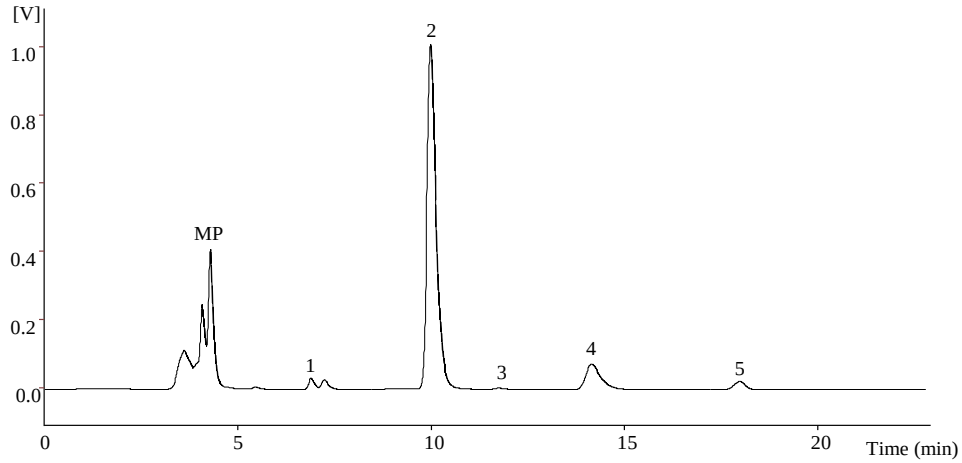


Figure 14. HPLC chromatogram with the profiles of tocopherols in borututu.
1-α-tocopherol; 2-β-tocopherol; 3- γ-tocopherol; 4- δ-tocopherol; 5-tocol (IS). MP- Mobile phase.

3.1.2. Nutritional characterization, antioxidant activity and hepatotoxicity of borututu dry material submitted to gamma irradiation

Nutritional characterization. Results regarding proximate composition and energetic value of borututu control (non-irradiated) and irradiated samples are present in Table 12.

Table 12. Proximate composition and energetic values of borututu samples submitted to different gamma irradiation doses.

	0 kGy	1 kGy	10 kGy
Ash (g/100 g dw)	7.9±0.6^{a}	7.5±0.7^{a}	6.9±0.2^{a}
Proteins (g/100 g dw)	2.92±0.01^{b}	2.99±0.05^{a}	2.88±0.06^{b}
Fat (g/100 g dw)	2.2±0.2^{a}	2.31±0.04^{a}	2.29±0.07^{a}
Carbohydrates (g/100 g dw)	86.97±0.55^{a}	87.2±0.5^{a}	87.9±0.2^{a}
Energy (kcal/100 g dw)	379.71±1.22^{b}	382±2^{ab}	383.8±0.9^{a}

dw- dry weight. In each row different letters mean significant differences (p<0.05).

The results are in agreement with the ones obtained in a borututu sample previously analysed (see Table 9). All processed samples revealed similar proximate composition with hardly modifications in their levels as a result of the irradiation.

Regarding fatty acids (Table 13), the composition was similar to the one determined in the borututu sample previously analysed (Table 9), although some differences in the

percentages of some punctual compounds were observed. Nevertheless, the same general patterns were observed, with a prevalence of SFA and followed by PUFA, and the presence of linoleic (C18:2n6) and palmitic acid (C16:0) as main fatty acids. Minor differences were observed in the proportions of PUFA and SFA among samples, whereas no significant differences existed in the percentages of MUFA. It was previously reported that unsaturated fatty acids, especially n-3 PUFAs, play an important role in the treatment or prevention of hepatic steatosis (Hanke et al., 2013). Being borututu consumed essentially for hepatic purposes, it is important to keep those fatty acids after irradiation and, in fact, no significant change was produced in the levels of α–linolenic acid (C18:3n3) as a result of the treatment even at the dose of 10 kGy.

In agreement with the results presented for borututu in *section 3.1.1*. (Table 11), in these samples the four vitamers of tocopherol were also found, with the prevalence of β-tocopherol (Table 13), although the concentrations determined in this case were lower. Interestingly, higher total tocopherols contents were observed in the irradiated samples (both with 1 kGy and 10 kGy), mostly due to an increase in the level of β-tocopherol. Thus, irradiation seems to preserve these compounds with critical antioxidant importance in the prevention of PUFA oxidation, especially α-tocopherol, considered the most effective chain-breaking lipid-soluble antioxidant in animal and human tissues (Valk and Hornstra, 2000; Fernandes et al., 2011; Fernandes et al., 2013a and b). Otherwise, control sample showed some degradation (Table 13), highlighting the efficiency of gamma irradiation in the preservation of these compounds.

Table 13. Lipophilic compounds in borututu samples submitted to different gamma irradiation doses.

	0 kGy	1 kGy	10 kGy
C6:0	0.13±0.04	0.18±0.02	0.16 ± 0.02
C8:0	0.25±0.04	0.15±0.04	0.34 ± 0.01
C10:0	0.15±0.02	0.094±0.007	0.20±0.02
C11:0	0.051±0.002	0.055±0.001	0.044±0.006
C12:0	1.09±0.03	1.27±0.01	1.09±0.02
C13:0	0.086±0.006	0.082±0.004	0.053±0.002
C14:0	2.77±0.08	2.81±0.04	2.67±0.06
C14:1	0.088±0.001	0.096±0.001	0.055±0.001
C15:0	0.68±0.04	0.63±0.02	0.63±0.01
C15:1	0.10±0.03	0.063±0.005	0.078±0.004
C16:0	21.7±0.1	21.5±0.2	21.71±0.09
C16:1	0.41±0.02	0.49±0.01	0.37±0.05
C17:0	0.97±0.01	1.00±0.01	0.95±0.01
C18:0	13.9±0.7	15.09±0.4	13.5±0.3
C18:1n9	16.4±0.1	16.07±0.08	17.0±0.1
C18:2n6	24.44±0.05	23.87±0.09	24.55±0.02
C18:3n3	7.89±0.05	7.81±0.07	8.21±0.04
C20:1	6.7±0.3	6.67±0.05	6.5±0.1
C22:0	0.9±0.2	0.85±0.03	0.98±0.06
C23:0	0.39±0.04	0.38±0.04	0.35±0.03
C24:0	0.9±0.1	0.89±0.05	0.66±0.04
Total SFA (relative %)	44.01±0.3^{ab}	44.9±0.3^{a}	43.3±0.4^{b}
Total MUFA (relative %)	23.7±0.3^{a}	23.38±0.06^{a}	23.9±0.2^{a}
Total PUFA (relative %)	32.34±0.01^{a}	31.7±0.2^{b}	32.75±0.03^{a}
α-tocopherol (mg/100 g dw)	2.49±0.05^{b}	2.69±0.02^{a}	2.47±0.04^{b}
β-tocopherol (mg/100 g dw)	280±1^{b}	307±4^{a}	313±3^{a}
γ-tocopherol (mg/100 g dw)	0.61±0.01^{b}	2±1^{a}	0.59±0.01^{b}
δ-tocopherol (mg/100 g dw)	23±2^{a}	21±1^{a}	21±1^{a}
Total (mg/100 g dw)	306±3^{b}	332±7^{a}	337±4^{a}

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecanoic acid (C11:0); Lauric acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); *cis*-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic aci d (C18:1n9); Linoleic acid (C18:2n6); γ-Linolenic acid (C18:3n6); α-Linolenic acid (C18:3n3); Eicosenoic acid (C20:1); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). In each row different letters mean significant differences ($p<0.05$).

Regarding free sugars, the samples showed lower total contents but similar sugars profile (Table 14) than the one previously analysed (see Table 10), with fructose and sucrose as the most abundant molecules. As observed in Table 14, the irradiation at 1 kGy (sample 1) produced a slight decrease of fructose, glucose, sucrose, and trehalose, although it was not significant when considered the content of total sugars. On the contrary, the sample irradiated at 10 kGy showed higher concentrations of those sugars and also higher total sugars content. This is in agreement with previous works with irradiated plants and it can be explained through the depolymerization or degradation of polysaccharide molecules, as observed for soybeans (Byun et al., 1996) and ginseng

products (Sung et al., 1982) submitted to gamma irradiation. Tissot et al. (2013) also reported the potential of ionizing radiation on facilitating the breakdown of cellulose into simple sugars.

Table 14. Contents of sugars and organic acids in borututu samples submitted to different gamma irradiation doses.

	0 kGy	1 kGy	10 kGy
Fructose (g/100 g dw)	2.01±0.08^{b}	1.79±0.01^{c}	2.14±0.04^{a}
Glucose (g/100 g dw)	1.31±0.01^{b}	1.17±0.08^{c}	1.47±0.02^{a}
Sucrose (g/100 g dw)	3.04±0.06^{b}	2.87±0.02^{c}	3.39±0.05^{a}
Trehalose (g/100 g dw)	0.96±0.02^{a}	0.83±0.02^{b}	0.83±0.02^{b}
Raffinose (g/100 g dw)	0.60±0.07^{b}	0.68±0.03^{b}	0.80±0.01^{a}
Total (g/100 g dw)	7.9±0.1^{b}	7.3±0.1^{b}	8.63±0.04^{a}
Oxalic acid (g/100 g dw)	0.24±0.01^{a}	0.23±0.01^{a}	0.29±0.08^{a}
Malic acid (g/100 g dw)	1.30±0.02^{a}	1.26±0.02^{a}	1.6±0.5^{a}
Shikimic acid (g/100 g dw)	0.009±0.001^{a}	0.010±0.001^{a}	0.013±0.004^{a}
Citric acid (g/100 g dw)	1.22±0.04^{a}	1.13±0.03^{a}	1.35±0.41^{a}
Fumaric acid (g/100 g dw)	0.011±0.001^{a}	0.021±0.008^{a}	0.023±0.008^{a}
Total (g/100 g dw)	2.78±0.04^{a}	2.65±0.06^{a}	3.31±0.98^{a}

dw- dry weight. In each row different letters mean significant differences (p<0.05).

With respect to organic acids (Table 14), oxalic, malic and citric acids were the most relevant compounds, as above observed (Table 10), although lower contents were determined in these samples than in the previously analysed one, which may be explained by the kind of material analysed, once those analyses were performed on a purchased preparation for infusion, while bulk borututu, from another supplier was used to test the irradiation effects. It was verified that the irradiation did not significantly affect these compounds; this is is of great interest once the presence and ratio of organic acids in plants can affect their chemical and sensory characteristics, such as pH, total acidity, microbial stability, sweetness and global acceptability; for instance, malic acid possesses a smooth lingering taste and also a tart taste, not as sharp as that of citric acid, but longer lasting (Tormo and Izco, 2004). Organic acids also play an important role on food technology, for example, citric and malic acids are used as acidulants and the latest one is also used as a flavor enhancer and as a potent growth inhibitor of yeasts

and some bacteria. On the other hand, the presence of fumaric acid is an important parameter to reveal microbial spoilage or processing of decayed food, and the concentration of some organic acids in plants can give information about the addition of synthetic preservatives to plant products, providing evidences of eventual authenticity issues (Gebre et al., 1994; Vaughan and Geissler, 1997).

Thus, irradiation treatment up to 10 kGy does not appreciably affect relevant components of nutritional interest in the studied plant, which could be explained by the low water content of dry herbs that limits the possibility of formation of free radicals (Venskutonis et al., 1996; Murcia et al., 2004; Brandstetter, et al., 2009). In fact, the sample irradiated at 10 kGy had the highest levels of total sugars, organic acids, total tocopherols, and PUFA.

Antioxidant activity. Four *in vitro* assays were used to evaluate antioxidant properties of infusions and methanolic extracts of the samples: scavenging effects on DPPH radicals, reducing power, inhibition of *β*-carotene bleaching and inhibition of lipid peroxidation in brain cell homogenates (as described in *section 2.6.2*).

As shown in Table 15, the infusion obtained from the sample irradiated at 10 kGy had a higher antioxidant activity in all the assays performed, with no statistical differences in the DPPH scavenging effect when compared to the control sample. On the other hand, the methanolic extract of this sample gave higher DPPH radical scavenging activity and reducing power, and similar *β*-carotene bleaching and lipid peroxidation inhibitions. The methanolic extracts revealed higher scavenging activity, reducing power and *β*-carotene bleaching inhibition than the infusion, which is in agreement with a previous study where the ethanolic extracts showed greater antioxidant activity than the corresponding infusions prepared from irradiated Korean medicinal herbs (Byun et al., 1999). Nevertheless, the antioxidant capacity of different borututu preparations was further evaluated (see *sections 3.2.1 and 3.2.2*), obtaining for the infusions lower EC_{50} values (0.02-0.6 mg/mL; Table 17) than the ones determined in the samples analysed in the present assay, and comparable to those obtained herein for the methanolic extracts.

Despite the results obtained in previous studies reporting that gamma irradiation did not influence the electron donating activity of Korean medicinal plants (Byun et al., 1999) and did not influence the free radical scavenging effect of some Korean soybean

fermented foods (Byun et al., 2002), in the present work the results obtained are more in agreement with Kim et al. (2009) that observed that the DPPH radical scavenging activity of gamma irradiated *Hizikia fusiformis* extracts was increased with increasing irradiation dose, which could be related to an increase in the levels of total polyphenolic compounds induced by the irradiation. Similarly to the referred observations, in the present study significantly higher contents of total polyphenols and flavonoids were observed in the infusions and methanolic extracts irradiated at 10 KGy than in the control, reaching respectively 26.85 mg GAE/g and 1.68 mg CE/g in the infusion, and 107.45 mg GAE/g and 33.77 mg CE/g in the methanolic extract (Figure 15), supporting the results obtained for the antioxidant activity.

Table 15. Antioxidant activity of infusions and methanolic extracts of borututu samples submitted to different gamma irradiation doses.

Assay	0 kGy	1 kGy	10 kGy	Positive control*
		Infusions		
DPPH	0.93±0.05b	1.27±0.09a	0.86±0.05b	0.04±0.00
Reducing	0.74±0.01b	0.90±0.01a	0.65±0.01c	0.03±0.00
β-carotene	3.0±0.1b	3.12±0.03a	1.3±0.1c	0.003±0.00
TBARS	0.035±0.001a	0.039±0.001a	0.033±0.002c	0.0036±0.00
		Methanolic extracts		
DPPH	0.24±0.01a	0.25±0.01a	0.212±0.001b	0.04±0.00
Reducing	0.16±0.01a	0.16±0.01a	0.119±0.003b	0.03±0.00
β-carotene	0.25±0.04a	0.25±0.04a	0.24±0.06a	0.003±0.00
TBARS	0.036±0.002b	0.058±0.001a	0.035±0.001b	0.004±0.00

nd- not detected . In each row different letters mean significant differences (p<0.05). *Trolox was used as positive control.

According to the results obtained for the antioxidant activity assays, the risk of decreasing antioxidative properties of dried borututu due to the irradiation at the tested doses can be excluded, with sample irradiated at 10 kGy revealing, in general, the highest antioxidant capacity in the performed assays.

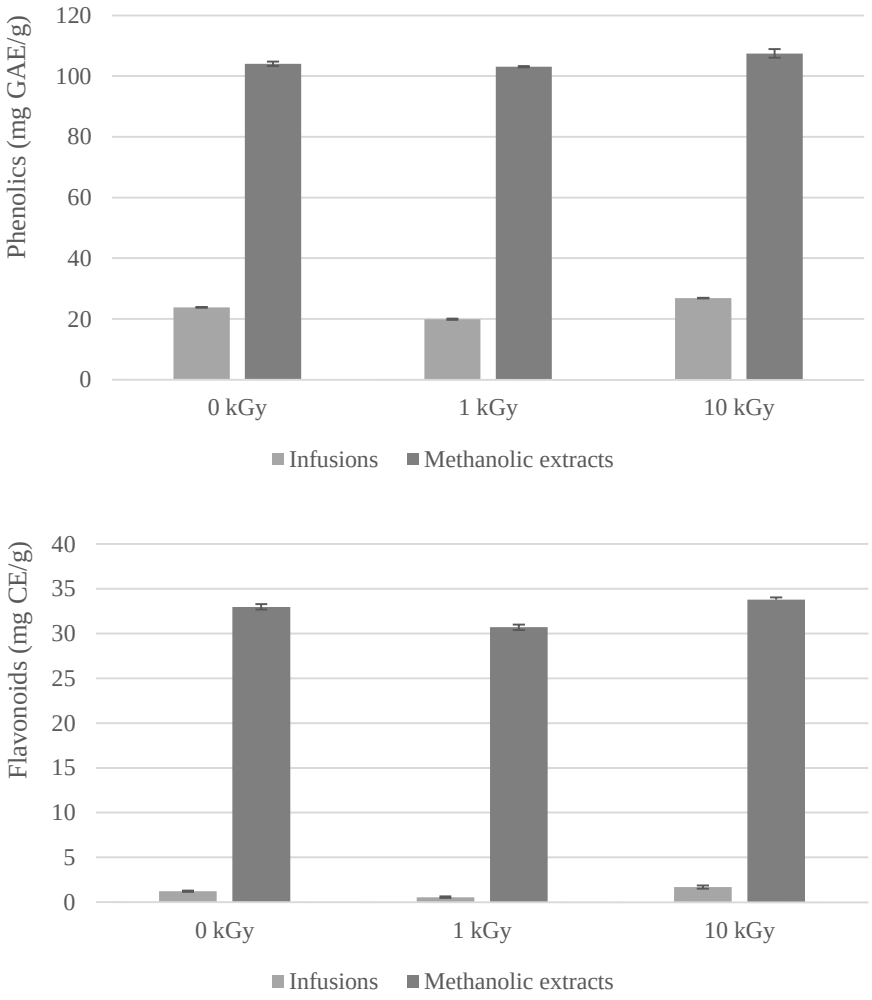


Figure 15. Total polyphenols and flavonoids contents in infusions and methanolic extracts obtained from borututu samples submitted to different gamma irradiation doses. GAE = gallic acid equivalents; CE = catechin equivalents.

Hepatotoxicity. The anti-hepatocellular carcinoma activity and hepatotoxicity of the infusions and methanolic extracts prepared from the three samples were also evaluated and the results are presented in Table 15. The methanolic extracts showed some anti-hepatocellular carcinoma activity that clearly decreased in the sample irradiated at 10 kGy (GI_{50}=189 µg/mL) relatively to the control (GI_{50}=160 µg/mL), whereas the infusions did not reveal anti-hepatocellular carcinoma activity in opposition to the results further obtained for a borututu sample whose activity is outlined in *section 3.2.1*, where some anti-hepatocellular carcinoma activity was found for the infusion with a GI_{50} value of 146 µg/mL (Table 17). This discrepancy, as those reported throughout the present discussion of results, may be explained by the different kind of plant material analysed, i.e., a preparation for infusion or bulk borututu, as also commented

previously. Moreover, the bioactivity of medicinal plants depends on numerous factors beyond the plant species, among others the origin of the sample, making it impossible to guarantee chemical homogeneity.

Neither the infusions or methanolic extracts revealed toxicity in non-tumor porcine liver cells (GI_{50} > 400 µg/mL).

Table 16. Hepatotoxicity of infusions and methanolic extracts of borututu samples submitted to different gamma irradiation doses.

	0 kGy	1 kGy	10 kGy	Positive control*
Infusions				
HepG2 (hepatocellular carcinoma)	>400	>400	>400	3.2±0.7
PLP2 (non-tumor liver primary culture)	>400	>400	>400	2.06±0.03
Methanolic extracts				
HepG2 (hepatocellular carcinoma)	160±13^{b}	159±8^{b}	189±6^{a}	3.2±0.7
PLP2 (non-tumor liver primary culture)	>400	>400	>400	2.06±0.03

In each row different letters mean significant differences (p<0.05). *Elipticine was used as positive control.

3.2. Antioxidant activity and hepatotoxicity of dietary supplements prepared from artichoke, borututu and milk thistle

3.2.1. Antioxidant activity and hepatotoxicity of infusions and pills

The results obtained for bioactive compounds, antioxidant activity, and hepatotoxicity, are presented in Tables 17 and 18, for infusions and pills, respectively. Borututu infusion gave the highest contents of total phenolics and flavonoids, as also the highest antioxidant activity in all the assays (Table 17). The infusions of milk thistle and artichoke revealed, in general, similar values in phenolics and antioxidant activity. The much higher content of total phenolics (132 mg GAE/g) and flavonoids (17.9 mg CE/g) observed in borututu infusion might be related to the part of the plant used in this case (bark).

Table 17. Bioactive compounds, antioxidant activity and hepatotoxicity of artichoke, borututu, and milk thistle infusions.

	Artichoke	Borututu	Milk thistle	Positive control*
Bioactive compounds				
Total phenolics (mg GAE/g)	20.8±0.1[b]	132±1[a]	23.3±0.2[b]	-
Total flavonoids (mg CE/g)	4.37±0.05[c]	17.9±0.2[a]	7.0±0.2[b]	-
Antioxidant activity (EC_{50} values, mg/mL)				
DPPH scavenging activity	2.1±0.4[a]	0.15±0.01[b]	2.5±0.2[a]	0.04±0.00
Reducing power	1.81±0.07[a]	0.174±0.003[b]	1.73±0.03[a]	0.03±0.00
β-carotene bleaching inhibition	1.7±0.2[a]	0.6±0.1[c]	1.36±0.08[b]	0.003±0.00
TBARS inhibition	0.14±0.01[b]	0.02±0.01[c]	0.35±0.01[a]	0.004±0.00
Anti-hepatocellular carcinoma activity (GI_{50} values, µg/mL)				
HepG2 (hepatocellular carcinoma)	52±5[b]	146±11[a]	>400	3.2±0.7
Hepatotoxicity (GI_{50} values, µg/mL)				
PLP2 (non-tumour liver primary culture)	72±6	>400	>400	2.06±0.03

n.p.- not possible to determine. In each row different letters mean significant differences ($p<0.05$). *Trolox and elipticine were used as positive control for antioxidant activity and hepatotoxicity assays, respectively.

positive control for antioxidant activity and hepatotoxicity assays, respectively.

Although there are some reports on phenolics, flavonoids and DPPH scavenging activity of artichoke (Jiménez-Escrig et al., 2003; Falleh et al., 2008; Kubić et al., 2008; Lutz et al., 2011; Gouveia and Castilho, 2012), the studies were performed in plant extracts and not in preparations for direct consumption (e.g. infusions and pills as herein). Data described in literature for methanolic (15 mg GAE/g dry weight, 9 mg CE/g and DPPH EC_{50}=53 µg/mL; Falleh et al., 2008), methanol:water (50:50, *v:v*) (50 mg GAE/g; Jiménez-Escrig et al., 2003), ethyl acetate (203 mg GAE/g and DPPH EC_{50}=22 µg/mL; Kubić et al., 2008), buthanol (62 mg GAE/g and DPPH EC_{50}=127 µg/mL; Kubić et al., 2008), ethanol (50 mg GAE/g and DPPH EC_{50}=157 µg/mL; Kubić et al., 2008) and water extracts (46 mg GAE/g and DPPH EC_{50}=173 µg/mL; Kubić et al., 2008), usually report higher phenolics content and DPPH radical scavenging activity than the ones determined for the infusion (actually a water extract) studied herein, although some authors also observed the opposite, such as Gouveia and Castilho (2012) in ultrasound-assisted methanolic extract of artichoke (0.23 mg GAE/g) or Lutz

et al. (2011) in aqueous and hydroalcoholic extracts (~30% of DPPH scavenging activity). Nevertheless, it should be highlighted that extracts do not mimetize the usually consumed forms of plants and also that most of the mentioned solvents present some degree of toxicity, so that they cannot be used for preparation of products intended for dietary consumption.

Regarding borututu, Costa et al. (2012) studied phenolics, flavonoids and DPPH scavenging activity in infusions, although their results are expressed referred to volume of infusion and not to dry weight (i.e., 1.4 mg GAE/100 mL, 0.34 mg epicatechin equivalents/100 mL, and DPPH EC_{50}=1.9 mg trolox equivalents/100 mL).

In the present work, artichoke and borututu infusions demonstrated anti-hepatocellular carcinoma activity with GI_{50} values of 52 and 146 µg/mL, respectively, but artichoke showed toxicity in normal cells (72 µg/mL). Despite the lower activity of borututu infusion, this sample did not show hepatotoxicity in normal cells (GI_{50}>400 µg/mL). Milk thistle infusion did not reveal anti-hepatocellular carcinoma activity nor hepatotoxicity in non-tumour liver cells, either (Table 17).

Table 18. Bioactive compounds, antioxidant activity and hepatotoxicity of artichoke, borututu, and milk thistle pills.

	Artichoke	Borututu	Milk Thistle	Positivecontrol*
Bioactive compounds				
Total phenolics (mg GAE/g)	3.35±0.01^{c}	30.7±0.8^{a}	20.9±0.5^{b}	-
Total flavonoids (mg CE/g)	2.8±0.1^{c}	5.65±0.03^{a}	3.9±0.1^{b}	-
Antioxidant activity (EC_{50} values, mg/mL)				
DPPH scavenging activity	10.1±0.6^{a}	1.27±0.01^{c}	8.2±0.5^{b}	0.04±0.00
Reducing power	2.28±0.09^{a}	0.58±0.01^{c}	1.10±0.02^{b}	0.03±0.00
β-carotene bleaching inhibition	n.p.	5.6±0.7^{b}	17.7±0.6^{a}	0.003±0.00
TBARS inhibition	1.49±0.05^{a}	0.34±0.05^{c}	0.78±0.03^{b}	0.004±0.00
Anti-hepatocellular carcinoma activity (GI_{50} values, µg/mL)				
HepG2 (hepatocellular carcinoma)	>400	>400	>400	3.2±0.7
Hepatotoxicity (GI_{50} values, µg/mL)				
PLP2 (non-tumour liver primary culture)	>400	>400	>400	2.06±0.03

In each row different letters mean significant differences (p<0.05). *Trolox and elipticine were used as positive control for antioxidant activity and hepatotoxicity assays, respectively.

Regarding pills, the amount of bioactive compounds and the degree of antioxidant activity was as follows: borututu > milk thistle > artichoke. These samples neither revealed anti-hepatocelullar carcinoma activity (HepG2 GI_{50} value>400 μg/mL), nor toxicity for normal liver cells (PLP2 GI_{50} value>400 μg/mL) (Table 18).

As it can be observed in Table 18, the bioactive properties (antioxidant and antitumour) of the infusions were positively correlated (p<0.05) with phenolics and flavonoids content. Similar observation was made for the biological activity of the pills that was positively correlated (p<0.05) with the total phenolics and flavonoids content (Table 19), but for the cases of phenolics/DPPH scavenging activity, flavonoids/reducing power and flavonoids/TBARS inhibition correlations. The relationship between the contents of total polyphenols, as determined by the Folin-Ciocalteu reagent, and antioxidant activity (especially the reducing power) is not surprising taking into account that this reagent reacts not only with phenolic compounds but also with total reducing substances in the samples.

Table 19. Correlations between bioactive compounds, antioxidant activity and hepatotoxicity of artichoke, borututu, and milk thistle infusions and pills.

		Infusions		Dietary supplements	
		Phenolics (mg GAE/g)	Flavonoids (mg CE/g)	Phenolics (mg GAE/g)	Flavonoids (mg CE/g)
DPPH scavenging activity	Linear equation	Y= -0.0194x+2.7156	Y= -0.1653x+3.1871	Y= 0.626x+2.445	Y= 15.319x-56.167
EC_{50} value (mg/mL)	R^2	0.9246	0.8570	0.3078	0.9288
Reducing power	Linear equation	Y= -0.0144x+2.0805	Y= -0.1266x+2.4657	Y=-0.0498x+2.2389	Y= -0.0289x+1.4481
EC_{50} value (mg/mL)	R^2	0.9970	0.9774	0.6047	0.3001
β-carotene bleaching inhibition	Linear equation	Y=-0.0083x+1.7099	Y= -0.0757x+1.9611	Y=1.2166x-19.730	Y= 6.7664x-20.581
EC_{50} value (mg/mL)	R^2	0.8551	0.9117	0.9718	0.9861
TBARS inhibition	Linear equation	Y=-0.0004x+0.0563	Y= -0.0041x+0.0719	Y= -0.0426x+1.6595	Y= -0.3914x+2.6643
EC_{50} value (mg/mL)	R^2	0.5447	0.6874	0.9885	0.4208
HepG2	Linear equation	Y= 0.8434x+34.512	Y= 6.9512x+21.71	-	-
GI_{50} value (µg/mL)	R^2	0.9899	0.9892	-	-

3.2.2. Antioxidant activity and hepatotoxicity of syrups and synergistic effects

Artichoke (A), borututu (B) and milk thistle (M) were assessed either as single plant syrup, as a combinations of the three plants in the same syrup ((A+B+M)S) or in a mixture of different plant syrups (AS+BS+MS). As for the label information, single plant syrups contained 1000, 100, and 26.4 mg/mL, for artichoke, borututu, and milk thistle, respectively, and the syrup with the three plants (i.e., (A+B+M)S) consisted of a mixture of artichoke and borututu at 150 mg/mL and milk thistle at 350 mg/mL. The sample (AS+BS+MS) was prepared in the laboratory by mixing the single plant syrups at the same concentrations indicated for the commercial syrup containing the three plants. The results obtained for antioxidant activity and hepatotoxicity, for individual and combined syrups, are shown in Tables 20 and 21, respectively.

Table 20. Antioxidant activity and hepatotoxicity of artichoke, borututu and milk thistle single plant syrups.

	Artichoke	Borututu	Milk thistle	Positive control*
Antioxidant activity (EC_{50} values, mg/mL)				
DPPH scavenging activity	225±10[a]	1.34±0.06[b]	0.32±0.01[c]	0.04±0.00
Reducing power	73.2±0.2[a]	1.06±0.01[b]	0.052±0.001[c]	0.03±0.00
β-carotene bleaching inhibition	17.4±0.4[a]	0.51±0.04[b]	0.018±0.002[c]	0.003±0.00
TBARS inhibition	21.26±0.04[a]	0.150±0.003[b]	0.049±0.002[c]	0.004±0.00
Anti-hepatocellular carcinoma activity (GI_{50} values, µg/mL)				
HepG2 (hepatocellular carcinoma)	n.a.	n.a.	280±18	1.44±0.02
Hepatotoxicity (GI_{50} values, µg/mL)				
PLP2 (non-tumour liver primary culture)	n.a.	n.a.	n.a.	2.14±0.01

n.a.- no activity up to 400 µg/mL. In each row different letters mean significant differences between the EC_{50} values ($p<0.05$). *Trolox and elipticine were used as positive control for antioxidant activity and hepatotoxicity assays, respectively.

Milk thistle syrup was the most powerful in all the assays, in spite of being the syrup containing the lowest plant concentration. Borututu syrup also revealed relevant antioxidant activity, whereas the artichoke syrup showed the weakest one, despite possessing the highest plant concentration. Regarding the hepatocellular carcinoma activity, only milk thistle syrup revealed some capacity to inhibit the proliferation of

HepG2 cell line, whereas none of the syrups showed hepatotoxicity for normal liver cells (Table 20).

Comparing infusions and pills with the studied syrups, the antioxidant activity followed the order: infusion>pills>syrup, infusion>syrup≈pills and syrup>infusion>pills for artichoke, borututu, and milk thistle, respectively. Nevertheless, this comparison should be considered carefully once the concentrations of the plants in the different preparations are different in each case.

Table 21. Theoretical *versus* experimental values of antioxidant activity and hepatotoxicity of syrups containing the three plants (artichoke, borututu and milk thistle).

		(A+B+M)S		AS+BS+MS	
	Theoretical*	Experimental	Effect	Experimental	Effect
Antioxidant activity (EC_{50} values, mg/mL)					
DPPH scavenging activity	52.29	5.77±0.02[a]	SN	2.16±0.02[b]	SN
Reducing power	17.10	4.14±0.06[a]	SN	0.78±0.01[b]	SN
β-carotene bleaching inhibition	4.13	1.36±0.02[b]	SN	7.1±0.2[a]	AN
TBARS inhibition	4.95	0.60±0.02[a]	SN	0.25±0.01[b]	SN
Anti-hepatocellular carcinoma activity (GI_{50} values, µg/mL)					
HepG2 (hepatocellular carcinoma)	280.48	342±27	AN	n.a.	AN
Hepatotoxicity (GI_{50} values, µg/mL)					
PLP2 (non-tumour liver primary culture)	n.a.	n.a.	-	n.a.	-

Different letters in each row mean significant differences between the experimental EC_{50} values (p<0.05).

Besides the syrups based on a single plant there are also available syrups containing mixtures of the three plants. Our research was guided by three main questions: 1) Are syrups based on mixed plants more bioactive than syrups with a single plant? 2) Have the syrups with mixed plants the same bioactivity of mixed plant syrups? 3) What are the main effects (synergistic, additive or antagonist) in the antioxidant and anti-hepatocellular carcinoma activity of the syrups containing the three plants?

Milk thistle syrup proved to have the highest antioxidant activity in all the assayed methods, providing the best results among single syrups and even better than mixed syrups (compare results in Tables 20 and 21). The same tendency was observed for borututu syrup that gave better antioxidant properties than mixed samples. The opposite

was observed for artichoke, whose syrup showed lower antioxidant values than (A+B+M)S and (AS+BS+MS) preparations; thus, artichoke was the plant material more favoured by the mixture of plants in the same syrup. Regarding antioxidant capacity, mixing syrups (i.e., AS+BS+MS) gave in general more favourable results than mixing plants in the same syrup, (i.e., (A+B+M)S) , resulting in higher DPPH scavenging activity, reducing power and TBARS inhibition, although *β*-carotene bleaching inhibition was better in the (A+B+M)S (Table 21).

The experimental results obtained for the antioxidant capacity of the mixtures were better than the theoretical values calculated from the results obtained for the individual syrups, with the exception of the preparation AS+BS+MS in the *β*-carotene bleaching inhibition assay (7.1 > 4.13 mg/mL; Table 21). Thus, in general, synergistic effects (increase of antioxidant capacity) were produced, but for the indicated case, in which an apparent antagonistic effect was observed. Nevertheless, these observations should be considered with caution as they might be influenced by the different proportion of the plants existing in every mixture.

Regarding the hepatoprotective effect, only the mixture (A+B+M)S revealed some anti-hepatocellular carcinoma activity in HepG2 (342 µg/mL), but none of the syrups revealed hepatotoxicity in normal cells. Thus, an apparent antagonistic effect is produced in the mixture of the three plants in (A+B+M)S (decrease of anti-hepatocellular carcinoma activity) and in AS+BS+MS (no activity) in comparison to the milk thistle syrup. Once again the possibility of this being influenced by the different plant concentrations in every preparation should be considered.

There are some studies involving combined raw materials, like mixtures of different mushrooms, dietary supplements (plant pills or capsules) or plant infusions and decoctions, in order to achieve synergistic effects in the bioactive properties. A previous study in our laboratory mixing different mushroom species (*Boletus edulis, Calocybe gambosa, Cantharellus cibarius, Craterellus cornucopioides and Marasmius oreades*) revealed that synergism was the most abundant effect in the antioxidant properties of the mixtures and that *M. oreades* was present in the best mixtures, while *C. cibarius* was present in the worst ones (Queirós et al., 2009). According to Vieira et al. (2012), different effects on reducing or scavenging properties are obtained mixing different proportions of mushrooms and the final result depends on the used proportions.

Regarding combinations of different dietary supplements (pills, capsules or bags with labeled antioxidant effects), studies performed by Almeida et al. (2011) showed that the synergistic interaction was also the main observed effect. Similar results were obtained in a study involving plant (fennel, lemon-verbena and spearmint) infusions and decoctions (Guimarães et al., 2011), concluding that these mixtures are in fact more effective than individual preparations. Nevertheless, as far as we know, there are no studies involving syrups interactions in the antioxidant and anti-hepatocellular carcinoma activity. The syrup of borututu is indicated to detoxify the organism and relieve liver disorders and it is also recognized for its potential antiviral effect; artichoke syrup is used in cases of fluid retention or malfunction of the liver and gallbladder, and milk thistle syrup is applied in cases of difficult digestion, fatty liver, jaundice, poisoning and biliary lithiasis (Longe, 2005). Due to their benefits, syrups combining the three plants are available in the market that claim to have beneficial effects in liver, stomach, spleen and the entire urinary tract diseases acting as detoxifiers and cleansers of the whole body, being also diuretic and efficient in removing grease and weight reduction (Longe, 2005). Thus, the combined syrup might be helpful in the treatment of health problems that the individual plants can not, and, if so, in those cases there would be, indeed, synergistic effects.

3.2.3. Effects of formulation and compositional mixtures

Antioxidant properties and hepatotoxicity. The effects of formulation type (F) and A:B:M ratio (R) were evaluated by fixing one of the factors; *i.e.*, the results are presented as the mean of each F, comprising values for all R in the formulation, as well as the mean for every R, containing the results for all the corresponding F. Accordingly, the standard deviation values should not be looked up as a simple measure of assays repeatability, since they reflect results from assays performed in different conditions.

As it can be seen in Table 22, each factor showed a significant effect *per se*, but the interaction among factors (F×R) was also a significant ($p<0.001$) source of variation for all parameters, indicating a strong interaction between the formulation and the percentages of each plant in the prepared mixtures. Therefore, although the least squares means are presented, the results for multiple comparisons became meaningless. Nevertheless, from the analysis of the plots of the estimated margins means (Figures 16 and 17), some particular tendencies can be observed.

For instance, pill formulation, regardless of the A:B:M ratio, gave lower antioxidant activity in all antioxidant assays (DPPH scavenging activity: EC_{50} = 1.2 mg/mL; reducing power: EC_{50} = 0.4 mg/mL; β-carotene bleaching inhibition: EC_{50} = 2 mg/mL; TBARS formation inhibition: EC_{50} = 0.3 mg/mL), and also lower contents in total phenolics (69 mg GAE/g) and total flavonoids (5 mg CE/g) contents. On the other hand, syrups and infusions presented similar antioxidant activity values, except for reducing power (lower on infusion), but total phenolics (469 mg GAE/g) and total flavonoids (78 mg CE/g) contents were higher in syrups.

Table 22. Bioactive compounds and antioxidant activity of the different formulations prepared with mixtures of artichoke (A), borututu (B) and milk thistle (M).

		Total phenolics (mg GAE/g)	Total flavonoids (mg CE/g)	DPPH scavenging activity	Reducing power	*β*-Carotene bleaching inhibition	TBARS formation inhibition
Formulation (F)	infusion	148±19	34±4	0.4±0.1	0.2±0.1	0.15±0.03	0.06±0.01
	pill	69±11	5±1	1.2±0.4	0.4±0.1	2±1	0.3±0.1
	syrup	469±164	78±32	0.3±0.2	0.06±0.02	0.10±0.02	0.03±0.01
	p-value (n=36)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
A:B:M ratio (R)	1:1:1	232±178	38±33	0.6±0.2	0.2±0.1	0.3±0.3	0.2±0.2
	2:1:1	172±112	31±20	1.0±0.5	0.3±0.2	1±1	0.1±0.1
	1:2:1	198±121	31±20	0.5±0.4	0.2±0.1	1±1	0.1±0.1
	1:1:2	312±301	55±54	0.6±0.4	0.3±0.2	2±2	0.1±0.1
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
F×R	*p*-value (n=108)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

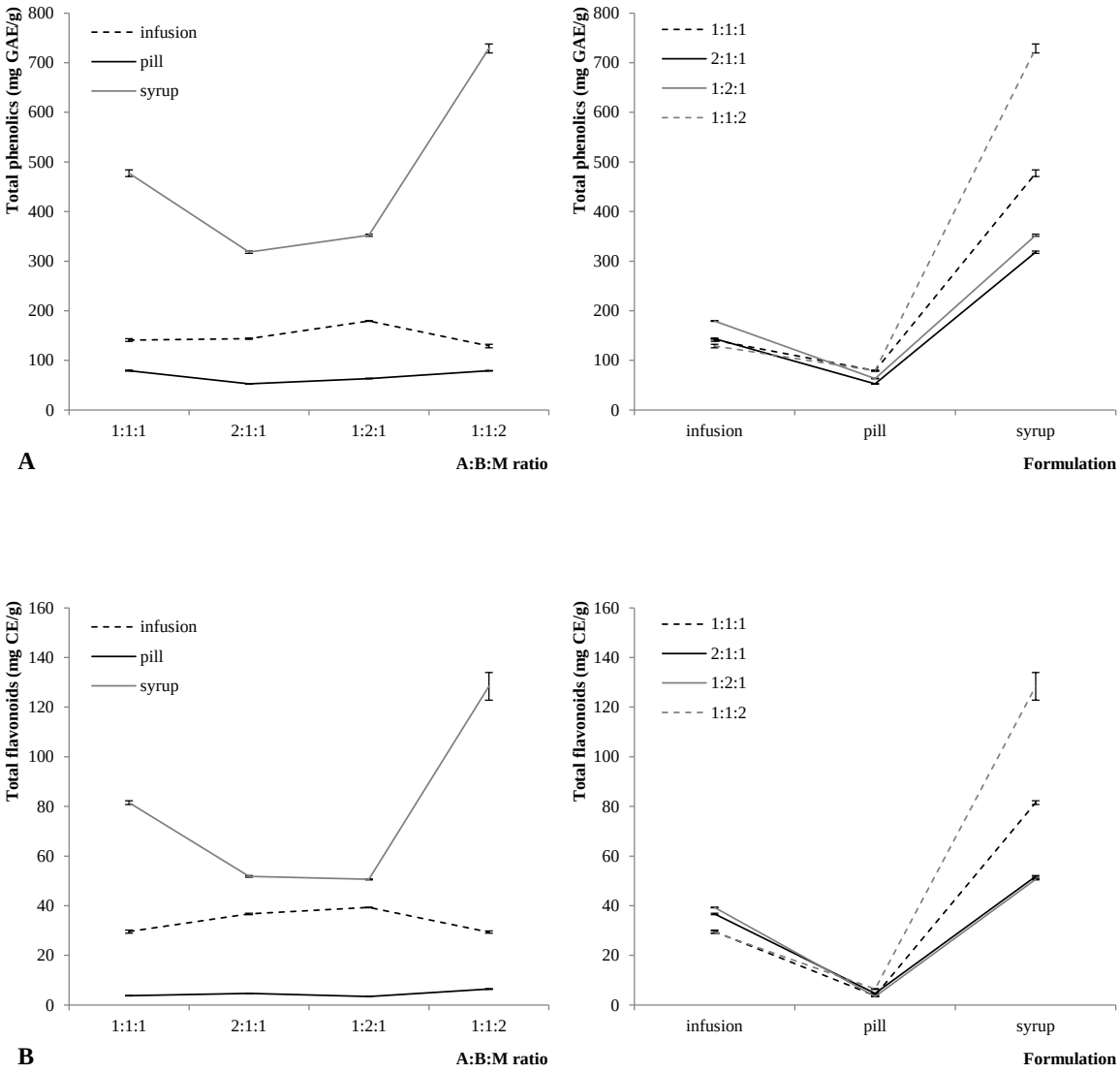


Figure 16. Interactions between formulation (F) and artichoke:borututu:milk thistle ratio (R). Effects on the contents of bioactive compounds. Total phenolics (A), total flavonoids (B).

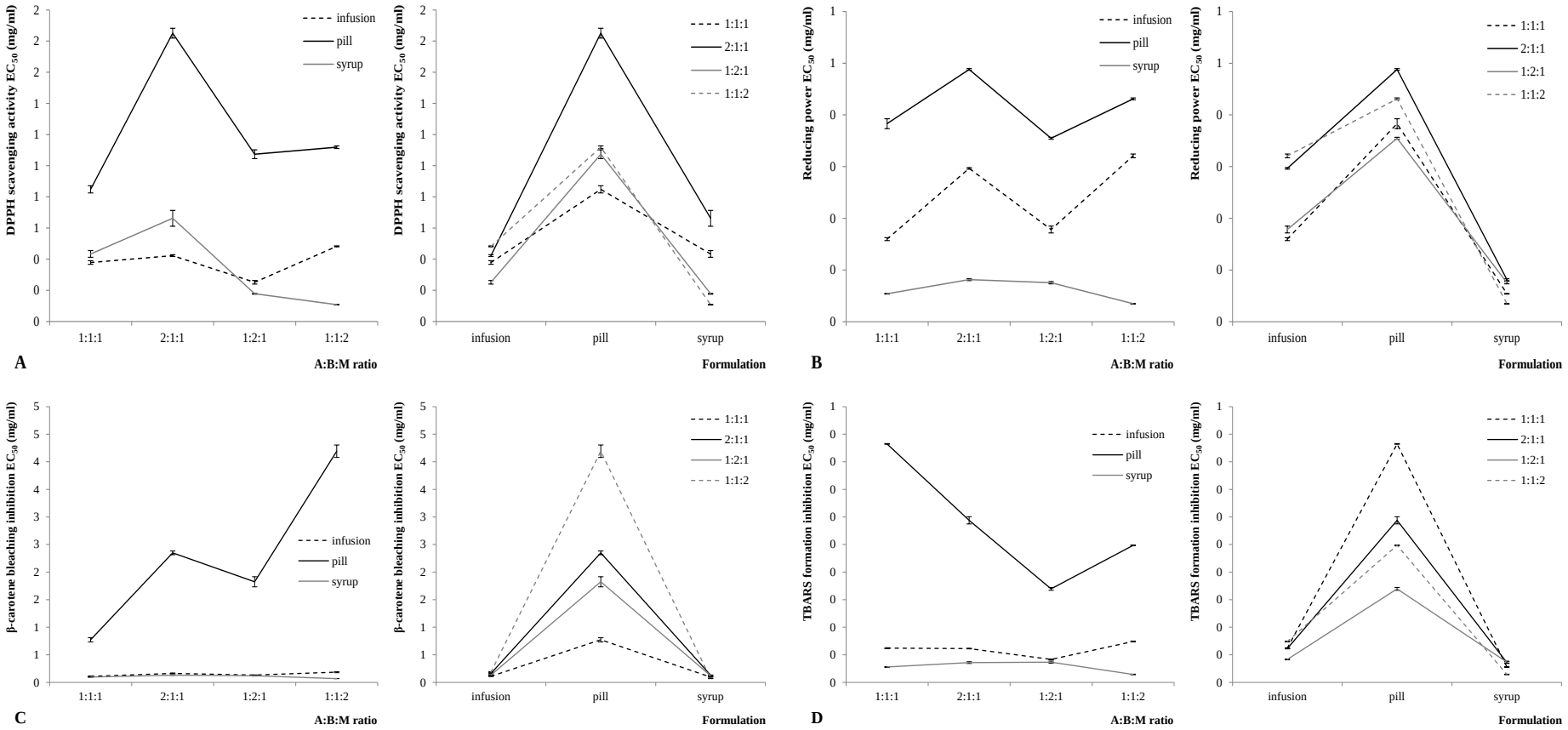


Figure 17. Interactions between formulation (F) and artichoke:borututu:milk thistle ratio (R). Effects on the antioxidant activity. DPPH scavenging activity (A), reducing power assay (B), *β*-carotene bleaching inhibition (C), TBARS formation inhibition (D).

In what concerns A:B:M ratios, the results did not reveal so pronounced differences, except for the lower DPPH scavenging activity (EC_{50} = 1.0 mg/mL), *β*-carotene bleaching inhibition (EC_{50} = 2 mg/mL) and TBARS formation inhibition (EC_{50} = 0.2 mg/mL) in mixtures 2:1:1, 1:1:2 and 1:1:1, respectively.

Besides the pointed differences, the assayed mixtures and formulations proved to have higher antioxidant activity than previously assayed formulations, namely syrups with a A:B:M ratio of 1:1:2.35, except in the case of *β*-carotene bleaching inhibition, for which the results were similar (see *section 3.2.2*).

Regarding the anti-hepatocellular carcinoma activity, more favourable effects were obtained in the herein assayed mixtures than those obtained from infusion, pills or syrups based on a single species (*section 3.2.1*) or in the mixtures previously checked (*section 3.2.2*). The antitumor activity was especially high in the infusions (1:1:1, GI_{50} = 24 µg/mL; 2:1:1, GI_{50} = 49 µg/mL; 1:2:1, GI_{50} = 63 µg/mL; 1:1:2, GI_{50} = 67 µg/mL; Table 22). None of the prepared mixtures showed hepatotoxicity (GI_{50} > 400 µg/mL, in all cases), which represents an important result considering the need of obtaining innocuous formulations.

Additive, synergistic or antagonistic effects. When comparing with the antioxidant activity and bioactive compounds content of the single plant preparations, the results obtained for the present mixtures and formulations are close to those reported for borututu, which is, by far, the plant with most active derived products among the three assayed species (see *section 3.2.1*). As a consequence, the possibility of having a synergistic effect within the prepared mixtures was raised. This hypothesis was mathematically verified by calculating the expected theoretical values for the different studied activities obtained by averaging the simple mean (for 1:1:1 mixture) or the weighted mean (in the remaining mixtures) of the distinct plants. The results of these calculations are indicated in the Table 23. Regarding the anti-hepatocellular carcinoma activity, the GI_{50} values higher than 400 µg/mL (the maximum assayed concentration) were included as being 400, since this is precisely the value that most hinder the possible synergistic effect; *i.e.*, if the result obtained for a given mixture when considering the GI_{50} value of a particular plant (or plants) as being 400 µg/mL instead of the actual (higher) experimental value, then the resulting effect would certainly be synergistic. With no exception, the mixtures of all formulations gave synergistic effects in the antioxidant activity. In fact, the highest activity of mixtures when compared to

the individual plants was previously observed in formulations of other plants, i.e., fennel, lemon-verbena and spearmint (Guimarães et al., 2011).

Nevertheless, the mixtures 2:1:1 and 1:1:2, for pills and syrups, and also mixture 1:2:1, for syrups, did not result in a synergistic effect in what regards the anti-hepatocellular carcinoma activity on HepG2. The hepatotoxicity, as evaluated on PLP2 cells, was always lower in the mixtures, when compared to the activity of single plants, which represents also a good result considering the previously stated objective of obtaining non-toxic mixed formulations.

Linear discriminant analysis of antioxidant properties. In order to have a complete perspective about the effect of F and R on the antioxidant activity and levels of bioactive compounds, two linear discriminant analyses (LDA) were applied. The significant independent variables (results for antioxidant activity assays and bioactive compound contents) were selected following the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis. The anti-hepatocellular carcinoma activity and hepatotoxicity results were not included, since there were some cases with GI_{50} >400 µg/mL that could not be used in the treatment.

In the case of F effect, two significant functions were defined, which included 100% of the observed variance (first, 58.2%; second, 41.8%). As it can be observed in the Figure 18, the tested groups (infusion, pill and syrup) were completely individualized (shadowed ellipses). Function 1 was primarily correlated to TBARS formation inhibition, DPPH scavenging inhibition and *β*-carotene bleaching inhibition, which were much lower in pill formulation. Actually, this function separated mainly pills from the remaining formulations, as confirmed by the means of canonical variance (MCV: infusion, -4.684; pill, 5.212; syrup, -0.529.

Function 2, by its side, was more correlated to reducing power (lower in syrup), total phenolics and total flavonoids (in higher concentrations in syrups). Accordingly, as it can be seen in the vertical axis, function 2 clearly separated syrup formulation (MCV: infusion, -2.806; pill, -2.031; syrup, 4.837). All samples were correctly classified, either for original grouped cases, as well as for cross-validated grouped cases.

Table 23. Theoretical *versus* experimental values of antioxidant activity and hepatotoxicity of the different mixtures and formulations.

Bioactivity	Theoretical*	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect†
	1:1:1			2:1:1			1:2:1			1:1:2		
Infusion												
DPPH scavenging activity	1.56	0.38±0.02	SN	1.72	0.42±0.04	SN	1.22	0.25±0.02	SN	1.80	0.48±0.03	SN
Reducing power	1.22	0.16±0.01	SN	1.38	0.30±0.02	SN	0.97	0.18±0.01	SN	1.36	0.32±0.02	SN
β-carotene bleaching inhibition	1.21	0.11±0.01	SN	1.34	0.16±0.01	SN	1.07	0.13±0.01	SN	1.26	0.18±0.01	SN
TBARS inhibition	0.17	0.06±0.01	SN	0.16	0.06±0.01	SN	0.13	0.04±0.01	SN	0.22	0.07±0.01	SN
HepG2 (hepatocellular	199.37	24±1	SN	162.55	49±8	SN	186.05	63±7	SN	249.53	67±2	SN
Pill												
DPPH scavenging activity	6.46	0.85±0.05	SN	7.43	1.85±0.03	SN	5.22	1.07±0.03	SN	6.94	1.12±0.04	SN
Reducing power	1.31	0.38±0.02	SN	1.56	0.49±0.02	SN	1.14	0.35±0.03	SN	1.27	0.43±0.02	SN
β-carotene bleaching inhibition	7.70	0.77±0.04	SN	5.84	2.35±0.05	SN	7.24	1.82±0.05	SN	10.27	4.19±0.05	SN
TBARS inhibition	0.86	0.43±0.02	SN	1.03	0.29±0.01	SN	0.74	0.17±0.01	SN	0.85	0.25±0.02	SN
HepG2 (hepatocellular	400	360±14	SN	400	>400	AD	400	340±2	SN	400	>400	AD
Syrup												
DPPH scavenging activity	74.89	0.43±0.02	SN	113.05	0.66±0.05	SN	57.07	0.18±0.02	SN	56.81	0.11±0.01	SN
Reducing power	24.51	0.05±0.01	SN	36.85	0.08±0.01	SN	18.83	0.08±0.01	SN	18.58	0.03±0.01	SN
β-carotene bleaching inhibition	5.91	0.10±0.01	SN	8.82	0.13±0.01	SN	4.61	0.12±0.01	SN	4.48	0.07±0.01	SN
TBARS inhibition	7.08	0.03±0.01	SN	10.68	0.04±0.01	SN	5.40	0.04±0.01	SN	5.38	0.02±0.01	SN
HepG2 (hepatocellular	360.16	317± 12	SN	370.12	>400	AN	370.12	>400	AN	340.24	>400	AN

*Theorical values were calculated from the results obtained for the individual plants considering their proportion in the prepared mixture

†Effects: SN=synergistic; AN=antagonistic; AD=additive

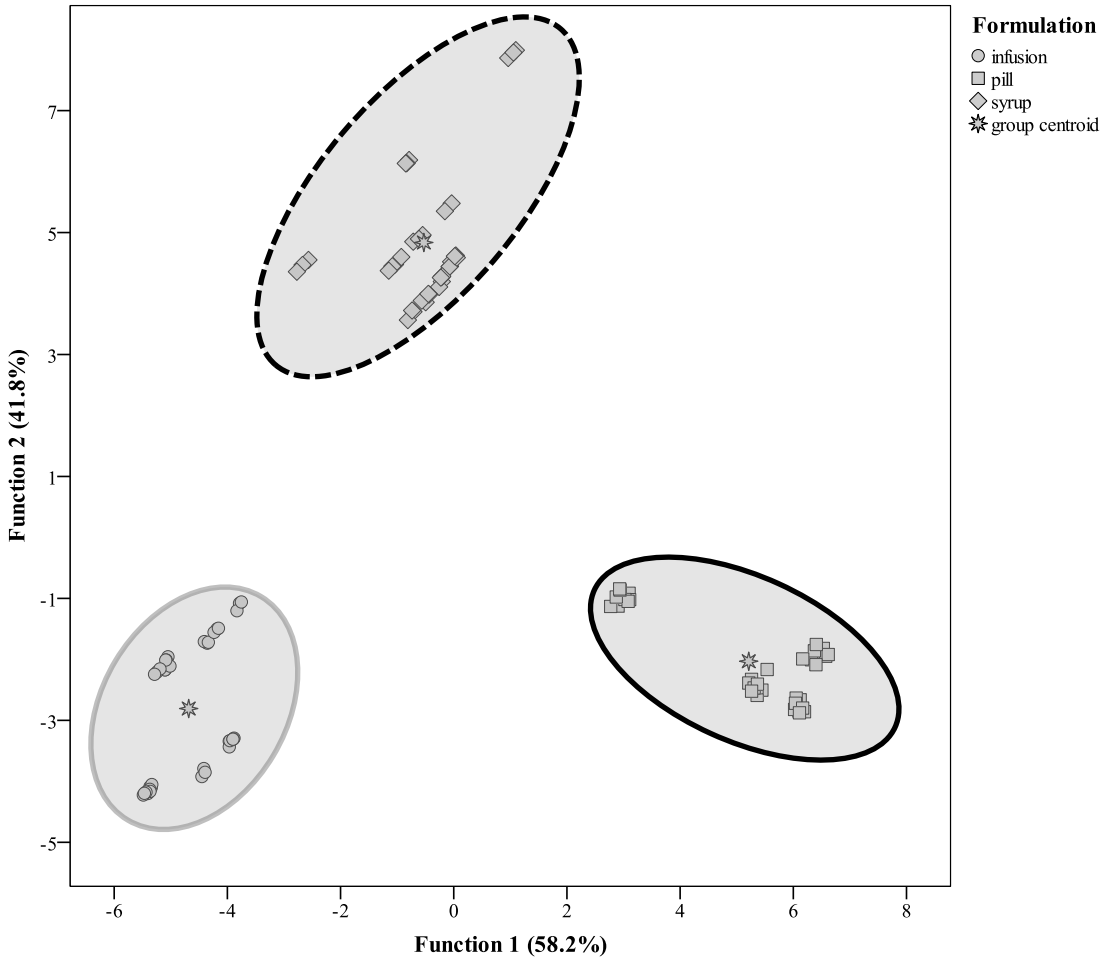


Figure 18. Discriminant scores scatter plot of the canonical functions defined for bioactive compounds content and antioxidant activity results according to formulation.

Regarding the A:B:M ratio, the discriminant model selected 3 significant functions (Figure 19), which included 100.0% of the observed variance (function 1: 65.3%, function 2: 20.9%, function 3: 13.8%). In this case, the tested groups (1:1:1, 2:1:1, 1:2:1 and 1:1:2) were not completely individualized, indicating that the differences in the antioxidant activity assays and bioactive compounds contents were not enough to discriminate the tested groups. The classification performance allowed 65% of correctly classified samples (sensitivity) and 64% of overall specificity within the leave-one-out cross-validation procedure (Table 24). Despite all variables were kept in the final analysis, it became obvious that the differences verified for the assayed ratios were not as significant as it would be necessary to obtain individualized groups. This can be clearly observed in Figure 19, in which several overlapping markers confirm the similarity among the assayed mixtures of artichoke, borututu and milk thistle.

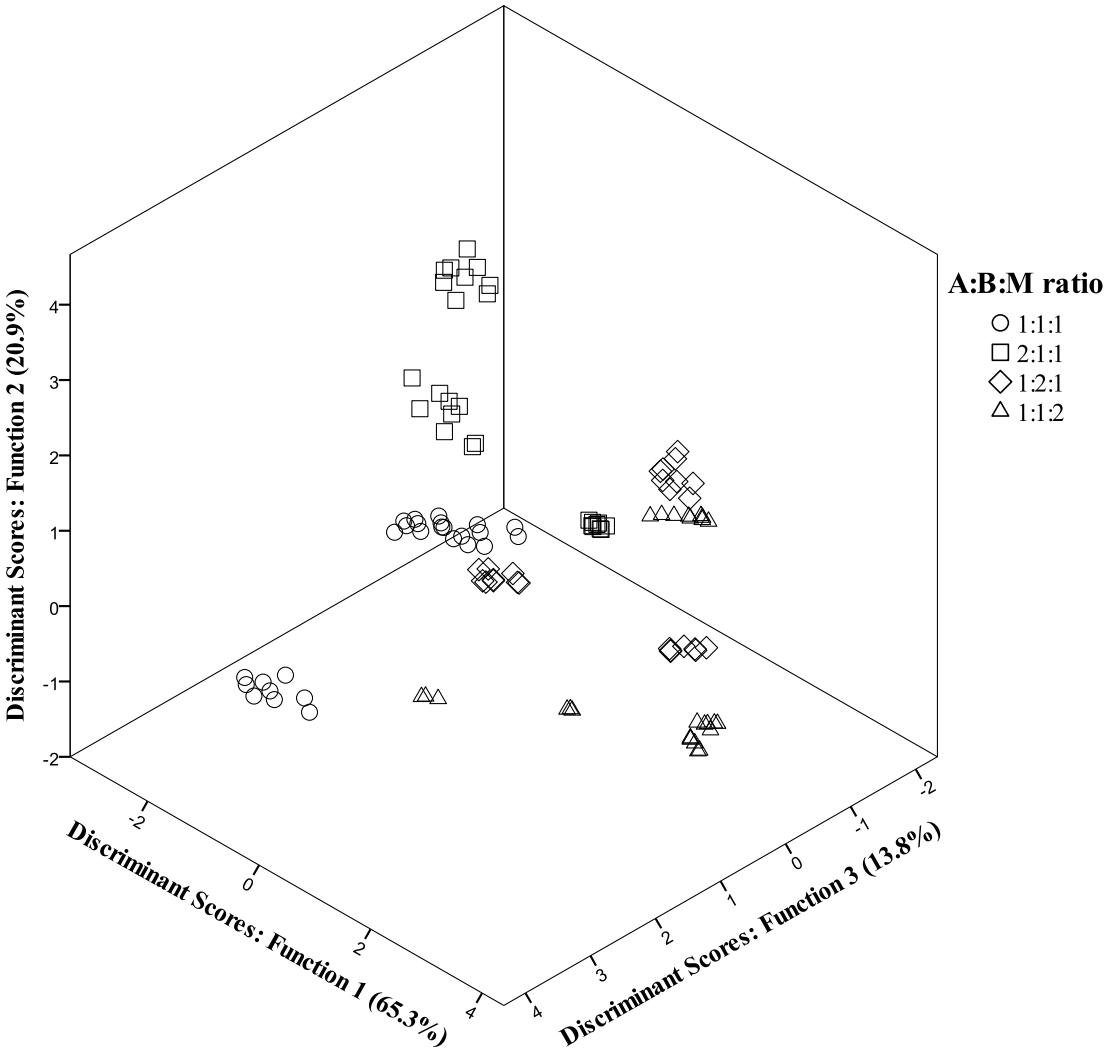


Figure 19. Discriminant scores scatter plot of the canonical functions defined for bioactive compounds content and antioxidant activity results according to the artichoke:borututu:milk thistle ratio.

Overall, the interaction among F and R was significant in all cases, indicating that the effects caused by each assayed formulation are related to the used proportion of each plant. Even so, syrups tended to be the formulation with the highest antioxidant activity and higher contents in total phenolics and flavonoids; this was specially verified when the mixture 1:1:2 was used, as it can be concluded from the estimated marginal mean plots. On the other hand, pills were the worst formulation, independently of the used mixture. In what concerns artichoke:borututu:milk thistle ratios, the results did not reveal so observable differences. The higher influence of F in comparison with R was clearly highlighted by the LDA outputs. In addition, the effects of each factor were

significantly different, since the correlations among discriminant functions and selected variables were different within each statistical test. The obtained outputs confirmed the existence of significant differences among infusions, pills and syrups, showing also that the A:B_M ratios used in the mixtures had much lower effects in the antioxidant activity assays and bioactive compounds contents.

Table 24. Contingency matrix obtained using LDA based on antioxidant activity and bioactive compounds content in different artichoke:borututu:milk thistle ratios.

	Predicted group membership				Total	Sensitivity (%)
	1:1:1	2:1:1	1:2:1	1:1:2		
1:1:1	19	7	1	0	27	70
2:1:1	9	9	0	9	27	33
1:2:1	6	0	21	0	27	78
1:1:2	0	0	6	21	27	78
Total	34	16	28	30	108	65
Specificity (%)	56	56	75	70	64	

3.2.4. Antioxidant activity and hepatotoxicity in infusions potentiated by mixing honey

Honey quality. The quality of honey is highly dependent on the botanical origin of the nectar source, and so, its properties. Dark honeys are generally assumed to present higher antioxidant activity than light-colored ones (Alves et al., 2013), which is in part explained by the presence of several phytochemicals in its composition, among them phenolic compounds. Chestnut honey is identified by its dark-reddish color and high electrical conductivity due to a high mineral content, what makes a good candidate to be used as nutraceutical. Recent studies indicated that the fortification of yogurts with chestnut honey leads to an increase in the antioxidant activity of the final product (Perna et al., 2014).

The melissopalynological results for the honey sample used in this study revealed a high content of *Castanea sativa* pollen close to 70%. This botanical classification was confirmed by its physicochemical features, the dark amber color and the high electrical conductivity above 1100 μs/cm (Table 25). Low acidity and high content in the amino

acid proline were also observed, as well as a ratio of fructose/glucose well above 1.2, characteristic of honeys with low tendency for crystallization. The sugar profile of chestnut honey presents typically higher content of the monosaccharide fructose compared to glucose, with some traces of oligosaccharides that arise from the collection of honeydew by the bees, due to the late season harvesting of this type of honey. These features can be observed in the footnote of Table 25, where the presence of some levels of oligosaccharides can be observed, including the trisaccharide melezitose, typlical of honeydrew. The other quality parameters, such as humidity, HMF, diastase and sugar contents (Table 24) all certify the sample as a good quality honey, with the values fitting within the international standards forhoney (Codex Alimentarius, 2001; European Honey Directive, 2001).

Table 25. Honey quality parameters.

Parameters	Honey sample	Standard Regulations
Color (mm Pfund)	Dark Ambar	Dark to very dark
Humidity (%)	14.6 ± 0.0	Less than 20
Conductivity (μs/cm)	1167.3 ± 0.6	Above 800
HMF (mg/kg)	0.7 ± 0.2	Below 40
Free acidity (meq/kg)	15.3 ± 0.6	Low values
Lactonic acidity (meq/kg)	11.3 ± 0.3	-
Total acidity (meq/g)	26 ± 1	-
Reducing sugars (g/100 g)	74.0 ± 0.4	Above 60
Proline (mg/kg)	1158 ± 42	High values
Diastase (DN)	28.3 ± 0.3	-
Sucrose (g/100 g)	0.7 ± 0.0	Below 5
Fructose/Glucose ratio*	1.36	High values

*The sugars detected (g/100 g) in the sample of honey were fructose (42.6 ± 0.2), glucose (31.4 ± 0.4), sucrose (0.7 ± 0.0), turanose (2.5 ± 0.1), maltulose (3.2 ± 0.1), maltose (0.2 ± 0.0), trehalose (1.6 ± 0.0) and melezitose (0.4 ± 0.1).

Antioxidant properties and hepatotoxicity. In order to evaluate the effect of adding honey to infusions of artichoke, borututu, and milk thistle, infusions were prepared using single plants, mixtures of two plants and also using the three plants together. A chestnut based honey was chosen according to its high antioxidant activity. Due to the quantities of dried plants and honey commonly used to prepare infusion-based or

decoction-based beverages, it is important to assess the maintenance/improvement of the antioxidant activity in the consumed products.

The concentrations of each component in the mixtures are shown in Table 8 (*section 2.4.6*). Initially, the infusions were prepared using individual components: honey (H), artichoke (A), borututu (B) and milk thistle (M), or mixtures: AB, AM, BM and ABM. The results obtained for the antioxidant activity of these preparations are presented in Table 26. In general, the antioxidant activity of the infusions prepared only with honey was weaker than that obtained using plant infusions. Among these, preparations containing borututu showed the highest antioxidant activity. The obtained values are in the expected range, considering previously reported results (*section 3.2.1*). As it can also be depicted from Table 26, only artichoke and two-plant mixtures containing artichoke showed some hepatotoxicity, as evaluated from the assays in the HepG2 cell line, although the prepared beverages might be considered as having low levels for this indicator. In fact, none of the samples showed to be hepatotoxic at the assayed concentrations in the assays carried on PLP2 cell lines, except honey, for which a GI_{50} = 2.2 mg/mL was found.

The same indicators (antioxidant activity and hepatotoxicity) were evaluated in infusions containing the same plant composition plus honey (AH, BH, MH, ABH, AMH, BMH and ABMH), in order to verify the practical effect of adding this component to each of the prepared infusions. The experimental results obtained were compared with the theoretically predicted values to verify the occurrence of antagonistic, additive or synergistic effects (Table 27).

As it can be reasoned from Table 27, the addition of honey to the infusions had a beneficial effect, producing a synergistic effect in all cases, except in the *β*-carotene bleaching inhibition assay for the AMH preparation. Regarding the specific effect on each antioxidant assay, it might be concluded that TBARS formation inhibition and DPPH scavenging activity were improved in a higher extent. Concerning the assayed preparations, BH and BMH showed the highest increase in antioxidant activity, independently of the tested assay.

As no GI_{50} values could be determined for borututu, milk thistle, BM and ABM (i.e., lack of hepatoxicity), it was not possible to calculate the theoretical values for BH, MH, BMH and ABMH mixtures. Nevertheless, considering the cases in which these

calculations were possible, a reduction in the potential hepatotoxicity seems to be produced in the prepared mixtures (except in the case of AH).

Table 26. Antioxidant activity and effect on HepG2 cell line of the honey solution and of the infusions prepared from individual or mixed artichoke, borututu, and milk thistle.

Sample/Mixture		DPPH scavenging activity (EC_{50} values, mg/mL)	Reducing Power (EC_{50} values, mg/mL)	β-carotene bleaching inhibition (EC_{50} values, mg/mL)	TBARS inhibition (EC_{50} values, mg/mL)	HepG2 (hepatocellular carcinoma, GI_{50} values, µg/mL)
Honey (H)		33.7±0.5[a]	6.5±0.1[a]	10.0±0.5[a]	5.2±0.1[a]	1.4±0.2[a]
Artichoke (A)		8.8±0.3[c]	3.8±0.1[d]	1.01±0.03[e]	3.43±0.03[c]	0.09±0.01[b]
Borututu (B)		1.5±0.1[f]	0.79±0.01[h]	1.31±0.05[d]	0.22±0.01[g]	NT
Milk thistle (M)		4.4±0.1[d]	5.0±0.1[c]	1.31±0.05[d]	4.1±0.1[b]	NT
AB		2.3±0.1[e]	1.1±0.1[g]	1.55±0.05[d]	0.27±0.01[g]	0.20±0.01[b]
AM		12.1±0.2[b]	5.3±0.1[b]	2.2±0.1[b]	2.49±0.04[d]	0.18±0.01[b]
BM		1.9±0.1[e]	1.3±0.1[f]	1.86±0.04[c]	0.48±0.02[f]	NT
ABM		2.2±0.1[e]	1.7±0.1[e]	1.05±0.04[e]	0.72±0.02[e]	NT
p-values	Homoscedasticity[2]	<0.001	0.047	<0.001	<0.001	<0.001
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001
Positive control[*]		41±1	41.7±0.3	18±1	22.8±0.7	1.10±0.08

[*]Trolox and elipticine were used as positive control for antioxidant activity and HepG2 assays, respectively.

Table 27. Theoretical *versus* experimental values of antioxidant activity and effects on HepG2 cell lines of mixtures containing honey and plant infusion(s) (artichoke, borututu, and milk thistle, individual or mixed samples).

	DPPH scavenging activity (EC_{50} values, mg/mL)			Reducing Power (EC_{50} values, mg/mL)			β-carotene bleaching inhibition (EC_{50} values, mg/mL)			TBARS inhibition (EC_{50} values, mg/mL)			HepG2 (hepatocellular carcinoma, GI_{50} values, µg/mL)		
	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect*	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect
Artichoke (A) + Honey (H)	21.5±0.3^{b}	19.0±0.3^{a}	SN	5.21±0.02^{c}	4.6±0.2^{b}	SN	5.5±0.2^{c}	4.7±0.2^{c}	SN	4.38±0.03^{b}	3.2±0.1^{a}	SN	0.8±0.1	0.65±0.01^{c}	SN
Borututu (B) + Honey (H)	17.6±0.3^{d}	5.3±0.1^{e}	SN	3.64±0.03^{g}	2.2±0.1^{f}	SN	5.7±0.2^{bc}	3.8±0.2^{d}	SN	2.70±0.04^{f}	0.49±0.02^{g}	SN	NT	-	-
Milk thistle (M) + Honey (H)	19.2±0.4^{c}	7.3±0.3^{cd}	SN	5.86±0.05^{b}	4.7±0.1^{b}	SN	5.7±0.2^{bc}	4.8±0.2^{bc}	SN	4.72±0.04^{a}	2.3±0.1^{b}	SN	NT	-	-
ABH	18.1±0.4^{d}	5.1±0.2^{e}	SN	3.82±0.05^{f}	2.7±0.1^{e}	SN	5.8±0.3^{bc}	5.0±0.2^{b}	SN	2.72±0.04^{f}	0.89±0.01^{e}	SN	0.8±0.1	0.97±0.04^{b}	AN
AMH	23.2±0.3^{a}	13.9±0.5^{b}	SN	6.0±0.1^{a}	4.8±0.1^{a}	SN	6.2±0.2^{a}	6.9±0.3^{a}	AN	3.89±0.05^{c}	1.51±0.01^{c}	SN	0.8±0.1	1.07±0.04^{a}	AN
BMH	17.9±0.3^{d}	7.0±0.4^{d}	SN	3.9±0.1^{e}	2.9±0.2^{d}	SN	6.0±0.2^{ab}	1.8±0.1^{f}	SN	2.83±0.05^{e}	0.72±0.01^{f}	SN	NT	-	-
ABMH	18.0±0.3^{d}	7.7±0.4^{c}	SN	4.1±0.1^{d}	3.3±0.2^{c}	SN	5.6±0.2^{c}	2.2±0.1^{e}	SN	2.96±0.05 d	1.06±0.03^{d}	SN	NT	-	-
p-values Homoscedasticity[2]	0.901	<0.001		0.005	0.507		0.970	0.001		0.185	<0.001		0.996	0.018	
p-values 1-way ANOVA[3]	<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		0.481	<0.001	

*Effect: SN=synergistic; AN=antagonistic

Linear discriminant analysis of antioxidant properties. In order to have a complete perspective about the effect of honey addition on the antioxidant activity, a linear discriminant analysis was applied (the hepatotoxicity results were not included, since the GI_{50} were not available for all cases). The basic purpose of this discriminant analysis was estimating the connection between a single categorical dependent variable (infusion formulation) and a set of quantitative independent variables (the EC_{50} values obtained in the antioxidant assays). The significant independent variables were selected following the stepwise method of the LDA, according to the Wilks' λ test. Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis.

In order to simplify the interpretation of results, and also to increase their scope of application, the 15 prepared formulations were aggregated in seven groups: honey (H), 1 plant (A, B and M), 1 plant + honey (AH, BH, MH), 2 plants (AB, AM, BM), 2 plants + honey (ABH, AMH, BMH), 3 plants (ABM) and 3 plants + honey (ABMH).

The discriminant model selected 4 significant functions, which included 100.0% of the observed variance. The graph representation (Figure 20) of the three first functions (function 1: 70.1%, function 2: 27.2%, function 3: 2.3%) was included to assess the association of the analyzed infusions based on their antioxidant activity.

The tested groups were not completely individualized, but it is interesting to verify that all markers corresponding to infusions added with honey (shadowed markers) were proximately distributed (despite the overlapping of a few markers corresponding to the "2 plants" group). This observation was corroborated by the corresponding contingency matrix (Table 28).

The classification performance allowed 56% of correctly classified samples (sensitivity) and 66% of overall specificity within the leave-one-out cross-validation procedure, which may be considered as acceptable values. The displayed results show that all samples including honey in its preparation were classified in groups corresponding to infusions prepared with this component (from the 27 "1 plant + honey" samples, 19 were correctly classified and 8 were classified as "2 plants + honey"; from the 27 "2 plants + honey" samples, 12 were correctly classified, 6 were classified as "1 plant + honey" and 9 were classified as "3 plants + honey"; all the "3 plants + honey" samples were correctly classified).

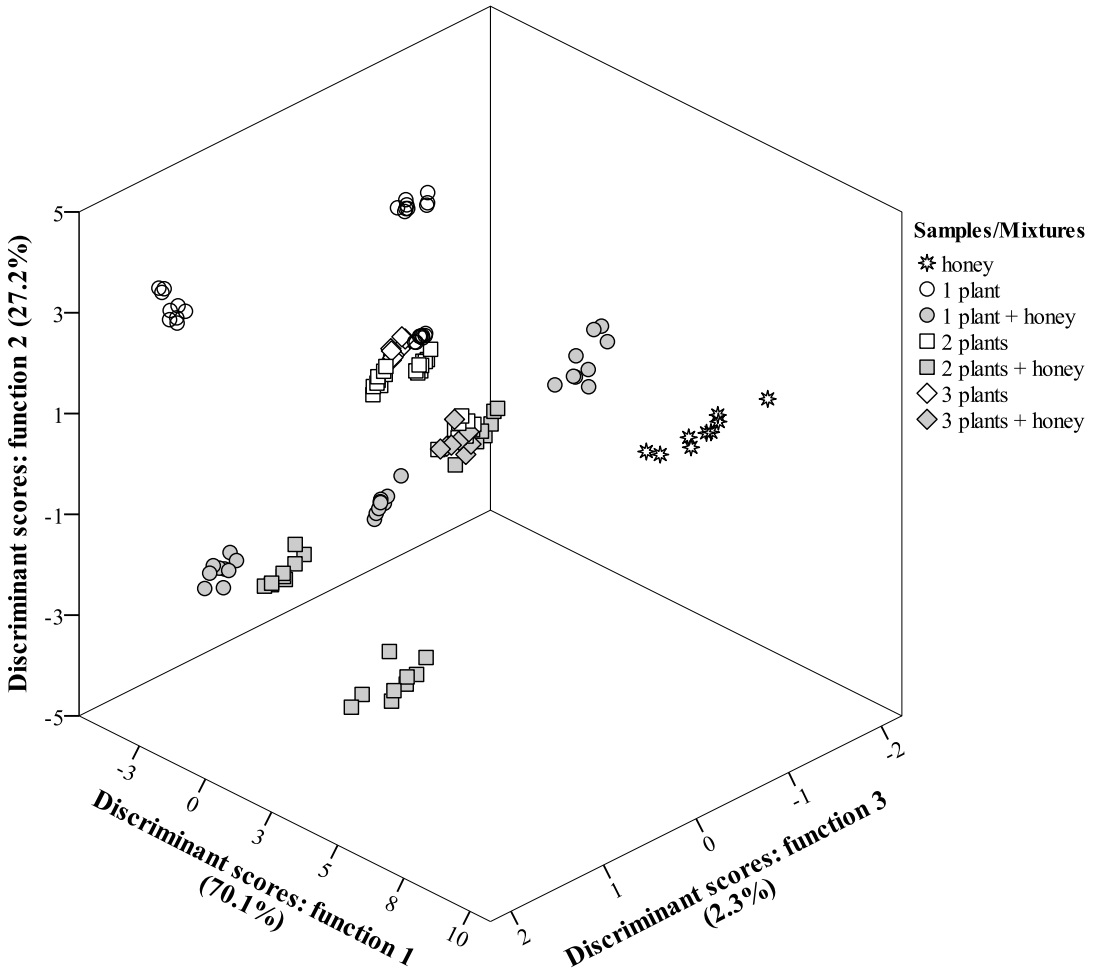


Figure 20. Mean scores of different samples/mixtures projected for the three first discriminant functions defined from antioxidant properties.

This result, together with the differences observed in Table 27, is a strong indication of the distinctively beneficial effect of honey addition in the antioxidant activity of these infusions. It is also noteworthy that 9 "1 plant" samples (out of 27) were classified as "3 plants", and that none of the "2 plants" samples was correctly classified as "2 plants". This might indicate that the enhancing effect induced by the addition of honey overcomes the potential effects of using one or two plants to prepare a determined infusion, which is so often reported. Furthermore, and despite the lack of scientific evidence, it could be considered that preparations added with honey have an improved flavor (increased sweetness and less bitterness), favoring the acceptance of a wider number of consumers.

Table 28. Contingency matrix obtained using LDA based on antioxidant activity EC50 hepatotoxicity GI50 values of mixtures containing honey and plant infusion(s) (artichoke, borututu and milk thistle, individual or mixed samples).

Sample/	Predicted Group Membership							total	Sensitivity (%)
Mixture	Honey	1 plant	1 plant	2 plants	2 plants	3 plants	3 plants		
Honey (H)	9	0	0	0	0	0	0	9	100
1 plant	0	18	0	0	0	9	0	27	67
1 plant + H	0	0	19	0	8	0	0	27	70
2 plants	0	0	0	0	0	18	9	27	0
2 plants + H	0	0	6	0	12	0	9	27	44
3 plants	0	0	0	0	0	9	0	9	100
3 plants + H	0	0	0	0	0	0	9	9	100
total	9	18	25	0	20	36	27	135	56
Specificity	100	100	76	-	60	25	33	66	

3.3. Phenolic composition and antimicrobial activity of the prepared dietary supplements

3.3.1. Phenolic compounds in artichoke and milk thistle extracts and infusions

Tables 29 and 30 present the data obtained from the HPLC-DAD-MS analysis (retention time, λ_{max} in the visible region, mass spectral data) used for the identification and quantification of phenolic compounds in artichoke and milk thistle, respectively. As an example, the HPLC phenolic profiles of their infusions, recorded at 370 nm, can be observed in Figures 21 and 22, respectively.

Phenolic acids. Protocatechuic acid (compound 3), 5-*O*-caffeolylquinic acid (compound 6), quinic acid (compound 7), caffeic acid (compound 8), and *p*-coumaric acid (compound 15) were positively identified according to their retention time, mass and UV-vis characteristics by comparison with commercial standards.

Hydroxycinnamic acid derivatives were detected in both samples, being mostly quinic acid derivatives, whose identities were assigned based on their MS spectra and fragmentation patterns taking into account the hierarchical keys developed by Clifford et al. (2003; 2005) Nomenclature of the different caffeoylquinic, feruloylquinic and *p*-coumaroylquinic acid isomers was made using the recommended IUPAC numbering system.

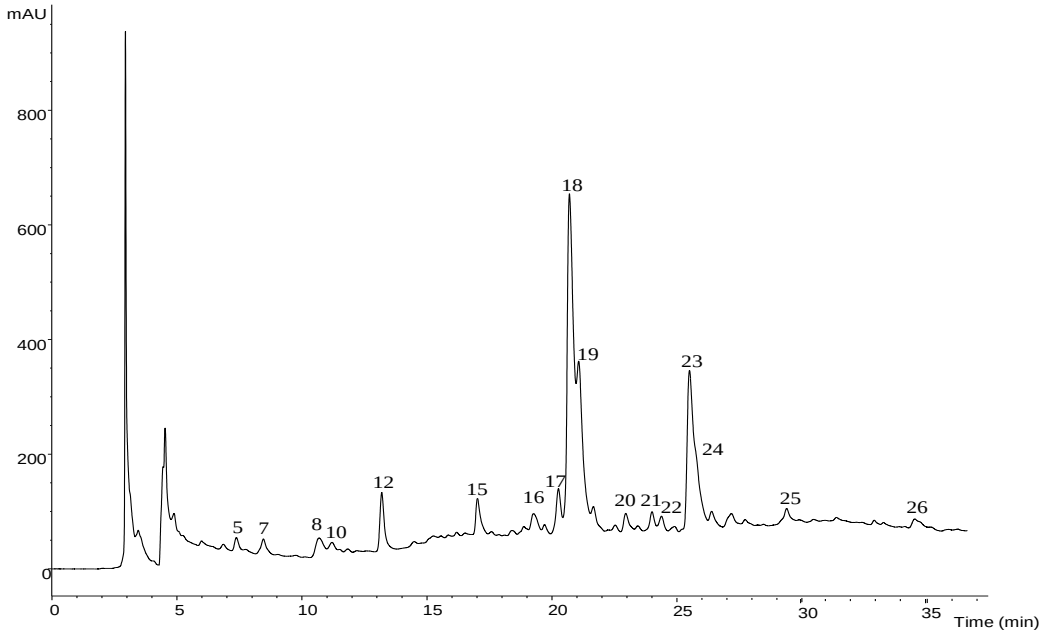


Figure 21. HPLC chromatogram recorded at 370 nm showing the phenolic profile of the infusion of artichoke.

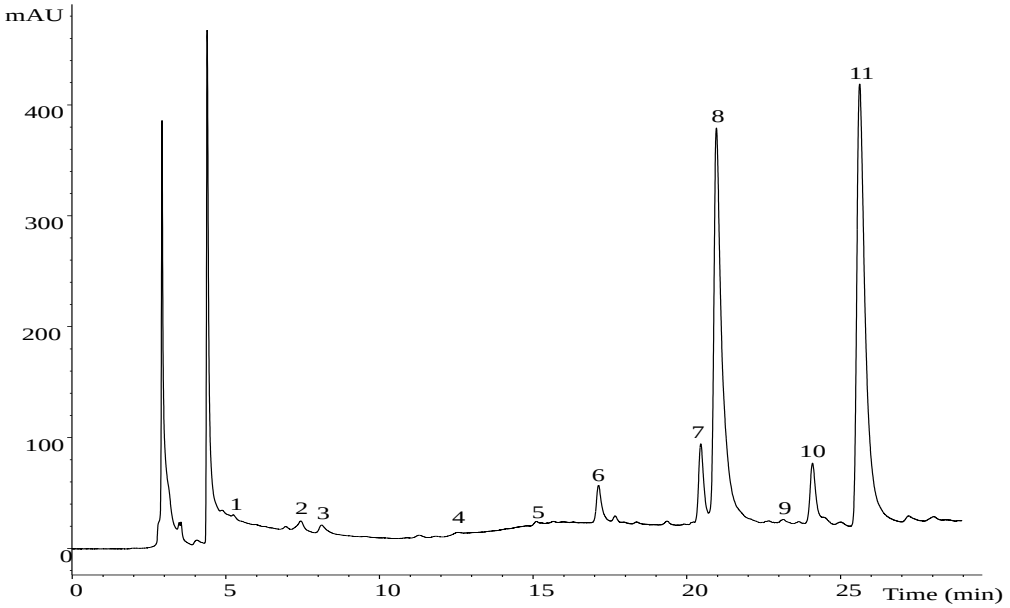


Figure 22. HPLC chromatogram recorded at 370 nm showing the phenolic profile of the infusion of milk thistle.

Compound 1 ($[M-H]^-$ at *m/z* 353) detected only in artichoke was identified as 3-*O*-caffeoylquinic acid, yielding the base peak at *m/z* 191 ($[quinic\ acid-H]^-$) and the product ion at *m/z* 179 ($[caffeic\ acid-H]^-$) with an intensity >63% base peak, characteristic of 3-

acylchlorogenic acids as reported by Clifford et al. (2003; 2005) Monocaffeoylquinic acids have been largely reported by many authors in different parts of artichoke, such as heads and leaves (Wang et al., 2003; Schütz et al., 2004; Ferracane et al., 2008; Lombardo et al., 2010; Pandino et al., 2010; Pandino et al., 2011a and b; Gouveia and Castilho, 2012; Farag et al., 2013; Wu et al., 2013), hearts (Abu-Reidah et al., 2013), wastes such as bracts, receptacles and steams from the fruit (Sánchez-Rabaneda et al., 2003), juices and pomace (Schütz et al., 2004; Gouveia and Castilho, 2012), and in dietary supplements (Gouveia and Castilho, 2012; Farag et al., 2013; Schütz et al., 2006).

Compound 22 present in milk thistle and artichoke was identified as 3,5-*O*-dicaffeoylquinic acid based on its fragmentation pattern similar to the one reported by Clifford et al. (2005). Its MS^2 base peak was at *m/z* 191, but also presented a very high relative abundance of the ion at *m/z* 353, produced by the loss of one of the two caffeoyl moieties $[M\text{-}H\text{-caffeoyl}]^-$, whose subsequent fragmentation yielded the same fragments as 5-*O*-caffeoylquinic acid at *m/z* 191, 179 and 135. Compound 10 (artichoke) was identified as 1,3-*O*-dicaffeoylquinic acid (cynarin) according to its MS^2 fragmentation and elution characteristics, being the most hydrophilic dicaffeoylquinic acid (Clifford et al., 2005). Dicaffeoylquinic acids have been extensively reported in hydroalcoholic extracts obtained from different parts of artichoke, as mentioned above (Sánchez-Rabaneda et al., 2003; Wang et al., 2003; Schütz et al., 2004; Schütz et al., 2006; Ferracane et al., 2008; Lombardo et al., 2010; Pandino et al., 2010; Pandino et al., 2011a and b; Gouveia and Castilho, 2012; Abu-Reidah et al., 2013; Wu et al., 2013).

Four peaks in artichoke (compounds 4, 9, 11 and 13) showed the same pseudomolecular ion ($[M\text{-}H]^-$) at *m/z* 337. They were assigned as the 3-acyl, 4-acyl and 5-acyl isomers of *p*-coumaroylquinic acid based on their HPLC retention and MS^2 fragmentation characteristics, as previously reported by Clifford et al. (2003; 2006). Thus, compound 4 (artichoke) was tentatively identified as 3-*p*-coumaroylquinic acid, yielding the base peak at *m/z* 163 ($[\text{coumaric acid-H}]^-$). Fragmentation of compound 9 with a majority MS^2 product ion at *m/z* 173 was coherent with 4-*p*-coumaroylquinic acid, whereas compound 13 (artichoke and milk thistle), yielding the base peak at *m/z* 191, was identified as *trans* 5-*p*-coumaroylquinic acid. This latter was also found in the analyzed milk thistle extracts. Compound 11 (artichoke) with a UV spectrum and MS^2 fragmentation pattern identical to that of compound 13 was tentatively assigned as the

cis isomer of 5-*p*-coumaroylquinic acid. This tentative assignment was supported by the observation that hydroxycinnamoyl *cis* derivatives are expected to elute before the corresponding *trans* ones, as previously observed in our laboratory after submitting hydroxycinnamic derivatives to UV irradiation (366 nm, 24 h), which induces the formation of the *cis* forms from the more usual *trans* isomers (Barros et al., 2012). Furthermore, compound 19 in milk thistle with a molecular weight 162+162 mu (two hexosyl moieties) higher than compound 13 was tentatively identified as 5-*p*-coumarolyquinic acid dihexoside. As far as we are aware, but for 3-*p*-coumaroylquinic acid identified in artichoke hearts by Abu-Reidah et al. (2013), any of these *p*-coumaroylquinic acid derivatives has been previously reported neither in artichoke nor in milk thistle.

Compound 14 in milk thistle was identified as 5-*O*-feruloylquinic acid taking into account its pseudomolecular ion ($[M-H]^-$ at *m/z* 367) and MS^2 fragmentation pattern similar to that of 5-*O*-caffeoylquinic acid. This compound was previously identified in artichoke samples (Abu-Reidah et al. 2013; Farag et al., 2013), but, as far as we know, it has not been reported in milk thistle.

Compound 5 present in milk thistle and artichoke showed a pseudomolecular ion $[M-H]^-$ at *m/z* 341, releasing an MS^2 fragment at *m/z* 179 ($[caffeic\ acid-H]^-$) from the loss of a hexosyl moiety (-162 mu) and was tentatively assigned as caffeic acid hexoside. A similar compound was identified in hydroalcoholic extracts of artichoke hearts by Abu-Reidah et al. (2013).

Finally, compound 2 (artichoke) with the same UV and mass characteristics as compound 3 (protocatechuic acid, i.e. 3,4-dihydroxybenzoic acid) was just tentatively assigned as a dihydroxybenzoic acid. Protocatechuic acid was previously reported in hydroalcoholic extracts of artichoke wastes (bracts, receptacles and steams from the fruit) by Sánchez-Rabaneda et al. (2003).

Flavonoids. Compounds 16, 21 and 29 in artichoke and compound 20 in both samples, were identified as luteolin derivatives according to their UV and mass spectra characteristics (Tables 28 and 29). Compounds 21 and 29 were positively identified as luteolin-7-*O*-glucoside (cynaroside) and luteolin, respectively by comparison with commercial standards, being also largely identified in artichoke hearts (Abu-Reidah et

al., 2013), leaves and heads (Wang et al., 2003; Schütz et al., 2004; Lombardo et al., 2010; Pandino et al., 2010; Pandino et al., 2011a and b; Gouveia and Castilho, 2012; Farag et al., 2013), juices and pomace (Schütz et al., 2004; Gouveia and Castilho, 2012), and dietary supplements (Schütz et al., 2004; Gouveia and Castilho, 2012; Farag et al., 2013).

Table 29. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in hydromethanolic extract and infusion of artichoke.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*) (% base peak)	Tentative identification	Quantification (mg/g)	
						Hydromethanolic	Infusion
1	5.18	326	353	191(100),179(63),135(25)	3-*O*-Caffeoylquinic acid	0.103±0.002	nd
2	5.58	262sh294	153	109(100)	Dihydroxybenzoic acid	nd	0.85 ± 0.02
3	6.18	262sh296	153	109(100)	Protocatechuic acid	0.25±0.01	0.23 ± 0.01
4	6.84	310	337	191(13),173(6),163(96), 155(6),119(33)	3-*p*-Coumaroylquinic acid	0.072±0.002	nd
5	7.37	328	341	179(100),135(89)	Caffeic acid hexoside	0.134±0.004	0.066 ± 0.002
6	7.92	326	353	191(100),179(2),161(2), 135(3)	5-*O*-Caffeoylquinic acid	0.49±0.01	nd
7	8.44	286/333	191	175(100),148(33),103(6)	Quinic acid	0.21±0.01	0.078 ± 0.004
8	10.66	324	179	135(100)	Caffeic acid	nd	0.51 ± 0.01
9	10.67	306	337	191(5),173(100),163(18), 155(5),119(10)	4-*p*-Coumaroylquinic acid	0.124±0.003	nd
10	11.21	324	515	353(95),191(100),179(65), 135(40)	1,3-Dicaffeoylquinic acid	0.37±0.02	0.90 ± 0.02
11	12.95	312	337	191(100),173(6),163(10), 119(4)	*cis* 5-*p*-Coumaroylquinic acid	0.33±0.02	nd
12	13.19	356	639	477(80),315(51)	Methylquercetin-*O*-hexoside-*O*-hexoside	nd	0.14 ± 0.01

13	13.90	306	337	191(100),173(3),163(4), 119(2)	*trans* 5-*p*-Coumaroylquinic acid	0.029±0.002	nd
15	16.81	310	163	119(100)	*p*-Coumaric acid	nd	0.401±0.002
16	17.02	350	623	461(7),285(100)	Luteolin-*O*-hexoside-*O*-glucuronide	0.26±0.01	0.46±0.01
17	19.26	350	477	301(100)	Quercetin-3-*O*-glucuronide	0.062±0.002	0.094±0.004
18	20.26	340	607	269(100)	Apigenin-4-*O*-hexoside-7-*O*-glucuronide	0.117±0.005	0.31±0.02
20	20.70	344	461	285(100)	Luteolin-7-*O*-glucuronide	0.70±0.02	5.64±0.28
21	21.07	348	447	285(100)	Luteolin-7-*O*-glucoside	0.49±0.01	2.88±0.05
22	22.95	330	515	353(68),191(100),179(20), 173(5),161(10),135(7)	3,5-*O*-Dicaffeoylquinic acid	nd	0.359±0.002
24	24.01	338	577	269(100)	Apigenin-7-*O*-rutinoside	0.09±0.02	0.16±0.02
25	24.38	352	623	315(16),300(56)	Methylquercetin-*O*-rutinoside	0.079±0.001	0.074±0.001
26	25.51	336	445	269(100)	Apigenin-7-*O*-glucuronide	0.201±0.001	1.24±0.12
27	25.67	338	431	269(100)	Apigenin-7-*O*-glucoside	0.21±0.01	0.68±0.02
28	29.30	340	431	285(100)	Kaempferol-*O*-deoxyhexosyl	0.04±0.01	0.059±0.004
29	34.51	346	285	175(8),151(8),133(5)	Luteolin	nd	0.14±0.01
					Total phenolic acids	2.12±0.04[b]	3.40±0.06[a]
					Total flavonoids	2.25±0.01[b]	11.9±0.4[a]
					Total phenolic compounds	4.37±0.05[b]	15.3±0.3[a]

Table 30. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in hydromethanolic extract and infusion of milk thistle.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]- (m/z)	MS2 (m/z) (% base peak)	Tentative identification	Quantification (mg/g)	
						Hydromethanolic	Infusion
3	6.19	262sh296	153	109(100)	Protocatechuic acid	0.443±0.001	0.08±0.01
5	7.44	328	341	179(100),135(22)	Caffeic acid hexoside	0.119±0.001	0.05±0.01
6	8.11	326	353	191(100),179(4),173(7),135(5)	5-*O*-Caffeolyquinic acid	0.56±0.02	0.15±0.02
13	13.19	312	337	191(100),173(7),163(9),119(5)	5-*p*-Coumarolyquinic acid	0.120±0.002	0.03±0.01
14	15.02	328	367	193(43),191(100),173(11),134(2)	5-*O*-Feruloylquinic acid	0.05±0.01	0.05±0.01
15	17.10	306	163	119(100)	*p*-Coumaric acid	0.108±0.002	0.11±0.01
19	20.27	322	661	499(100),337(11),179(11),173(87), 163(14),119(8)	5-*p*-Coumarolyquinic acid dihexoside	0.111±0.004	0.38±0.03
20	20.77	350	461	285(100)	Luteolin-7-*O*-glucuronide	0.58±0.01	1.17±0.09
22	23.03	330	515	353(71),191(100),179(6),173(6), 135(6)	3,5-*O*-Dicaffeolyquinic acid	0.13±0.01	0.09±0.02
23	23.95	336	591	269(100)	Apigenin-*O*-deoxyhexosyl-glucuronide	0.10±0.01	0.36±0.02
26	25.48	338	445	269(100)	Apigenin-7-*O*-glucuronide	1.26±0.01	3.1±0.1
					Total phenolic acids	1.65±0.04a	0.91±0.09b
					Total flavonoids	1.94±0.01b	4.66±0.18a
					Total phenolic compounds	3.56±0.05b	5.57±0.27a

Compound 20 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 461 releasing a fragment ion at *m/z* 285 ($[M-176]^-$, loss of a glucuronyl moiety), although the position of the glycosyl moiety could not be established it was assigned to luteolin 7-*O*-glucuronide, owing to the identification of that compound in different artichoke-derived products (Sánchez-Rabaneda et al., 2003; Schütz et al., 2004; Schütz et al., 2006; Ferracane et al., 2008; Pandino et al., 2010; Gouveia and Castilho, 2012; Pandino et al., 2011a and b; Abu-Reidah et al. 2013; Farag et al., 2013). Compound 16 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 623, yielding fragment ions at *m/z* 461 (-162 mu; loss of a hexosyl residue) and 285 (-176 mu; loss of a glucuronyl residue), which allowed its assignment as luteolin-*O*-hexoside-*O*-glucuronide. Two compounds with similar characteristics were reported by Abu-Reidah et al. (2013) in artichoke hearts, also without assigning the position of substitution of the glycosyl residues.

In accordance with their UV and mass spectra characteristics, different apigenin derivatives were also detected in the analysed samples. Compounds 24, 27 (artichoke) and 26 (artichoke and milk thistle) showed pseudomolecular and fragment ions coherent with deoxyhexosyl-hexoside, hexoside and glucuronide derivatives of apigenin, respectively. The presence of apigenin-7-*O*-rutinoside, apigenin-7-*O*-glucuronide and apigenin-7-*O*-glucoside in different parts of artichoke was consistently reported by the previously mentioned authors, so that those identities could also be tentatively assumed for the compounds detected herein. Furthermore, the identity of apigenin-7-*O*-glucoside (compound 27) was here confirmed by comparison with a commercial standard.

The pseudomolecular ion of compound 18 in artichoke ($[M-H]^-$ at *m/z* 607) released a fragment ion at *m/z* 269 ($[M-162-176]^-$; apigenin), which allowed its tentative identification as an apigenin-*O*-hexoside-*O*-glucuronide. A compound with similar characteristics was identified as apigenin-4-*O*-hexoside-7-*O*-glucuronide by Abu-Reidah et al. (2013) in artichoke hearts. Another apigenin derivative (compound 23) was detected in the sample of milk thistle, whose mass characteristics ($[M-H]^-$ at *m/z* 591 releasing a fragment ion at *m/z* 269 ($[M-146-176]^-$) from the loss of deoxyhexosyl and glucuronyl moieties) pointed to an apigenin-*O*-deoxyhexosyl-glucuronide. To our knowledge, this compound was not previously described in milk thistle.

The following compounds were only present in artichoke. Compound 17 ($[M-H]^-$ at *m/z* 477) presented a UV spectrum with λ_{max} around 350 nm and an MS^2 product ion at *m/z*

301. The comparison with a standard obtained in our laboratory (Dueñas et al., 2008) allowed its identification as quercetin 3-*O*-glucuronide. Compound 12 ([M-H]$^-$ at *m/z* 639) released fragment ions at *m/z* 477 and 315, from the consecutive losses of 162 mu (two hexosyl moieties). The ion at *m/z* 315 can be attributed to a methylquercetin, whilst the high abundance of the ion at *m/z* 477 indicated that each hexosyl group was located on different position of the aglycone. Therefore, the compound was tentatively assigned as methylquercetin-*O*-hexoside-*O*-hexoside. Compound 25 ([M-H]$^-$ at *m/z* 623) released fragment ions at *m/z* 315 and 300 (further loss of a methyl group) also suggesting a methylquercetin. In this case, the loss of 308 mu (146+162 mu) to yield the aglycone suggested the existence of deoxyhexose and hexose as glycosylating substituents, probably constituting a disaccharide owing to their joint loss. Although there was not further indication about the type of sugar, it might be a rutinose, taking into account the previous identification of quercetin-3-*O*-rutinoside in hydroalcoholic extracts of artichoke samples by Sánchez-Rabaneda et al. (2003) and Abu-Reidah et al. (2013). Thus, the compound was tentatively assigned as methylquercetin *O*-rutinoside. Finally, compound 28 with a pseudomolecular ion [M-H]$^-$ at *m/z* 431 yielding a product ion at *m/z* 285 (-146 mu, loss of a dexoyhexosyl moiety) could be associated to a kaempferol-*O*-deoxyhexoside. As far as we know, none of these latter four compounds has been previously described in artichoke.

In both species, infusions presented higher phenolic contents than hydromethanolic extracts. Milk thistle presented the same composition in both types of extracts with only quantitative differences between them. Nevertheless, different phenolic profile between infusions and hydromethanolic extracts was obtained in the case of artichoke, which might be due to the heat treatment to which infusions were subjected. Apigenin-7-*O*-glucuronide was the major flavonoid found in milk thistle (Table 29), while luteolin-7-*O*-glucuronide was the most abundant in artichoke (Table 28).

In the literature, milk thistle phenolic composition is characterized by the presence of a mixture of flavonolignans (called silymarin) (Bilia et al., 2001; Wang et al., 2003; Zhao et al., 2005; Lee et al., 2006; Kéki et al., 2007; Lee et al., 2007; Cai et al., 2009; Wang et al., 2010; Brinda et al., 2012; Calani et al., 2012; Althagafy et al., 2013). These compounds are known to be normally present in seeds of milk thistle (Calani et al., 2012; Althagafy et al., 2013). Therefore, it can be supposed that the sample studied by

us did not contain seeds, but only the other parts of the plant, even though the label mentioned the whole plant material.

Despite the many articles reporting phenolic composition of artichoke hydroalcoholic extracts (Sánchez-Rabaneda et al., 2003; Wang et al., 2003; Schütz et al., 2004; Schütz et al., 2006; Ferracane et al., 2008; Lombardo et al., 2010; Pandino et al., 2010; Pandino et al., 2011a and b; Gouveia and Castilho, 2012; Abu-Reidah et al., 2013; Farag et al., 2013; Wu et al., 2013), the present work also characterizes the phenolic composition in infusions, which is a common form to consume this plant. The literature reports mainly the existence of caffeoylquinic acids, and luteolin and apigenin derivatives; however, in the present study other compounds have also been detected and tentatively identified in artichoke. Regarding milk thistle, to our knowledge, this is the first study presenting results for the whole plant material and not just seeds.

3.3.2. Phenolic compounds in artichoke and milk thistle pills and syrups and antimicrobial activity of infusions, pills and syrups

Phenolic compounds. Tables 31 and 32 present the data obtained from HPLC-DAD-MS analysis (retention time, λ_{max} in the visible region, mass spectral data) used for the identification and quantification of phenolic compounds in artichoke and milk thistle formulations.

Table 31. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in syrup and pills formulations of artichoke (mean ± SD).

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g)
				Syrup		
1[A]	14.1	338	607	269(100)	Apigenin-*O*-hexoside-*O*-glucuronide	0.29±0.01
2[A]	15.5	264,296sh	167	123(100)	Vanillic acid	5.58±0.02
3[A]	19.7	350	461	285(100)	Luteolin-7-*O*-glucoronide	0.72±0.04
4[A]	20.6	354	447	285(100)	Luteolin-7-*O*-glucoside	2.2±0.1
5[A]	25.1	336	431	269(100)	Apigenin-7-*O*-glucoside	0.49±0.02
					Total phenolic acids	5.58±0.02
					Total flavonoids	3.7±0.1
					Total phenolic compounds	9.3±0.1
				Pill		
6[A]	5.2	326	353	191(100),179(82),135(73)	3-*O*-Caffeoylquinic acid	1.854±0.005
7[A]	5.6	324	341	179(85),135(100)	Caffeic acid hexoside	2.0±0.1
8[A]	7.2	328	353	191(59), 179(64), 173(100), 161(10), 135(77)	4-*O*-Caffeoylquinic acid	13.3±0.3
9[A]	7.9	328	353	191(100), 179(32), 173(20), 161(6), 135(12)	5-*O*-Caffeoylquinic acid	28.2±0.7
10[A]	11.4	324	179	135(100)	Caffeic acid	1.7±0.2
11[A]	12.4	324	515	353(85), 335(11), 191(100), 179(90), 173(6), 161(12), 135(49)	1,3-Dicaffeoylquinic acid	24±1
1[A]	14.5	334	607	431(43), 269(43)	Apigenin-*O*-hexoside-*O*-glucuronide	1.36±0.06

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g)
13[A]	17.1	312	163	119(100)	*p*-Coumaric acid	0.041±0.009
14[A]	19.6	350	593	285(100)	Luteolin-7-*O*-rutinoside	1.37±0.07
3[A]	20.3	348	461	285(100)	Luteolin-7-*O*-glucuronide	2.59±0.08
16[A]	20.7	326	515	353(49), 335(17), 191(47), 179(58), 173(83), 161(22), 135(46)	3,4-*O*-Dicaffeoylquinic acid	6.1±0.1
17[A]	22.7	326	515	353(31), 335(10), 191(100), 179(20), 161(8), 135(5)	3,5-*O*-Dicaffeoylquinic acid	7.1±0.1
5[A]	25.0	332	445	269(100)	Apigenin-7-*O*-glucuronide	3.07±0.05
					Total phenolic acids	85±3
					Total flavonoids	8.4±0.3
					Total phenolic compounds	93±2

Table 32. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in syrup and pill formulations of milk thistle (mean ± SD).

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g)
				Syrup		
1^{MT}	8.0	326	353	191(100), 179(20), 173(10), 135(8)	5-*O*-Caffeolyquinic acid	1.90±0.02
2^{MT}	11.4	350	771	625(50), 301(33)	Quercetin-*O*-deoxyhexoside-*O*-dihexoside	0.73±0.02
3^{MT}	12.5	352	785	623(100), 315(33)	Isorhamnetin-*O*-hexoside-*O*-rutinoside	0.60±0.02
4^{MT}	12.8	356	639	477(58), 315(57)	Isorhamnetin-*O*-hexoside-*O*-hexoside	1.13±0.07
5^{MT}	14.0	350	755	609(100), 285(36)	Kaempferol-*O*-deoxyhexoside-*O*-dihexoside	1.55±0.02
6^{MT}	14.6	356	785	639(100), 315(26)	Isorhamnetin-*O*-deoxyhexoside-*O*-dihexoside	3.64±0.04
7^{MT}	15.7	358	755	301(100)	Quercetin-*O*-deoxyhexoside-rutinoside	0.33±0.02
8^{MT}	16.5	358	609	463(13), 447(33), 301(13)	Quercetin-3-*O*-rutinoside	0.33±0.03
9^{MT}	17.3	310	163	119(100)	*p*-Coumaric acid	0.39±0.03
10^{MT}	19.4	358	769	623(72), 461(16), 315(28)	Isorhamnetin-*O*-deoxyhexoside-*O*-rutinoside	2.25±0.07
11^{MT}	19.9	354	623	477(50), 461(37), 315(75)	Isorhamnetin-*O*-deoxyhexoside-*O*-hexoside	7.26±0.04
12^{MT}	22.0	356	593	461(33), 315(33)	Isorhamnetin-*O*-pentosyl-*O*-deoxyhexoside	0.26±0.02
13^{MT}	23.1	354	623	315(100)	Isorhamnetin-*O*-deoxyhexoside-hexoside	0.69±0.03
14^{MT}	23.7	356	623	315(100)	Isorhamnetin-3-*O*-rutinoside	5.75±0.04
15^{MT}	24.9	356	477	315(100)	Isorhamnetin-*O*-hexoside	0.59±0.02
16^{MT}	25.2	354	477	315(100)	Isorhamnetin-3-*O*-glucoside	1.21±0.02
					Total phenolic acids	2.30±0.01
					Total flavonoids	26.3±0.4

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g)
					Total phenolic compounds	28.6±0.4
				Pill		
17^{MT}	5.3	326	353	191(100), 179(60), 173(5), 135(80)	3-*O*-Caffeolyquinic acid	0.020±0.001
1^{MT}	8.0	328	353	191(100), 179(60), 173(35), 135(26)	5-*O*-Caffeolyquinic acid	0.0314±0.0001
18^{MT}	12.4	328	515	353(44), 191(100), 179(56), 173(5), 161(33), 135(56)	1,3-*O*-Caffeolyquinic acid	0.049±0.002
19^{MT}	19.3	290,336sh	303	285(97), 259(15), 241(15), 217(12), 199(21), 177(38), 150(15), 125(100)	Taxifolin	0.284±0.007
20^{MT}	25.2	286	481	463(10), 453(10), 337(5), 325(5), 301(8), 299(10), 283(5), 179(13), 151(23), 125(10)	Silymarin derivative	0.131±0.005
21^{MT}	28.8	288	481	463(10), 453(14), 337(10), 325(10), 179(43), 151(19), 125(48)	Silymarin derivative	0.27±0.02
22^{MT}	29.2	278	481	463(5), 453(20), 301(8), 299(3), 283(3), 179(38), 151(30), 125(24)	Silymarin derivative	0.33±0.01
23^{MT}	29.6	290	481	463(7), 453(12), 325(3), 301(7), 179(24), 169(17), 153(17), 151(19), 125(17)	Silymarin derivative	0.12±0.02
24^{MT}	30.6	284,336sh	497	453(86), 435(57), 317(71), 181(57), 151(28), 125(29)	Hydroxylated silibinin	1.565±0.007
					Total phenolic acids	0.1003±0.0005
					Total flavonoids	2.70±0.06
					Total phenolic compounds	2.80±0.06

The phenolic compounds identified in artichoke formulations obviously presented similarities with the infusions and hydroalcoholic extracts previously analyzed (section 3.3.1), being all the detected compounds already described and tentatively identified, with the exception of peaks 2^{A}, 8^{A} and 16^{A}. Compound 2^{A} was positively identified as vanillic acid, according to its retention time, mass and UV-vis characteristics by comparison with a commercial standard. To the best of our knowledge, vanillic acid has not been described in artichoke, thus, it might be present in the syrup due to its addition as a flavouring agent, even though nothing was mentioned in the label. Compounds 8^{A} and 16^{A} were tentatively identified as 4-*O*-caffeoylquinic acid and 3,4-*O*-dicaffeoylquinic acid, respectively, taking into account the fragmentation patterns reported by Clifford et al. (2003) and (2005) and previous identifications in other materials in our laboratory (Dias et al., 2013; Guimaraes et al., 2013; Souza et al., 2015).

Different monocaffeoylquinic and dicaffeoylquinic acids have been largely reported by many authors in different parts of artichoke (Sánchez-Rabaneda et al., 2003; Schütz et al., 2004; Ferracane et al., 2008; Lombardo et al., 2010; Pandino et al., 2011a; Pandino et al., 2011b; Gouveia and Castilho, 2012; Abu-Reidah et al., 2013; Farag et al., 2013; Wu et al., 2013). The syrup did not present this type of compounds, but showed apigenin and luteolin derivatives also very characterictic of artichoke (with the exception of vanillic acid), being the most abundant compound luteolin-7-*O*-glucoside. The main compounds present in the pills were chlorogenic acid (i.e., 5-*O*-caffeoylquinic acid) and cynarin (i.e, 1,3-*O*-dicaffeoylquinic acid); these molecules are also the most characteristic compounds found in artichoke.

On the contrary of artichoke, both formulations of milk thistle hardly presented similarities to the infusions and hydroalcoholic extract previously described, with the exception of compounds 1^{MT} (5-*O*-caffeolyquinic acid) and 17^{MT} (3-*O*-caffeolyquinic acid), that were previously described and identified (section 3.3.1). In literature, milk thistle phenolic composition is characterized by the presence of a mixture of flavonolignans (silymarin), which are known to be normally present in its seeds (Bilia et al., 2001, 2002; Zhao et al., 2005; Kéki et al., 2007; Lee et al., 2006 and 2007; Cai et al., 2009; Wang et al., 2010; Brinda et al., 2012; Calani et al., 2012; Althagafy et al., 2013). Nevertheless, the syrup formulation presented flavonol derivatives, mainly isorhamnetin, quercetin and kaempferol glycoside derivatives, as the main compounds,

with the exception of compound 9^{MT}, that was positively identified as *p*-coumaric acid, according to its retention, mass and UV-vis characteristics by comparison with commercial standards, and compound 1^{MT} (5-*O*-caffeolyquinic acid).

Compounds 3^{MT},4^{MT},6^{MT},10^{MT}, 11^{MT}, 12^{MT}, 13^{MT},14^{MT},15^{MT} and 16^{MT} were identified as isorhamnetin derivatives owing to the product ion observed at *m/z* 315 and UV spectra (λ_{max} around 352-358 nm). Compounds $14^{\mathbf{MT}}$ (isorhamnetin-3-*O*-rutinoside) and $16^{\mathbf{MT}}$ (isorhamnetin-3-*O*-glucoside) were positively identified by comparison with commercial standards. Peaks 3^{MT} and 6^{MT} presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 785, but revealed a different MS^2 fragmentation pattern. Compound 3^{MT} presented the consecutive losses of hexosyl (product ion at *m/z* 623; -162 u) and deoxyhexosyl-hexoside (ion at *m/z* 315; -308 u), while peak 6^{MT} presented the losses of deoxyhexosyl (*m/z* at 639; -146 u) and dihexosyl (*m/z* at 315; -324 u). For both peaks, no information about the identity of the sugar moieties and location in the aglycone could be obtained, so, these compounds were tentatively identified as isorhamnetin-*O*-hexoside-*O*-(deoxyhexosyl-hexoside) and isorhamnetin-*O*-deoxyhexoside-*O*-dihexoside, respectively. Moreover, the positive identification of different rutinosides, including isorhamnetin-3-*O*-rutinoside, in the analyzed sample may suggest a rutinose identity for the deoxyhexosyl-hexose residue present in peak 3^{MT}. Similarly, peak 10^{MT} ($[M-H]^-$ at *m/z* 769) was assigned as isorhamnetin-*O*-deoxyhexoside-*O*-rutinoside. Mass characteristics of compound 4^{MT} ($[M-H]^-$ at *m/z* 639) indicated that it corresponds to an isorhamnetin derivative bearing two hexosyl residues. The observation of MS^2 fragments at *m/z* 477 (-162 u) and 315 (-162 u), also indicated the consecutive loss of each of the hexosyl moieties, pointing to their location on different positions of the aglycone. Thus, this compound was tentatively identified as isorhamnetin-*O*-hexosyl-*O*-hexoside. Moreover, peaks 11^{MT} and 13^{MT} presented the same pseudomolecular ion as compound 14^{MT} (isorhamnetin-3-*O*-rutinoside), indicating that they should correspond to other isorhamnetin derivatives bearing deoxyhexosyl and hexosyl residues. Nevertheless, the fragmentation pattern of compound 11^{MT}, with the alternative losses of the deoxyhexosyl (ion at *m/z* 477) and hexosyl (ion at *m/z* 461) moieties indicated that the sugars were located on different positions of the aglycone, whereas in compound 13^{MT}, for which only one ion at *m/z* 315 corresponding to the aglycone was produced, the two sugars residues should be constituting a disaccharide. Thus, these compounds were tentatively assigned as isorhamnetin-*O*-deoxyhexosyl-*O*-

hexoside (peak 11^{MT}) and isorhamnetin-*O*-deoxyhexosyl-hexoside (peak 13^{MT}). This later could correspond to an isorhamnetin *O*-neohesperidoside or to an *O*-rutinoside bearing the sugar residue on a location different to peak 14^{MT} (isorhamnetin-3-*O*-rutinoside). Following a similar reasoning, peak 12^{MT} ($[M-H]^-$ at *m/z* 593) was assigned as isorhamnetin-*O*-pentosyl-*O*-deoxyhexoside, and peak 15^{MT} ($[M-H]^-$ at *m/z* 477) as a isorhamnetin-*O*-hexoside differing from peak 15^{MT} (isorhamnetin-3-*O*-glucoside) in the type of sugar or position of substitution.

Peaks 2^{MT}, 7^{MT} and 8^{MT} were identified as quercetin glycosides based on their UV spectra (λ_{max} around 358 nm) and the production of an MS^2 fragment ion at *m/z* 301. Similarly, peak 5^{MT} was identified as a kaempferol glycoside (λ_{max} around 348 nm, MS^2 fragment at *m/z* 285). Tentative identities of these compounds were assigned based on their pseudomolecular ions using a similar reasoning as for isorhamnetin derivatives. Thus, peaks 2^{MT} ($[M-H]^-$ at *m/z* 771) and 5^{MT} ($[M-H]^-$ at *m/z* 639) could correspond to quercetin-*O*-deoxyhexoside-*O*-dihexoside and kaempferol-*O*-deoxyhexoside-*O*-dihexoside, respectively, whereas peak $\mathbf{7}^{MT}$ ($[M-H]^-$ at *m/z* 755) was tentatively assigned as quercetin-*O*-hexoside-*O*-rutinoside. Compound $8^{\mathbf{MT}}$ was positively identified as quercetin-3-*O*-rutinoside according to its retention time, mass and UV-vis characteristics by comparison with a commercial standard.

Pills of milk thistle revealed the presence of five (peaks 20^{MT}, 21^{MT}, 22^{MT}, 23^{MT} and 24^{MT}) flavonolignans (silymarin), assigned based on their UV spectra (λ_{max} around 286-290 nm) and the observation of the ion at *m/z* 481 and a very characteristic fragmentation pattern observed in many studies (Bilia et al., 2001, 2002; Zhao et al., 2005; Kéki et al., 2007; Lee et al., 2006 and 2007; Cai et al., 2009; Wang et al., 2010; Brinda et al., 2012; Calani et al., 2012; Althagafy et al., 2013). Nevertheless, and due to the lack of commercial standards and difficult to interpret fragmentation, the full identification of these compounds was not possible, being just named as silymarin derivatives. These compounds were quantified using a taxifolin calibration curve. Compound 24^{MT} was identified as a hydroxylated silibinin taking into account its pseudomolecular ion, 16 u higher than peaks 20^{MT}-23^{MT}, and the findings of Venisetty et al. (2011). Finally, peak 18^{MT} (1,3-*O*-caffeolyquinic acid) was identified taking into account the retention time and fragmentation pattern observed for compound 11^{A}, and peak 19^{MT} was positively identified as taxifolin according to its retention, mass and UV-vis characteristics by comparison with a commercial standard.

Isorhamnetin-*O*-deoxyhexoside-*O*-hexoside and isorhamnetin-*O*-rutinoside were the main phenolic compounds in milk thistle syrup, while hydroxyl silybin was the most prominent compound in the pills.

Antimicrobial activity. The screening of antimicrobial activity of artichoke and milk thistle was performed in the three different formulations (infusions, pills, and syrups) and the results are presented in Table 33.

Table 33. Antimicrobial activity of infusions, pills and syrups of artichoke and milk thistle against bacteria clinical isolates (MIC values, mg/mL).

	Artichoke			Milk thistle		
Bacteria	Infusion	Pills	Syrup	Infusion	Pills	Syrup
Escherichia coli	125	>15	>100	125	>15	0.3
Escherichia coli ESBL	125	>15	>100	31.3	15	0.2
Proteus mirabilis	>1000	>15	>100	>1000	>15	>2.6
MRSA	31.3	1.9	>100	31.3	>15	0.2
Pseudomonas aeruginosa	500	>15	>100	500	>15	1.3

MIC - Minimum inhibitory concentration; ESBL - spectrum extended producer of *β*-lactamases; MRSA - methicillin-resistant *Staphylococcus aureus*.

Among the artichoke-based samples, the infusion revealed the best results, showing the capacity to inhibit *E. coli*, *E. coli* ESBL, *S. aureus* MRSA, and *P. aeruginosa*, with MIC values of 125, 125, 31.3, and 500 mg/mL, respectively. The pills only revealed activity in *S. aureus* MRSA, although at lower concentrations than the infusion (MIC value 1.9 mg/mL). In contrast with the results obtained by Alves et al. (2013), where vanilic acid inhibited the growth of *E. coli* and *P. mirabilis*, among others, the syrup of artichoke, presenting the referred acid, did not inhibit the bacterial growth of the studied cultures, which might be due to its low concentration in the sample.

Regarding milk thistle, the syrup presented the highest antimicrobial activity, with lower MIC values, and proved to be able to inhibit the growth of *E. coli*, *E. coli* ESBL, *S. aureus* MRSA and *P. aeruginosa*, with MIC values ranging from 0.2 to 1.3 mg/mL. The infusion revealed capacity to also inhibit these bacteria, but in significantly higher concentrations than the syrup (31.3-500 mg/mL). The pills showed antimicrobial activity for *E. coli* ESBL at lower concentration (MIC: 15 mg/mL) than the infusion, but did not inhibit the growth of the remaining bacteria. Among the studied

formulations of these plants, none of them was able to inhibit *P. mirabilis* at the studied concentrations.

As far as we know, there are no reports on the antimicrobial activity of infusions, pills or syrups containing artichoke or milk thistle, although there are studies performed with hydroalcoholic extracts of artichoke, which exhibited antimicrobial activity against *E. coli* and *Salmonella abony enterica* (Ionescu et al., 2013). Moreover, Zhu et al. (2004) performed a study using several fractions obtained from artichoke leaves, showing that at least six kinds of bacteria were sensitive to these extracts, including *B. subtilis*, *S. aureus*, *Agrobacterium tumefaciens*, *Micrococcus luteus*, *E. coli*, and *S. typhimurium*. On the other hand, ethanolic extracts from milk thistle seeds revealed antimicrobial activity in several clinical bacterial isolates, such as *E. coli*, *S. aureus*, *Staphylococcus saprophyticus*, and *Klebsiella pneumoniae* when mixed with agar media (Abed et al., 2015). Furthermore, several compounds isolated from artichoke also showed antimicrobial activity against microbial strains, including some of the bacteria used in the present study, namely *E. coli*, *S. aureus*, and *P. aeruginosa* (Zhu et al., 2004). One of the compounds isolated and tested by Zhu et al. (2004) that revealed antimicrobial activity was 3,5-*O*-dicaffeoylquinic acid, found in the infusion and pills of artichoke studied herein, which could explain the high activity of the infusion, once the concentration of the referred compound was significantly higher in this formulation. The same observation can be made for luteolin and apigenin derivatives that were present in all of these plant formulations, but in higher amounts in the infusion. Protocatechuic (detected in artichoke and milk thistle infusions) and *p*-coumaric acids (detected in all artichoke formulations and milk thistle infusion and syrup) were tested by Lou et al. (2012) and revealed activity against *E. coli*, *S. aureus*, *S. typhimurium*, and *Shigella dysenteriae*; nonetheless, in the present study not all of the formulations containing these molecules presented antimicrobial activity. On the other hand, Cetin-Karaca and Newman (2015) reported the antimicrobial capacity of 5-*O*-caffeoylquinic acid and quercetin-3-*O*-rutinoside against *E. coli* and several other microbial cultures, which might justify the activity of the syrup and the infusion of milk thistle that presented, respectively, the first and both these molecules. The antimicrobial capacity of the milk thistle pills could also be related to the presence of silibinin and silymarin, which revealed this kind of activity in a study performed with *E. coli*, *S. aureus*, and *P. aeruginosa* (de Oliveira et al., 2015).

3.3.3. Phenolic compounds and antimicrobial activity of borututu infusions, pills and syrups

Phenolic compounds. Table 34 presents the data obtained from HPLC-DAD-MS analysis (retention time, λ_{max} in the visible region, mass spectral data) used for the identification and quantification of phenolic compounds in borututu formulations.

Table 34. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in infusion extracts, pills, and syrup of borututu.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (µg/g)		
						Infusion	Pills	Syrup
1[B]	5.8	276	483	331(41), 313(25), 169(30)	Digalloyl glucoside	117±6	27±2	nd
2[B]	6.0	259, 296sh	153	109(100)	Protocatechuic acid	1782±40	nd	nd
3[B]	7.8	280	453	313(11), 169(8)	Hydroxymethoxyphenyl-galloyl-hexoside	245±4	76±1	nd
4[B]	15.4	282	497	313(31), 169(25)	Eucaglobulin/globulusin B	492±51	430±5	381±11
5[B]	17.6	270	457	305(5), 169(10)	(Epi)gallocatechin-*O*-gallate	1040±8	777±15	155±15
6[B]	18.7	254, 362sh	477	315(64), 300(20)	Methyl ellagic acid hexoside	172±8	72±1	tr
7[B]	19.2	248, 362sh	433	301(100)	Ellagic acid pentoside	50±8	nd	nd
8[B]	20.7	246, 364sh	301	284(10), 245(3), 185(4), 173(5), 157(3), 145(6)	Ellagic acid	216±17	tr	tr
9[B]	21.8	250, 362sh	447	315(43), 300(12)	Methyl ellagic acid pentoside	159±1	55±1	tr
10[B]	22.3	284, 340sh	433	271(100)	Naringenin-*O*-hexoside	nd	653±32	nd
11[B]	22.9	282, 338sh	609	301(100)	Hesperitin-*O*-rutinoside/ hesperetin-*O*-neohesperidoside	nd	111±7	nd
12[B]	24.7	248, 366sh	447	315(54), 300(15)	Methyl ellagic acid pentoside	343±20	181±3	55±4
13[B]	26.9	250, 360sh	461	315(95), 300(41)	Methyl ellagic acid deoxyhexoside	111±7	238±2	tr
					Total phenolic compounds	4726±7[a]	2618±58[b]	590±30[c]

Compounds 2^B and 8^B were positively identified as protocatechuic acid and ellagic acid, respectively, according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Compound 1^B showed a fragmentation pattern characteristic of a digalloyl hexoside, with a precursor ion at *m/z* 483 and product ions at *m/z* 313 $[M-H-170]^-$, 331 $[M-H-162]^-$ and 169 $[M-H-162-152]^-$ from the losses of gallic acid and hexosyl and galloyl moieties, respectively. Compound 3^B presented a pseudomolecular ion $[M-H]^-$ at *m/z* 453 releasing MS^2 fragment ions at *m/z* 169 and 313 associated to a galloyl moiety and a galloylglucose group, respectively. A compound with similar characteristics was reported in *Rhus coriaria* by Abu-Reidah et al. (2015) and identified as hydroxymethoxyphenyl-galloyl-hexoside, so that this identity was tentatively assigned to our peak. Compound 4^B ($[M-H]^-$ at *m/z* 497) also yielded fragment ions at *m/z* 169 and 313, this latter from the loss of 184 mu indicated as characteristic of oleuropeic acid (Hasegawa et al., 2008); these mass features are coherent with the structure of eucaglobulin or globulusin B (Figure 23), previously reported in the leaves of *Eucalyptus globulus* (Hasegawa et al., 2008; Boulekbache-Makhlouf et al., 2013).

Compound 5^B was assigned as (epi)gallocatechin-*O*-gallate owing to its pseudomolecular ion ($[M-H]^-$ at *m/z* 457) and fragment ions at *m/z* 305 and 169, corresponding to the deprotonated ions of (epi)gallocatechin and gallic acid, respectively.

Compounds 6^B, 9^B, 12^B and 13^B presented similar UV-vis and mass spectra characteristic of ellagic acid derivatives All of them produced a major MS^2 fragment ion at *m/z* 315, from the loss of hexosyl (-162 u, compound 6), pentosyl (-132 u, 9 and 12) or deoxyhexosyl moieties (-146 u, 13), which can be interpreted as corresponding to deprotonated methyl ellagic acid; the second product ion at *m/z* 300 would derive from the further loss of the methyl group (-15 u). Compound 7, with a molecular mass 14 u lower than 9 and 12 and a fragment ion at *m/z* 301 (-132 u, loss of a pentosyl moiety; ellagic acid) was assigned as ellagic acid pentoside. All these compounds, together with ellagic acid, have been previously reported as majority phenolics in hydromethanolic extracts obtained from the bark of borututu (Ferreres et al., 2013).

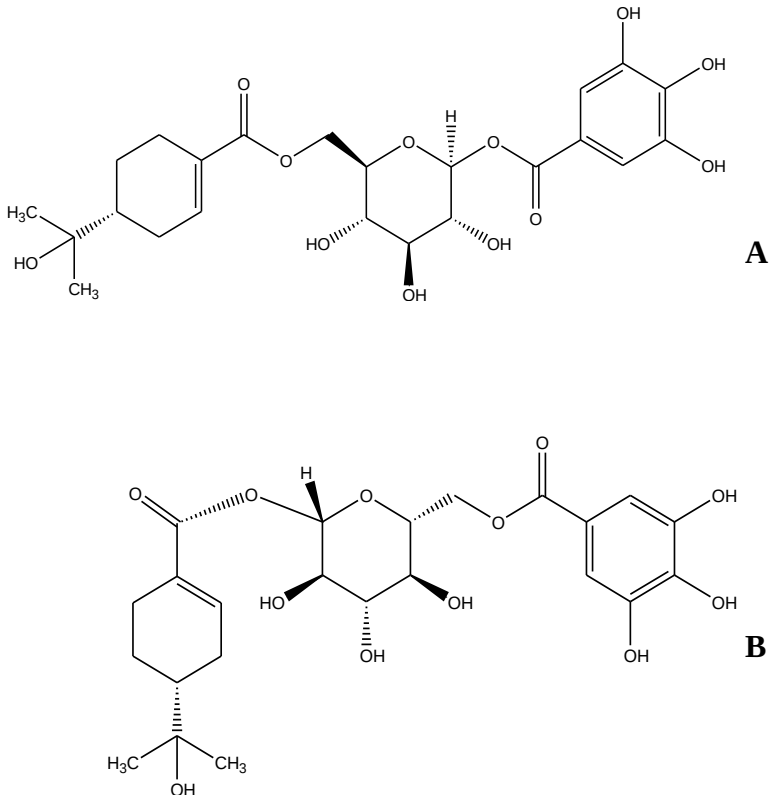


Figure 23. Chemical structures of eucaglobulin (A) and globulusin B (B).

Compounds 10^B and 11^B were assigned as flavanone glycosides based on their UV-vis and mass spectra. Compound 10^B ($[M-H]^-$ at *m/z* 433 and MS^2 fragment ion $[M-H-162]^-$ at *m/z* 271) was tentatively identified as a naringenin-*O*-hexoside, whereas compound 11 ($[M-H]^-$ at *m/z* 609 and MS^2 fragment at *m/z* 301 $[M-H-308]^-$) could be associated to a hesperetin-*O*-rutinoside or hesperetin-*O*-neohesperidoside. As far as we are aware, these compounds have not been previously reported in borututu.

Furthermore, a compound with a pseudomolecular ion $[M-H]^-$ at *m/z* 211 was present in the syrup that corresponded to propyl gallate added as a preservative, as described in the label, which also explained its high levels (data not shown).

Ellagic acids, methyl ellagic acids, eucaglobulin/globulusin B and (epi)gallocatechin-*O*-gallate were the common compounds present in all the different formulations. The highest concentration of phenolic compounds was found in the infusion extract (Table 34). Protocatechuic acid was the most abundant phenolic compound in the infusions,

the only preparation where it was detected, whereas (epi)gallocatechin-*O*-gallate was the main phenolic in the pills and eucaglobulin/globulusin in the syrup.

Antimicrobial activity. The results obtained in the screening of antimicrobial activity of the different formulations of borututu against the studied bacteria are shown in Table 35. The infusion extract revealed the highest antimicrobial activity, with lower MIC values and proved to be able to inhibit the growth of *E. coli*, *E. coli* ESBL, *S. aureus* and *P. aeruginosa*, with MIC values of 50, 6.2, 1.6 and 25 mg/mL, respectively. The effectiveness of this formulation against *E. coli* and *S. aureus* could be due to the presence of protocatechuic acid that revealed antimicrobial activity against *E. coli* (*β*-lactamases positive) in a previous study, when isolated from *Ficus ovata* (Kuete et al., 2009). Pills revealed activity against *E. coli* ESBL and *S. aureus* MRSA in concentrations of 15 and 1.9 mg/mL, respectively. Lastly, the syrup did not reveal antimicrobial activity at the studied concentration (10 mg/mL). None of the tested formulations inhibited *P. mirabilis*. As far as we know, there are no reports on the antimicrobial activity of pills or syrups containing borututu.

Table 35. Antimicrobial activity of infusion, pills and syrup of borututu against bacteria clinical isolates (MIC values, mg/mL).

Bacteria	Infusion	Pills	Syrup
Escherichia coli	50	>15	>10
Escherichia coli ESBL	6.2	15	>10
Proteus mirabilis	>100	>15	>10
MRSA	1.6	1.9	>10
Pseudomonas aeruginosa	25	>15	>10

MIC - Minimum inhibitory concentration; ESBL - spectrum extended producer of *β*-lactamases; MRSA - methicillin-resistant *Staphylococcus aureus*.

Conclusions and Future Perspectives

The present study aimed to stregthen the knowledge of three medicinal plants widely used by their hepatoprotective properties: artichoke, milk thistle, and borututu. For that purpose, they were chemically characterized, and the bioactivity of different formulations available on the market (infusions, pills, and syrups) was evaluated.

1) Regarding the chemical characterization, borututu gave the highest content of carbohydrates and fat, sucrose and total sugars, shikimic and citric acids, α-, *β*-, δ- and total tocopherols. Artichoke had the highest ash and protein contents, oxalic acid, saturated fatty acids (SFA), mainly palmitic acid acid, and γ-tocopherol, as also the best n6/n3 fatty acids ratio. Milk thistle showed the highest levels of fructose and glucose, quinic acid and total organic acids, polyunsaturated fatty acids (PUFA), mainly linoleic acid, and the best PUFA/SFA ratio. The bioactivity of lipophilic compounds, namely unsaturated fatty acids and tocopherols, is lost in the infusions, but it can be still significant in pills and syrups containing the plants that can be marketed as dietary supplements. As far as we know this is the first report on detailed composition of molecules with nutritional features in these plant materials.

2) From the study related to the application of an alternative method for dry borututu preservation (gamma irradiation), it was possible to conclude that, in general, this treatment did not appreciably affect the proximate composition of this plant material, actually the highest contents of total sugars, organic acids, tocopherols, and PUFA were found in the sample irradiated at 10 kGy. Furthermore, this sample presented also the highest levels of total phenolics and flavonoids and, in general, the highest antioxidant activity (either in infusions or methanolic extracts prepared from the irradiated material). Irradiated samples also kept the anti-hepatocellular carcinoma (HepG2 cell line) activity, but a decrease was observed in the methanolic extract prepared from the sample irradiated at 10 kGy. All in all, gamma irradiation at the assayed doses proved to be a suitable technique for preservation of dried herbs without affecting the bioactive compounds.

3) In order to study the bioactivity of different formulations obtained from a single plant, infusions, pills and syrups prepared from each of the three plants were evaluated. All the samples revealed good antioxidant properties, but infusions and syrups showed higher antioxidant activity than pills. Despite artichoke presented the best results in antitumor activity, its infusion also revealed some toxicity for normal cells in similar concentrations. Borututu infusion and milk thistle syrup gave the best results in the

antioxidant activity with similar EC_{50} values, but regarding the capacity to inhibit the proliferation of HepG2 cell line, the infusion showed best results than the syrup. Thus, to achieve the mentioned benefits of these plants, it seems unnecessary to acquire expensive syrups instead of the cheaper infusions, unless there are other benefits associated to the syrups besides the herein studied.

4) Different formulations containing mixtures in different proportions of the three considered plant materials were also evaluated. With no exception, the mixtures for all formulations gave synergistic effects for the antioxidant activity, and in some cases also regarding anti-hepatocellular carcinoma activity, when compared to the formulations based on single plants. Moreover, none of the samples showed toxicity for liver primary cells. The obtained results should represent a real asset in the choice of the best formulation and mixing proportions to be used in the preparation of non-toxic products derived from artichoke, borututu and milk thistle.

5) The effects of the addition of chestnut honey to the infusions were further tested, once this supersaturated sugar solution is frequently added to infusions to enhance their flavor. The results obtained proved the utility of honey addition to potentiate the antioxidant and cytoprotective properties of medicinal plant based infusions. The increase in the antioxidant activity was verified independently of using one, two, or three plants based infusions, potentiating their effects in all cases (except for β-carotene bleaching inhibition in the preparation containing artichoke, milk thistle, and honey). The enhanced antioxidant activity coupled to the lower hepatotoxicity showed by formulations containing honey might be helpful to define the most suitable practice in terms of infusion preparation. Actually, the observations made also suggested that the enhancing effect in the assayed bioactive properties induced by incoporating honey to the infusions could even overcome the potentiation/synergistic effect obtained from mixing the plants based infusions.

6) In what concerns the phenolic composition of the studied plants, artichoke, milk thistle and borututu proved to represent a good source of bioactive compounds, especially phenolic acids and flavonoids. Regarding artichoke and milk thistle, phenolic compounds are higher in the infusion preparations when compared to the hydromethanolic extracts. Comparing the different formulations of the three plants, the following tendencies were observed concerning the concentrations of phenolic compounds and antioxidant capacity: infusion>pills>syrup for artichoke and borututu,

and infusion>syrup>pills for milk thistle. Luteolin-7-*O*-glucuronide and luteolin-7-*O*-glucoside were the major flavonoids found in artichoke infusion, apigenin-7-*O*-glucuronide, luteolin-7-*O*-glucuronide, and apigenin-O-deoxyhexosyl-glucuronide were the main constituents of milk thistle infusion, and protocatechuic acid was the most abundant compound in borututu infusion.

7) The antimicrobial activity of these formulations was also assessed and, in a general way, the samples containing the higher amounts of phenolic compounds also presented the most potent antimicrobial activity at the tested concentrations, which could be explained by the well-known antimicrobial properties of these compounds.

From the present study, it can be concluded that artichoke, borututu, and milk thistle represent important natural sources of phytochemicals with antioxidant, hepatoprotective and antimicrobial properties, which can easily be included in diet, namely in the form of supplements, thereby contributing to prevent chronic diseases. This work also contributes to support the reasons for the traditional and current uses of these plants in different formulations (dry material, pills and syrups), by stregthing the knowledge of the main responsible bioactive compounds. Nevertheless, future studies are needed to clarify specific mechanistic pathways of these compounds. Among others, *in vivo* studies should be performed in order to confirm that the herein reported *in vitro* effects can be produced in the human organism.

References

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ANNEXES

ANNEX I

Extraction, identification, fractionation and isolation of phenolic compounds in plants with hepatoprotective effects

ANNEX II

Analytical tools used to distinguish chemical profiles of plants widely consumed as infusions and dietary supplements: artichoke, milk thistle, and borututu

ANNEX III

Effects of gamma radiation on chemical and antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity of borututu

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ANNEX IV

Antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity of artichoke, milk thistle and borututu

ANNEX V

Synergisms in antioxidant and anti-hepatocellular carcinoma activities of artichoke, milk thistle and borututu syrups

ANNEX VI

New insights into the effects of formulation type and compositional mixtures on the antioxidant and cytotoxic activities of dietary supplements basedon hepatoprotective plants

ANNEX VII

Is honey able to potentiate the antioxidant and cytotoxic properties of medicinal plants consumed as infusions for hepatoprotective effects?

ANNEX VIII

Infusions of artichoke and milk thistle represent a good source of phenolic acids and flavonoids

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ANNEX IX

Phenolic profile and antimicrobial activity of different dietary supplements based on Cochlospermum angolensis *Welw.*

ANNEX X

Resumen